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Regulation of gastric emptying and food intake

by

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Abstract

The present dissertation explores the relationship between regulation of gastric emptying and regulation of food intake. A modification of standard scintigraphic techniques was developed that allowed continuous measurement for 7-26 hours of cumulative food intake, gastric filling and gastric emptying during as well as between multiple meals in free-feeding parabiotic rats. Gastric filling and emptying characteristics in two different rat models were investigated: a control parabiotic preparation, as well as the one-way crossed intestine model, which is characterized by a relatively minor intestinal surgery, leading to large differences in food intake between the two partners of a pair.

The main findings were that gastric emptying of the liquid diet Ensure plus, that was used in the experiments, takes place at a fairly constant rate throughout the day. No differences were found between emptying in daytime vs. nighttime, in spite of large fluctuations in food intake and gastric filling over 26 hours. It was shown that gastric distension does not act alone as a signal that can terminate meal intake. It remains possible that gastric distension is used in synergy with other factors to inhibit food intake. Based on the present results, a circadian modulation of its expression would have to be hypothesized.

The data from the crossed-intestine experiments show that the rate of gastric emptying differs considerably between the two partners. This difference is similar to the threefold difference in food intake that characterizes this model. The data also showed that this difference appears to be present within minutes after the first meal and is not caused by differences in gastric filling.

Also, it was shown that an elevation for several hours of the plasma levels of amino acids, and to a lesser extent glucose, inhibits gastric emptying. Infusion of lipids into the bloodstream was ineffective in decreasing the rate of gastric emptying within the seven hours of the study. The differences in emptying rate in the cross-intestine rats may be caused by inhibitory intestinal feedback signals or by post-absorptive signals as shown in the nutrient infusion studies.

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I would like to dedicate this thesis to my parents. Thank you for all your love, support and patience throughout these years. Vadertje, moedertje, bedankt voor alles!

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Chapter 1 : Introduction

Food intake, ultimately directed to secure the delivery of sufficient amount of energy substrates to the body tissues, is a behavior of remarkable complexity. Although over the last century a great deal of work has been devoted to this research subject, resulting in the acquisition of a vast body of knowledge, the task of finding "the" regulatory mechanisms that govern food intake has remained an elusive one. This is understandable if one takes into account that, especially in higher vertebrates, the decision when to eat, what to eat and how much, is based on the interaction between a variety of genetic, ecological, psychological and physiological factors. The central nervous system (CNS) therefore needs to integrate a great deal of information from internal and external sources to finally generate or terminate feeding behavior. This implies that certain factors that have a major effect on food intake under a given set of circumstances can seem to be of only minor importance under different conditions; indeed, many findings appear to be contradicted by other studies that were sometimes performed under only slightly different experimental conditions. With these limitations in mind, considerable progress has been made, especially in the last few decades, in the understanding of the various mechanisms that are involved in the regulation of this behavior.

From a theoretical point of view, food intake could be controlled by different regulatory systems involving a variety of feed-back and feed-forward mechanisms. On the level of the body cells, all food intake could be considered as feed-forward regulated (except for a situation of severe starvation): not only does an animal rarely have all food emptied from its gastrointestinal (GI) tract before the next meal is initiated, but it also has considerable reserve energy stores in the form of glycogen storage and body fat tissue. Therefore, apart from specific diet-related needs such as sodium-appetite, it seems unlikely that food intake

under normal conditions is regulated on the basis of acute metabolic needs. Consequently, regulation of food intake must involve integration of available energy stores within the body with projected energy requirements in the near future. Feeding behavior would be initiated when the total body energy level (or the directly available amount of some specific substance or energy source) drops below a certain threshold and terminated after an upper threshold has been reached. The difference between the level of these two thresholds would serve as a positive reinforcement on feeding behavior and would allow activation in-between meals of other behaviors such as sleep for extended periods. Regulation of meals via a simple homeostatic feed-back system based on repletion of used energy stores (i.e. a single-threshold model) would instead cause small and frequent meals throughout the day-night period.

Over the years many different general theories about food intake behavior and specific central and peripheral mechanisms involved in its regulation have been proposed and, in the following chapters, the main theories and regulatory systems will be discussed. In the first half of the century, the attention of research was mainly focused on exploring the peripheral mechanisms that were assumed to be the origin of the sensations of hunger and satiety. One of the oldest theories and of particular importance within the framework of this thesis can be singled out here: it hypothesizes that the stomach is a main source of satiety signals and therefore has major importance in the regulation of meal patterns. And indeed, from a theoretical point of view the stomach is in an eminent position to play a pivotal role in the regulation of meal patterns and body weight. One of the functions of the stomach is to act as a reservoir for ingested nutrients. By creating an internal buffer of undigested food the animal could extend its feeding activity beyond the fulfillment of its immediate energetic needs by filling up the stomach and could also switch to other behavioral systems for extended periods by reliance on this internal source for its ongoing

energy requirements. Considering that the capacity of the stomach is limited, a certain level of gastric distension caused by the volume of the incoming meal could serve as a satiety signal that could be involved in meal termination; a decline in stomach contents below a certain level could trigger a new meal. Regulation of both short-term meal patterns and long-term body weight gain could be possible via regulation of the rate of gastric emptying: a fast emptying rate during and after a meal would bring the stomach contents quickly below the lower threshold, allowing a new meal to be taken and simultaneously causing a higher influx of nutrients per hour into the general circulation via absorption from the intestines, resulting in a relatively fast body weight development. Conversely, a lower emptying rate would keep the stomach filling level relatively high for extended periods, thus inhibiting further food intake and simultaneously slowing body weight gain by lowering the amount of nutrients that are made available for the body. This concept of the rate of gastric emptying serving as a central regulatory mechanism has been used as a basic working hypothesis for the design of the experiments that are described in this dissertation.

The quick expansion of neurophysiology has shifted the focus of research in the beginning of the second half of this century more to the elucidation of brain mechanisms in the regulation of food intake and the interactions between the CNS and the gastrointestinal system. Although great progress has been made in this field, for instance on how the brain regulates GI function, the understanding of what causes hunger and satiety still remains incomplete, and over the last decades some of the attention seems to be shifting back to a renewed investigation of peripheral satiety mechanisms, partly inspired by the availability of a number of new techniques.

Most relevant for the present manuscript is the development of gamma camera scintigraphy, a technique that utilizes radioactive markers that can be ingested or injected,

whereupon their distribution throughout the body can be monitored at regular intervals. Although in a relatively short period this technique has become the method of choice for measurement of gastric emptying in a clinical diagnostic setting, its use for animal research has still been somewhat restricted, partly due to the practical problems that arise when working with uncooperative, unanaesthetized subjects.

For the present series of experiments a modified scintigraphic technique was developed that can accurately measure gastric emptying in a free-feeding parabiotic rat model. Contrary to standard scintigraphic measurements, this adaptation allows measurement of stomach emptying for several consecutive meals over extended time periods, while simultaneously measuring stomach contents and food intake. This addresses a void in the literature: most gastric emptying studies are restricted to measurement of the emptying rate of one single meal, generally after a food deprivation period. 24-hour emptying rates must then be estimated via extrapolation of the single-meal results. Another advantage of the modified design is that it enables accurate determination of the rate of gastric emptying during meals, which is not possible under standard scintigraphic protocols.

This technique has been used to acquire more detailed information about the regulation of gastric emptying under natural, free-feeding conditions. Also these results were used to test the hypothesis that regulation of gastric emptying is a major common pathway in the regulation of food intake, so that mechanisms that affect the rate of stomach emptying may also directly affect food intake or visa versa. For that purpose the gastric emptying characteristics were investigated of two different rat models that are extensively used in our laboratory (Dr. H.S. Koopmans): a standard parabiotic preparation as well as the one-way crossed intestines rats.

Chapter 2: Literature review

An overview will be given of relevant literature about the mechanisms that regulate gastric emptying, of factors that can modulate food intake and gastric emptying (highlighting some of the similarities between these systems), and of nuclear medicine techniques.

2.1 Gastrointestinal motility

2.1.1 General regulation

The gastrointestinal system is designed to process a variety of foods in such a way that their nutrient contents become available for further metabolism in the various body tissues. This process involves a coordinated series of events with respect to motility and secretion: 1. Food is being moved through the GI system, while regulation takes place of the amounts retained in specific areas and the speed of transport through the different parts of the GI tract. 2. Digestive enzymes are synthesized, secreted and mixed with acidic or alkaline juices that create an optimal pH for enzymatic action. 3. Food particles are mechanically reduced into smaller particles in order to increase their surface area available for the digestive enzymes, and are mixed with these enzymes. 4. After being reduced to a more elementary form, the nutrients are distributed over the lining of the GI tract and absorbed through the mucosa into the bloodstream, followed by transport to the various body tissues.

A general coordination of basic motility and secretion patterns takes place via intrinsic reflexes, mediated through interconnected intramural ganglia of the enteric nervous

system (ENS) within the mucosa ("submucosal or Meissners plexus") and smooth muscle coat ("myenteric or Auerbach's plexus") of the digestive tract (66); interstitial cells of Cajal appear to be involved in mediation of basic motility in the intestines as a relay station between neural activity and smooth muscle cells (63;358) and may also be directly active as pacemakers in the generation of the gastrointestinal electrical rhythms (358). The role of the ENS in gastric motility has not been fully elucidated: in a number of species the submucosal plexus is poorly developed, and the electrophysiological characteristics of the myenteric plexus appear to be different from their counterparts in the intestines (93). The ENS itself consists of a large number of intrinsic neurons. These neurons receive synaptic contacts from the processes of both afferent (spinal and cranial sensory neurons) and efferent (sympathetic and parasympathetic) extrinsic neurons (66). Local circuits within this autonomic neural network can respond to sensory information from intrinsic gastrointestinal receptors as well as signals from the central nervous system (451).(450) The role of the extrinsic nerves is mainly to modulate the intrinsic reflexes according to the overall needs of the organism and to achieve an integration of activity in widely separated regions of the alimentary canal (335). Overall, parasympathetic activation tends to stimulate GI motility, while sympathetic input inhibits it (238;259). A considerable amount of integration of incoming sensory information and the primary response of the GI tract, however, occurs within the ENS itself.

2.1.2 Electrical basis of GI motility

At any given point in time the major part of the GI tract does not display motor activity; contractile activity takes place only at certain times at any site. The basic underlying mechanism of gastrointestinal motility is the generation by pacemaker cells of a cyclic potential change over the smooth muscle cell membrane (pacesetter potentials or slow

waves), probably arising from the interstitial cells of Cajal (358); muscle contractions are triggered by the occurrence of action potentials.

In the stomach two distinct regions can be discriminated: the fundus is an electrically stable muscle, which is mainly characterized by tonic alterations in volume (183;431). Its activity appears to be mainly regulated via excitatory cholinergic, and inhibitory non-adrenergic non-cholinergic vagal regulation; also sympathetic nerve fibers inhibit gastric activity. The tonic contractions (1-3 minutes in duration) result from small membrane depolarization by mechanisms that are poorly understood.

Smooth muscle cells in more distal parts of the stomach exhibit a fluctuating membrane potential: slow waves, or pacesetter potentials. More proximally located muscle cells have less negative resting membrane potentials and a higher frequency of spontaneous depolarization than cells from more distal sites (259). This induces a higher frequency of action potentials and a concomitant higher frequency of muscle contractions at the oral side, resulting in a circumferentially spreading contraction wave directed from the gastric corpus towards the pylorus; the bands of depolarization are generated by a pacemaker zone at the boundary between fundus antrum. A depolarization consists of an initial spike, followed by a plateau potential. Contractions are generated in association with this electrical activity, however, not every band of depolarization causes a contraction; antral contractions are generated only when the plateau potential exceeds a certain threshold. The amplitude and duration of a contraction are related to the time that the plateau potential remains above the threshold. Peristaltic contractions are thus organized by the orderly propagation of the contraction wave (324). Peristaltic waves occur at a rate of 0-3 / min in humans (or 0-5 / min. in dogs) (182;409).

The pyloric sphincter forms a functional unit with the distal antrum and the duodenum, but can also generate its own rhythm of contraction, that is maintained when the activity

of stomach and duodenum is blocked. Pyloric contractions ("isolated pyloric pressure waves") occur over a narrow distance and respond to neural and hormonal stimuli differently from the antrum (325).

The small intestine also displays an intrinsic myoelectric slow wave activity, and is characterized by irregular segmental contractions. The slow wave frequency decreases in a series of frequency plateaus from duodenum to ileum (in humans declining from 12 / min to 8 / min), resulting in proximal cells imposing their contraction rhythm on more distal cells (433). The slow wave is propagated rapidly in a circumferential direction, creating the conditions for the development of a ring constriction; propagation in a longitudinal direction is slower (in humans 60-100 cm / minute). The higher contraction frequency results in a pressure gradient that is aborally directed, and is thought to generate a net propulsive force on intestinal contents. Coordination of the contractions appears to be more important for propulsion than regulation of contraction strength (433). Also propulsive contractions of longitudinal muscle bundles occur ("pendular contractions"); the effect is to move the bowel over its contents. Peristalsis is accomplished by a reflex mechanism, where local distention of the intestinal wall leads to a contraction of circular muscle at the oral side of the bolus ("propulsive segment"), and of a relaxation on the aboral side ("receiving" segment) (450). The innervation by the ENS ensures that contractions of circular muscle occur in synchrony around the circumference of the gut.

The organization of peristaltic circuits into functional blocks is probably responsible for the fact that propulsive behavior in small intestine usually takes place in isolated segments and propagates over short, variable distances. A single block is hypothesized to be "hardwired" for relaxation, followed by contraction of an intestinal segment during the passage of a propagating complex. A "gating" mechanism within the ENS is thought to be responsible for continuation of peristalsis along the gut (450). Also mechanical or

chemical stimulation applied to the mucosa evoke reflex changes in circular muscle activity. These reflex mechanisms can be elicited by stimulation of sensory receptors within an area of a few millimeters wide at any point along the intestine (115).

2.1.3 Interdigestive patterns: MMC's

Two distinct motility patterns can be discriminated in the small intestine: the fed and the interdigestive pattern. The interdigestive pattern, or migrating motility complex, is characterized by regular cycles (in dogs every 90 to 120 min) of contractile activity, followed by a period of mechanical quietude. It can be initiated in the distal oesophagus, stomach or upper gut (359), and sweeps through the GI tract in aboral direction. It is believed to function as a "housekeeper" of the GI tract: contrary to its normal activity during antral contractions, the pylorus does not close at the arrival of a pressure wave after a MMC is initiated in the stomach, so that also larger particles are emptied (esp. the indigestible fibres and larger particles that would otherwise remain in the stomach) (450). Disorders of MMC patterns can result in bacterial overgrowth in the small intestines. The velocity of intestinal transit appears to be variable (313;340;434).

Four different phases can be recognized in the MMC:

Phase I: A relatively low myoelectric spiking activity, associated with a period of mechanical quiescence.

Phase II: Persistent but random spiking activity, with increasing bursts of action potentials and irregular muscle contractions, resembling the activity that normally occurs after a meal.

Phase III: An organized motor pattern, characterized by action spikes superimposed on every slow wave; the number of action potentials per slow wave determines the force of the muscle contraction. Phase 2 and 3 are considered to

be responsible for the propulsion of intestinal contents.

Phase IV: A transition period to the return of phase 1. Sometimes a phase 3 contractile activity is abruptly abolished and a phase IV can not be clearly defined.

The occurrence of MMC's is synchronized with secretatory activity throughout the GI tract. The mechanisms regulating the MMC cycles are not yet fully elucidated: neural factors (extrinsic or intrinsic), but also hormonal regulation, local enteric factors, or a combination of these have been proposed (359;361). The possible role of motilin is controversial: the injection of motilin into the blood stream at physiological levels is known to induce premature MMC's, and peak values for plasma motilin are synchronized with the occurrence of Phase III activity. Recent evidence shows that extrinsic neural connections may be more important (53;54); however, extrinsic denervation experiments suggest a role for hormonal regulation of MMC's in the stomach, and a role for the ENS in the initiation of MMC's in the jejunum of dogs (359). In the intestinal tract recent evidence suggests an important, but not exclusive, role in the regulation of MMC's by nitric oxide as a nonadrenergic noncholinergic neurotransmitter (360). Vagal blockade has also been shown to disrupt gastric MMC's (54;361). Food intake, or infusion of nutrients, is generally believed to abolish the MMC pattern; however, a more detailed analysis of the motility pattern after feeding revealed that still some cyclic motor activity remains (464). There is also some evidence for circadian regulation of MMC propagation velocity, independent of luminal content (201).The mechanism behind MMC initiation and disruption thus remains unclear: species differences may partly account for some of the discrepancies found in the literature.

2.1.4 Fed pattern: movement of food through the GI tract

After a meal the interdigestive pattern changes abruptly into the fed pattern, characterized by irregular contractile activity. Distention of the stomach appears to play a role in the disruption of MMC's (34), but the physiochemical composition of the meal is thought to be of more important than its volumetric or caloric properties (34;74). Even sham-feeding has been shown to disrupt MMC's (75), suggesting the possible involvement of vagal pathways.

Contraction of a small segment of circular muscle, in absence of contractile activity directly oral or aboral of that segment, leads to segmentation of intestinal contents without much net propulsion, thereby continuously mixing, separating and recombining the intestinal contents. Intestinal peristalsis (movement of gut content in aboral direction) is the result of muscle contraction at adjacent loci in an oral to aboral sequence.

2.2 Regulation of gastric emptying

The stomach plays a central role in the conversion of ingested food into a form that can be handled efficiently by the intestines. This process involves a sequence of events in the different anatomical regions of the stomach: first of all reception and temporarily storage of freshly arriving food in the fundus, followed by propulsion of food from the fundus to the body of the stomach and secretion of gastric juices by glands in the corpus, mixing of the food and juices and grinding of solid food particles into smaller size in the antrum, and finally the emptying of chyme (a semi-liquid phase) into the duodenum, controlled by integrated motor activity of fundus, antrum, pyloric sphincter and duodenum (158;160;182;190;237;240;259;260;324;385;418). In spite of numerous studies measuring stomach emptying of a variety of meal substances, many aspects of the regulation of gastric emptying are still poorly understood. Gastric emptying is a complex but regulated process that is controlled via various feed-back mechanisms

(15;16;160;190;248;249;252;321;324;325). The regulatory mechanisms can be of neural as well as of hormonal character: vagal efferents can have excitatory or inhibitory effects on gastric motor activity; they can increase the frequency and force of contractions by changing the amplitude of the slow waves. Sympathetic activity reduces GI motility. Many hormones have a site-specific effect: all gastrointestinal hormones, except motilin, relax the fundus, while CCK and gastrin stimulate antral contractions (260;433).

2.2.1 Gastric accommodation of meals

During and after feeding, the incoming meal is first stored in the proximal part of the stomach. The fundus adapts actively to the increase in intragastric volume via receptive and adaptive relaxation. Receptive relaxation, which is initiated by stimulation of oesophageal mechanoreceptors (72;91;462) and adaptive relaxation, initiated via mechanoreceptors in the muscularis mucosa of the stomach wall (68), are mainly transmitted via a nitric oxide-dependent, nonadrenergic, noncholinergic vagal pathway (13;76;258); they may be mediated by dopamine (190) or vasoactive intestinal peptide (VIP) (71;72;129;417). Electrical stimulation of afferent fibres in the vagus nerve originating from the stomach can artificially produce gastric relaxation; division of the vagal nerves to the stomach abolishes the reflex (120;141;167).

Also other intragastric feed-back loops exist: application of acid, alkali and hypertonic saline to the antral mucosa is capable to produce a reflex decrease in intragastric pressure, mediated by vagal afferent and non-adrenergic, non-muscarinic pathways (317); distention of the antrum of the anaesthetized cat causes reflex relaxation and inhibition of motor activity of the proximal stomach. Vagal, but also sympathetic pathways appear to be involved (136;380). Intestinal distention produces relaxation of the fundus via a vagal pathway (72). The receptive relaxation reflex diminishes the effects of ingestion of food

on intragastric pressure, but, possibly because the amount of food passing the oesophagus can not be precisely measured by oesophageal mechanoreceptors, the adjustment of fundic tone is imprecise: swallowing of small amounts may cause a small drop in intragastric pressure, where a fast intake of food may exceed the capacity of this reflex mechanism to adjust gastric tone, resulting in a smaller decrease or even an increase of intragastric pressure (223). Adaptive relaxation occurs as a reaction on an increase in intragastric pressure, leading to a concomitant increase in activity of slowly adapting mechano-receptors in the stomach wall (68;165;166;304-306). A heterogenous population of such mechanoreceptors is apparently able to monitor a wide range of pressure changes (68): this would create the possibilities for a precise feed-back regulation. However, in humans and in the dog an initial rise in intragastric pressure of 5 to 10 cm H2O appears to be required for initiation of the reflex (182;237). After an initial fundic relaxation, a gradual increase in tonic contractions occurs, reestablishing intragastric pressure. This results in a steady, continuing compression of the gastric contents.

A number of excitatory stimuli acting from within the stomach have also been described: release of gastrin by G-cells in the pyloric region stimulates antral motor activity. This is stimulated by antral distention, peptides or amino acids, but inhibited by acidity below pH = 3 (380). Also inflation of the whole stomach or a corpus pouch induced antral contractions in the ferret which where abolished by vagal section. For these coordinated patterns intrinsic reflexes (380), as well as vago-vagal reflexes (8;20) have been proposed. In dogs with separated corpus and antrum, distending the corpus caused vago-vagally mediated contractions of the corpus and also (potentiated by modest antral distention) of the antrum. Stronger distention of the antrum abolished corporal contractions, both during a migrating motor complex or as a result of corpus distention (380). Solid particles in the antrum induce a strong motor response (237;380). This suggests that stimulation of

mechanoreceptors in the pyloric region, as would occur when solid particles of sufficient size would be present when a strong antral contraction reaches this region, would reflexively induce higher motor activity of the antrum, which would be highly functional in further reduction of particle size. Both sympathetic (120) and vagal (120;334) innervation of the distal stomach are also involved in the regulation of antral motility.

2.2.2 Stomach emptying of liquids and solids

After a mixed meal fluids empty faster than solids; this may also allow better mixing of the remaining solids with gastric juices (324). Until recently a commonly held view suggested that contractions of the proximal stomach are responsible for emptying of liquids, whereas antral contractions regulate the emptying of solids (after grinding them down to small particles). Pyloric and duodenal activity were generally considered to be mainly important for control on the particle size of emptied solids. However, over the last decade a number of experiments have cast doubt about these separate functions (36-38;85;96;103;104;200;259;260): a pressure gradient has never been convincingly demonstrated (324), and the normal intragastric pressure would not be high enough to cause emptying of liquids (308). It has been demonstrated that liquids do not leave the stomach in a steady stream, but in gushes during contractions of the antrum, pylorus and duodenum, (155;312); It is therefore more likely that emptying of liquids and solids are regulated by a combination of pressure differences and resistances between fundus, antrum, pylorus and duodenum. Pyloric (both tonic and phasic contractions) and duodenal phasic contractions, and their coordination with antral contractions, are important in the regulation of fluid emptying by changing the resistance over the antroduodenal junction, and also can influence the emptying of solids and liquids separately (57;324). The timing of pyloric closure in relation to phasic gastric antral and corpus contractions is probably

regulated via antral intramural nerves (11). Nitric oxide is involved in the regulation of pyloric tone (10).

Gastric emptying of isotonic, non-caloric liquids is volume-dependent and follows an exponential pattern (240;248;260), whereas emptying of caloric liquids is slower and follows a more linear pattern (248), illustrating that the resistance of the antroduodenal region (in concert with inhibitory feed-back on gastric motility) can effectively influence this process. Experiments using implantation of barostats (260) support that the fundic contractions and intragastric pressure are not the only driving force in stomach emptying of liquids, but that duodenal resistance is also an important contributing factor.

Fundic tonic contractions are also to a certain extent involved in the regulation of emptying of solids: a gradually increasing contractile activity propels food towards the body of the stomach. Since antral contractions are evoked by stimulation of mechano-receptors in the antro-pyloric region (238;435), fundic contractions (forcing a new supply of larger particles into the corpus) can indirectly stimulate antral activity. Also the existence of direct reflex loops in and between fundus and antrum have been suggested (380). Solid particles are processed in the antrum by a grinding and mixing process until they have reached a diameter small enough to allow passage of the pylorus, suspended in a semi-liquid phase. Immediately after a meal, however, most particles have not been sufficiently reduced in size to pass the pyloric sphincter (indigestible particles larger than one millimeter in diameter do not pass the pylorus during normal contractions) and are therefore retained in the stomach for a certain amount of time: the solid "lag phase" (38). The notion of a lag phase as a result of larger particle size is widely accepted, but has recently also been challenged: solid food can also be retained in the fundus until the liquid phase of the mixed meal has been emptied, at which time it moves forward into the antrum and immediately begins to empty (324). The lag-phase would thus be the result of

retention of food in the fundus, and the duration of the lag-phase would be directly influenced by the caloric value of the liquid being emptied (57).

The actual emptying of solid particles from the stomach is mainly driven by antral contractions, but this requires coordination with pyloric and duodenal contractions (260). The presence of solid food in the distal antrum stimulates the generation of regular contraction waves originating in the body of the stomach. When these contractions reach the midpoint of the terminal antrum, the pylorus closes, any material trapped between the antral contraction wave and the pylorus is propelled back into the stomach (assisted by a concomitant relaxation of the fundus) until the next pressure wave moves it again forward, and only a small amount of chyme is emptied into the duodenum (178). Solid food evokes a stronger motor reaction in the distal antrum than a homogenized meal (119;237;326), possibly via stimulation of the mechanoreceptors in the pyloric region (165;166;305;306).

In humans or dogs, on regular times between meals the normal fed pattern of the stomach changes into MMC activity (182;190;237;335). During these contractions the pylorus remains open, and the larger particles are allowed to leave the stomach. In the rat the occurrence of MMC's in the stomach is not well described.

2.2.3 Intestinal feed-back regulation of stomach emptying

Several studies have demonstrated that after a meal feed-back control of gastric motility results in the delivery of a fixed rate of calories into the intestines. Different populations of receptors exist in the duodenum: osmoreceptors, fat receptors and receptors for specific amino acids (161;324), as well as mechanoreceptors (27). Also the existence of specific intestinal glucoreceptors has been proposed (253-255;324), yet no clear morphological evidence for their existence has been found. The physical and physiological properties of the receptors and their signal transduction have not been fully elucidated yet. Hunt (158)

hypothesized a mechanism based on the osmolarity of nutrients in the gut; changes in intraluminal volume would thus activate mechanoreceptors. This model, however, fails to explain the weaker inhibitory effects of equimolar amounts of fructose, compared to glucose. An alternative possible mechanism for their action could be an indirect stimulation of vagal nerves: the interaction of nutrients with enteroendocrine or taste cells in the epithelium would release transmitters that may circulate as hormones or exert a paracrine action, stimulating afferent nerve terminals (25;26) to slow gastric emptying and/or induce satiety (321). CCK may act in such a paracrine action on mucosal vagal afferents (330). Intestinal receptors would monitor the physical and chemical properties of the chyme that enters the gut and could activate inhibitory feed-back mechanisms on gastric emptying if required (27;179;240;248;249;259;381). It has been shown that nutrient (especially amino acids) infusion into the proximal duodenum elicits a stronger inhibition of gastric emptying than equimolar non-nutrient infusion; the signal is transduced by gastroduodenal load-sensitive vagal afferents (375;377). Direct intramural pathways are probably not involved in the feed-back signal: transection and re-anastomosis of the distal antrum did not change the gastric response to duodenal nutrients (11).

Some other factors which can slow down stomach emptying (237) include:

1. The acidity of the food: the ability of an acid to inhibit emptying decreases with higher molecular weight.
2. Osmolarity: isotonic solutions are emptied faster than either hypertonic or hypotonic solutions.
3. Energy density: meals with a higher energy density are emptied slower (in ml per minute), maintaining a similar caloric rate of emptying (in calories per minute); however, beyond a certain concentration the precision of this mechanism begins to

fail and the number of calories emptied per minute increases (160;162;249).

4. Size and density of solid particles: larger food particles empty more slowly since it takes the antrum longer to grind them down to smaller size. Smaller particles leave the stomach dissolved in the liquid phase.
5. Temperature, when different from body temperature (407;414).

Nutritive substances in the small intestine (or products of digestion of nutrients) inhibit both tonal contractions of the fundus (16;129) and antral motility (202), as well as coordination between pyloric and duodenal contractions (179;200)via stimulation of chemo- and mechanoreceptors in the intestines (33;96;136;150;200;381); this results in a modulation of the rate at which stomach contents are passed into the duodenum (160;214-218;248;249;252). Different nutrients may act via different mechanisms: inhibition of gastric motility by duodenal stimulation with fat or protein appears to be mediated by a capsaicin-sensitive vagal pathway and CCK (26), whereas the effects of duodenal glucose involve a vagal pathway (268;316) and possibly also CCK (269). Inhibition with maltose involves interaction between CCK and capsaicin-sensitive vagal and spinal pathways (317). Also serotonin (5-HT) can directly stimulate mucosal vagal afferents in the rat jejunum (147), via a direct action or indirectly as a response to serotonin-mediated contractile activity (149). Different populations of mesenteric afferents have been described that are sensitive for either CCK or 5-HT (148).

From a theoretical point of view, a higher level of inhibition of gastric emptying should be created if nutrients reach parts of the intestinal tract beyond the point where most of it would have been absorbed under normal conditions. This could be accomplished by creating a cumulative effect from all sites so that if more receptors are stimulated a stronger inhibitory signal is generated, or by creating a stronger feed-back signal from more distal sites, or by a combination of the two. The last option seems to be supported

by experimental evidence: the inhibitory feed-back signals from the intestines appear to be nutrient- and site- specific (214-219;320;324;325), although different methodology and the use of different species appears to generate contradictory results. Some studies find stronger inhibitory signals in the distal gut (218), while others claim the opposite effect (436). An early regulatory response could also take place via load-dependent receptors in the upper intestines (375;377), so that a high influx of nutrients into the duodenum could cause an early inhibitory response.

Fat may be more effective in the distal parts of the intestines (33;150;219;324;325;436), although it seems to exert some influence all along the gut (16;145;146;260). Fat has a number of effects on stomach motility: it has a strong inhibitory effect on gastric pressure by relaxation of the fundus. Furthermore it inhibits antral peristalsis, thus reducing further delivery of food into the duodenum, and narrows the diameter of antrum, pylorus and duodenum (thus increasing the resistance). It also inhibits the coordination of contractions between antrum and duodenum. For fatty acids, the potency to inhibit gastric motility increases with molecular chain length, up to C14 (16;260). The physiological significance of fat infusion into the distal gut has been questioned, however: the satiating effects of fat via oral intake and normal processing with gastric juices and bile are considerably smaller, and the effects of ileal infusion may be due to damage inflicted on the gut (111;152;315).

Certain amino acids, especially tryptophan and phenylalanine, appear to be acting on special duodenal receptors (136) and are particularly effective in inhibiting gastric emptying. Generally proteins are most effective in the ileum (325). Carbohydrates, that are usually absorbed mainly in the proximal parts of the gut (28), also have a greater effect in the ileum (16;214;218;325), mediated by products of starch digestion. Inhibition of amylase activity delays stomach emptying by increasing the carbohydrate load in the ileum (237).

Nutrient stimulation of the distal ileum has strong inhibitory effects on gastrointestinal motility (33;96;150;218;325). This "ileal break" not only inhibits intestinal motility but also causes a relaxation of the fundus; this can induce a redistribution of stomach contents from antrum to fundus (96;325). The ileal break could be considered as an emergency system: arrival of nutrients in the ileum would indicate that the rate of gastric emptying exceeds intestinal absorption capacity.

2.2.4 Post-absorptive feed-back regulation

Also post-absorptive metabolic events can be involved in feed-back regulation of gastric emptying: hyperglycaemia or an elevated glucose level in the hepatic portal vein can inhibit stomach emptying, likely via a glucose-sensing mechanism in the liver and a hepatic vagal afferent pathway that affects vagal pancreatic efferents, adrenal and splanchnic-hepatic efferents via known reflex circuits in the hindbrain (103;104;290-294;303). Hyperglycaemia is also thought to be the main factor in the observed slow gastric emptying in diabetic patients (47;103;105;154;356). Hypoglycaemia leads to accelerated gastric emptying (23;246;366;368), probably mediated via cholinergic vagal pathways (23). Interestingly, in non-symptomatic diabetes a faster emptying of liquids has been observed (98;186;224); also obese Zucker diabetic rats show accelerated gastric emptying (125).Hepatic gluco- and osmoreceptors could be involved in the transmission of information about glucose levels in the blood stream via vagal afferents (3;289-292;295-297).

Few studies have dealt with the effects of other intravenously infused nutrients on gastric motility; mixed parenteral nutrients (35;235) inhibit gastric emptying, an effect partly attenuated by extra infusion of branched-chain amino acids. Fat infusion (Intralipid) overnight has been shown to delay gastric emptying (43).

2.2.5 Neural factors

Although the general motility patterns of the gastrointestinal system are generated by the enteric nervous system, gastrointestinal function is continually modified via circuits in the brain stem, depending on information that the higher centers of the CNS continually receive from the GI tract and beyond. The extrinsic innervation of the GI tract consists of vagal and sympathetic nerve fibers, that also exert mutually modulatory influences. Afferent splanchnic sympathetic fibers, totaling 20- 40 % of all fibers in the splanchnic nerve (120;136;335), may convey nociceptive (generated by stronger stimuli, such as painful distension) but also innocuous information to the brainstem, where a first integration of sensory input takes place (329). The response of gastric mechanoreceptors is dependent on the inflation rate of an intragastric balloon: the slower the stomach is distended the lower is the threshold to perception and pain (187). This is thought to give support to the concept that mechanoreceptors subserving splanchnic afferents are in parallel with the gastric musculature. Apparently the musculature of the stomach behaves as a viscoelastic rather than elastic material, and reduces its resistance to stretch with time (187).

Splanchnic afferent information, directly affected by vagal input (likely via a descending vagal pathway) (411), can be relayed via the spinal cord to hypothalamic nuclei, where further integration with other information and activation of effector pathways can occur. Activation of sympathetic efferents, for instance by stressful stimuli, delay gastric emptying, reduce gastric tone, and inhibit antral and pyloric motility (411). A spinal intestino-intestinal reflex loop has been described, that appears to maintain a certain sympathetic restraint on intestinal motility via input on the ENS (120). From proximal to distal regions of the GI tract the ratio of vagal to splanchnic afferents decreases (136); whether this changes the balance in feedback signaling between vagal and splachnic nerves

is not known; the phenomenon of the ileal brake could suggest that this may happen, or alternatively that a specific upregulation of receptor sensitivity in the ileum takes place.

The vagus nerve itself is for the major part involved in transmission of sensory information: ca 90 percent of vagal fibers are afferent (120;136). The remaining efferent fibers, however, have a profound influence on gastrointestinal functions, possibly via a general "gating" mechanism where vagal excitatory or inhibitory activity affects the hard-wired circuits in the ENS that control GI function, and changes the sensitivity of the GI tract for other, non-vagal, stimuli or even completely switches on or off a specific function in response to an appropriate stimulus (67). Gating is accomplished via the adjustment of the level of vagal activity: the vast majority of vagal efferent fibres are spontaneously active, providing a background vagal tone. The level of vagal tone is under the influence of afferent input from the periphery and descending cephalic influences. Modulation of vagal tone may then allow or block responses mediated via non-vagal pathways (133).

Many of the vagal functions are exerted via reflex loops: one of the main pathways consists of vagal afferents projecting on the nucleus of the solitary tract (NTS) and the area postrema (AP) in the dorsal region of the medulla. Second-order neurons from the NTS (and possibly even primary vagal afferents) project on the dorsal motor nucleus (DMX), and from there excitatory or inhibitory motor pathways, that may reciprocally modulate each other (69) innervate the GI tract (67). From the NTS other projections relay information to higher brain areas, such as the hypothalamus, including the lateral hypothalamus (LH) and the paraventricular nucleus (PVN) where integration is believed to take place from information from various sources; reciprocal projections from LH and PVN to the NTS then modulates the autonomic outflow to the GI system (67). Other projections from the NTS supply motor nuclei that are associated with oro-facial muscle activity (67), and may be involved in a basal regulatory feeding response to oral stimuli

that takes place at the level of the caudal brainstem (131). Specific stimuli associated with the intake of a meal cause a stronger, partially vagally mediated, activation of regions in the NTS and AP than sham-feeding or gastric distension alone, independent of CCK release, suggesting that some integration takes place in the hindbrain from oropharyngeal, oesophageal and gastric input (100;102).

Direct vago-vagal reflex loops are directly involved in the control of intragastric pressure via the receptive and adaptive relaxation reflexes (see 2.2.1). Vagotomy abolishes the reflexes, so that intragastric pressure rises quickly when the stomach is filled with water, air or an intragastric balloon (169;259;388;389). Again, considerable interaction occurs with the sympathetic system. The splanchnic nerves are not directly involved in the adaptive relaxation reflex of the stomach: splanchnic section does not influence the intragastric pressure response to graded distention. However, after initial vagotomy, the increase in intragastric pressure is higher after an additional splanchnic section (133). Apparently the sympathetic system is involved in a reflex mechanism regulating intragastric pressure, but its influence can be suppressed by vagal activity. In the ferret an antral reflex has been described: distension of the proximal; stomach transected from the antrum increased antral motility (8;380); vagotomy abolished the reflex. Vagal efferent outflow is also modulated by stimulation of in-series tension receptors in the stomach wall activating afferent vagal nerves (69). A gastroenteric reflex appears to be involved in the finding that distension of the stomach diminished the flow of liquids into the intestines (262); this was not affected by vagotomy. Also enterogastric reflexes have been demonstrated: distension of the duodenum or colon inhibit gastric tone and peristalsis (259) via inhibition of vagal efferent activity (135); this reflex is, again, relatively unaffected by vagotomy or by splanchnectomy, but can be blocked completely by a combined vagotomy and splanchnectomy. The reflex appears to consist of splanchnic

afferents and vagal efferents.

Vagotomy can affect stomach motility in different ways, depending on which vagal fibers were severed. Truncal vagotomy involves resection of both the anterior and posterior vagal trunks at the cardia. This deprives not only the stomach, but also most of the rest of the gastrointestinal tract of its parasympathetic innervation, and therefore has the most profound effects on the total functioning of the gastrointestinal system. Secondly, a selective vagotomy only cuts the anterior and posterior gastric nerves, and leaves the extragastric vagus intact. The third possibility is a dissection of the vagal nerve supply of the proximal stomach; in this case a complete vagotomy is more difficult to perform, since a high number of nerve fibres have to be cut (6). In all cases however, the completeness of the vagotomy has to be checked in order to validate eventual conclusions of the study. One possible procedure is to test for the absence of gastric contractions during electrical stimulation of the cervical vagus (300).

One of the most direct effects of vagotomy of the gastric fundus is the loss of the receptive and adaptive relaxation reflex. This means that the intragastric pressure increases more rapidly with graded distention, and since liquids are mainly emptied as a result of pressure differences between fundus and pylorus, the effect is a faster emptying (dumping) of liquids directly after a meal. Also the rhythmic contractions in the muscle layer of the proximal stomach are reduced in strength (6), and since this reflects the transport function of the fundus to deliver food to the distal part of the stomach, this may slightly diminish the rate of delivery of solid food into the intestines. Antral motility is preserved after vagotomy of the proximal stomach.

Selective vagotomy involves denervation of both the antrum and fundus of the stomach. In this case (or in comparative studies where vagotomy of the fundus is subsequently converted to selective vagotomy of the whole of the stomach) the effects on the fundus

are the same, but in the antral region contractions are disorganized with lower amplitude. The initial rapid emptying of fluids remains unchanged. The final emptying of fluids or semifluid homogeneous meals, however, appears to be faster after selective vagotomy, while the change after vagotomy of the fundus is only minimal (6). Since pyloric tone is under vagal control (133), this may reflect a decrease of resistance in the pyloric region. Solids empty much slower after selective vagotomy ("gastric stasis").

The effects of truncal vagotomy on stomach motility are basically the same as after selective vagotomy: liquids empty rapidly after a meal, while solid food accumulates in the stomach. Since this type of surgery affects the functioning of the whole gastrointestinal system, the results of such studies are more difficult to interpret.

A major part of the regulation of gastric and intestinal motility thus appears to be taking place on the level of enteric nerves and the brain stem, kept in check via feed-back regulation involving both the sympathetic and the parasympathetic system, and a variety of reflex mechanisms. However, the before mentioned connections between brain stem and higher brain centers, especially in the hypothalamus, allow for a further integration of information and adaptation of the GI motility patterns. For instance, it has been shown that the PVN is involved in modulation of vagovagal reflexes and in regulation of gastric motility: stimulation of the PVN inhibits gastric motility (351), lesioning the PVN induces a transient increase in basal gastric motility (94;245), followed by an inhibition of gastric tone and motility (245), and also diminishes the sensitivity of the NTS for incoming vagal sensory information (245). Distension of the stomach causes neural activity in the LH (7;382). Conversely, unilateral electrical stimulation of the LH causes a gastric motor response; this effect is mediated via both vagal trunks (92). Lesions of the VMH cause an increased rate of gastric emptying (87;88). This effect is acute, demonstrated within hours after the lesions, therefore the increased emptying rate does not appear to be the effect of

excessive food intake, pushing food through the GI tract after the stomach is filled to capacity. Also the nucleus suprachiasmaticus (SCN), involved in the generation of circadian rhythms, may exert some influence on vagal activity (402).

The various effects of the sympathetic and parasympathetic system on gastrointestinal motility, and the subtle balance that appears to be maintained between these systems and their interaction with the ENS makes a straightforward description of their general function difficult. This issue becomes even more complicated, however, when the interactions between neural and hormonal factors are taken into account.

2.2.6 Hormonal factors

A number of gastrointestinal hormones and peptides appear to exert influence on GI motility. However, a physiological role in the regulation of gastric emptying is not always established. The physiologically effective dose of systemically infused substances do not necessarily represent the mechanism of their action: relatively small amounts would need to be released locally to establish paracrine effects. However, often the dosages that are needed for an effect appear to be out of range from the concentrations that are found in the normal animal. Also complex interactions can occur between peptides and neural reflexes; sometimes sites and mechanisms of action of peptides are not identical in vivo and in vitro (97). Cholecystokinin (CCK), secreted by nerves and endocrine cells in the upper gut, has been connected with the regulation of the gastrointestinal response to a meal: the secretion of CCK by mucosal I-cells in the duodenum is stimulated by the arrival of fresh food, especially proteins and fatty acids, in the GI tract. The effects of CCK are mediated via stimulation of 2 sub-populations of receptors: CCK-A receptors have a high affinity for CCK, and are mainly located in the periphery, as well as in terminals of the abdominal vagal nerve fibers (273), in the brain stem in the medial nucleus of the solitary

tract and the area postrema (where a hormonal effect of CCK would be possible, due to the lack of an effective blood-brain barrier in that region), as well as in sites in the forebrain (270-272). CCK-B receptors are widely distributed in the PVN, the VMH, as well as other brain regions (271). Also CCK-B receptors are found in vagal fibers (60). Exogenous CCK release causes a direct and potent inhibition of antral motility and gastric emptying via stimulation of CCK-A receptors: binding sites for CCK in the GI tract of the rat are limited to the gastroduodenal region, and concentrated in the pyloric region (387). Populations of mechanoreceptors in the stomach can respond to distension as well as CCK (318): systemic infusion of CCK caused a relaxation of the stomach, as well as a decrease in activity in the selected afferent fibers (134). Mucosal CCK-sensitive receptors can be stimulated by relatively low CCK-concentrations, which could be an indication for a possible paracrine action.

Recently evidence has been found for the possibility that vagal afferent activity can be potentiated by exogenous CCK (68;99;101;369-374;376;377). Since CCK is released by the duodenum as a reaction on protein digestive products or fat, this opens up the possibility that this mechanism is more sensitive for meal-induced gastric distention than for non-nutritive stimulation (370;371;376;377) between studies measuring the effects of stomach distention while allowing or preventing nutrient stimulation of the intestines. The action of CCK on gastric motility also appear to take place at least partially via interaction with vagal reflex pathways (26): exogenous CCK can induce c-fos activity in the area postrema and NTS if the vagal afferent connection are intact (99), also CCK-induced inhibition of gastric motility is attenuated by capsaicin treatment of vagal afferent C-fibers (319).

Some of the further actions of CCK are relaxation of the sphincter of Oddi and contraction of the gall-bladder (in many species), and stimulation of the release of

pancreatic hormones (including insulin, glucagon, somatostatin and peptide P).

Insulin is released by the β-cells of the pancreas as a direct reaction to food intake. An early insulin response takes place via vagal activation in the cephalic phase of feeding, stimulated by the sight, smell and taste of food (393;395;399;404). A further release of insulin takes place via a rise in plasma glucose levels, and additionally via nutrient stimulation of the GI tract, possibly via paracrine action of gastric inhibitory peptide or CCK (19)or via direct interaction between the myenteric plexus and the pancreas (405). Insulin can have an indirect effect on gastric emptying via regulation of the blood glucose levels. Gastric emptying in diabetic patients tends to be slower, an effect that is widely contributed to the elevated plasma glucose levels (310;367;412), but may also be influenced by peripheral autonomic neuropathy (186).Gastric emptying rates in early non-insulin dependent diabetics may be faster or slower (173); also early streptozotocin-induced in rats appears to stimulate gastric emptying, an effect that is reversed by insulin infusion (301). Insulin may also have a direct effect on GI motility: hyperinsulinaemia under euglycaemic conditions also appears to slow gastric emptying (191).

Amylin (islet amyloid polipeptide) is generally co-secreted with insulin by the β-cells of the pancreas, although under certain conditions changes may occur in the insulin/amylin ratio (226). Only recently it has been recognized as a very potent inhibitor of gastric emptying at physiological concentrations (55;192;278;458-460), although hypoglycemia (which itself accelerates gastric emptying) appears to block this effect (117). Spontaneously diabetic BB/Wistar rats are amylin-deficient and have rapid gastric emptying; amylin inhibits the emptying rate in a dose-dependent manner (459). Amylin also suppresses glucagon release; it is now thought that one of its main roles may be the regulation of glycemia via its effect on gastrointestinal motility (232). The mechanisms of its action have not been established. There is no evidence for an action directly on the

stomach: gastric amylin receptors have not been demonstrated. Part of its action seems to be mediated by the vagal nerve: after a vagotomy-induced inhibition of gastric emptying no further reduction in emptying rate could be evoked by amylin injections (232). Amylin receptors in the brain have been demonstrated in the area postrema (22); since the effects of vagotomy suggest that amylin does not have a major effect via the general circulation, it is likely that the inhibition of gastric emptying is mediated via an interaction with the basic regulatory circuits in the brainstem. A hypothetical feedback-regulation of its action in combination with the release of the incretins glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP), as well as monitoring of the blood glucose level by the CNS, has also been proposed (457).

Secretin, synthesized in S cells in the duodenum and jejunum and released by the presence of acid in the duodenum and proximal jejunum, has a primary role in stimulation of pancreatic bicarbonate secretion, but also may have a physiological role in inhibition of gastric emptying via relaxation of the fundus and inhibition of antral contractions, and stimulation of pyloric contractions (189;420;421;427); infusion of antibodies against secretin increase the rate of gastric emptying (172). Secretin release is independent from vagal activity.

Gastrin is synthesized by G cells in the pyloric glands, and to a lesser extent in the duodenum; it is released in response to the presence of peptides and certain amino acids in the gastric lumen (or proteins in the duodenum) or via vagal input on gastric ganglion cells, by a pH above 3. Its main effect is stimulation of the secretion of HCl; in pharmacological doses it can relax the fundus or, via stimulation of gastrin receptors on the smooth muscle of the distal stomach, increase the force and frequency of gastric peristaltic contractions (59;427).

Motilin stimulates gastric motor activity and gastric emptying, especially of liquids in

dogs and of glucose or solids in humans (51;52). Its mechanism of action is probably via a direct effect on receptors in the proximal and distal stomach. Peaks in plasma motilin concentration appear to coincide with phase III contractions of the MMC cycle: cause and effect are still awaiting clarification (427).

Peptide YY (PYY) is released by endocrine cells in the ileum and is released after a mixed meal, especially via the presence of fatty acids in the distal gut (12). It inhibits gastric and pancreatic secretion (4;307), possibly in synergy with neurotensin (17). Low doses of PYY inhibit gastric emptying; based on this effect, its presence in the ileum, and the increase in the rate of intestinal transit after immunoneutralization of PYY, it has been implicated in the "ileal brake" response (220;311). Large doses of PYY may also inhibit intestinal motility. It may exert its inhibitory effect on digestive function via direct inhibition of cholinergic vagal efferent neurons of the dorsal motor nucleus of the vagus (48;427).

A number of gastrointestinal peptides that appear to have some effect on gastrointestinal motility, especially in pharmacological doses, can be added to the list of potential regulators of gastric emptying, directly or via indirect effects. A clear physiological role in the regulation of gastric emptying still needs to be established for this group. Neurotensin is released by N cells in the intestinal mucosa, especially the ileum, as a reaction to the presence of fat in the duodenum or jejunum. It may act as a hormonal mediator, in combination with other peptides, to inhibit gastric acid secretion. It appears to inhibit gastric and duodenal motility and gastric emptying, and also can induce an intestinal fed motor pattern in fasted animals via a vagal effect (427). Somatostatin is released by D-cells in the mucosa throughout the intestinal tract and the pancreatic islets (and is also contained in nerve terminals and ganglion cells in the ENS, as well as in the central nervous system, as a neuropeptide). Presence of nutrients in the intestine stimulate its

release. Its main actions are inhibition of gastric acid and pepsin secretion and of gastrin, insulin and glucagon release. Also it appears to inhibit the generation of the interdigestive MMC cycles, and inhibits MMC initiation by motilin (427). GIP is released by the middle and lower gut in response to nutrient stimulation. It stimulates insulin release during hyperglycemia, can inhibit gastric acid secretion, and may inhibit gastric motility in the dog (427). Pancreatic polypeptide (PP) is located in pancreatic islets and F-cells, and is released as a reaction to (protein) meals. It inhibits pancreatic exocrine secretion and increases gastric and intestinal motility and gastric emptying, as well as increased rate of intestinal transit (427). Glucagon has strong metabolic effects, generally opposite to the actions of insulin, such as stimulation of glycogenolysis, and may indirectly influence gastrointestinal motility via post-absorptive feed-back signals. In pharmacological doses it can also inhibit intestinal motility (427). Enteroglucagons, including GLP-1, are released as a reaction to nutrient stimulation in the gut; GLP-1 potently stimulates insulin release (even in absence of hyperglycemia), inhibits glucagon release (even under hypoglycemic conditions) and can inhibit gastric emptying (427). Bombesin can inhibit gastric emptying in primates (363;364), but when peripherally administered its effectivity in the rat is controversial (171;362).

2.2.7 Gastric emptying during feeding

In a fed animal the above described post-prandial regulation of stomach emptying ensures that calories leave the stomach with a constant rate. However, this precise regulation seems absent during feeding (177;325): liquid food leaves the stomach at a much higher rate, more dependent on the volume of the meal than on the caloric value of the food. Emptying of a glucose meal therefore shows in that phase more similarity with emptying curves of a non-caloric load like saline (177). Furthermore, the rate of emptying

of liquids appears to be not only dependent on the intragastric volume (248-250;252), but also on the history of the meal: a closer examination of the data from ref (248) reveals that a single infusion of 150 ml empties faster from the stomach than the last 150 ml from a total infused volume of 300 ml.

A possible explanation for rapid emptying during a meal after an extended period of food deprivation could be that, early in the meal, inhibitory post-gastric signals may be reduced, and have to be generated first, before stomach emptying can slow down (30;252). The higher influx of nutrients into the duodenum will rapidly increase the nutrient concentration in the proximal intestines, and this may result in saturation of proximally located absorption sites and subsequent stimulation of receptors in more distal regions. The earlier (paragraph 2.2.1) described imprecise regulation of intragastric pressure during a meal may be involved here: rapid intake of liquids can cause a transient increase in intragastric pressure (223), and therefore of stomach emptying. Direct intragastric infusion of the meal could even exacerbate this increase in gastric pressure by bypassing the oesophagus, so that the gastric receptive reflex would not be induced. During the meal or infusion the increased emptying rate thus would cause extra delivery of nutrients into the duodenum (177;248;252), and in this phase stomach emptying would be directly correlated with the rate of ingestion rather than with calories delivered to the intestines. Inhibitory feed-back signals should be active soon after the start of the meal: within a few minutes glucose is already being absorbed into the bloodstream (391) and therefore must be stimulating intestinal receptors. Also at some point the increase in intragastric pressure will activate the adaptive relaxation reflex: after gastric branch vagotomy this reflex is impaired, and the emptying rate during glucose infusion is further elevated (176). Adaptive relaxation would, however, only prevent the intragastric pressure from rising further above an already elevated level that would still cause accelerated

emptying of liquids. Also this would only counteract a rise in intragastric pressure for a finite infused volume, due to the physical limits on gastric expansion. This may partly explain the fact that a high emptying rate is maintained during the full course of a glucose infusion (177). Therefore, interpretative problems remain if the meal is directly instilled into the stomach, especially so when the infusion rate is high and the total infused volume exceeds the maximum gastric volume by a wide margin.

2.2.8 Circadian rhythms and gastric emptying

A few studies indicate that over the full 24-hour day, under restricted feeding conditions, stomach emptying may show circadian variation (123;415;419), suggesting that even in postprandial periods the regulation of delivery of calories to the intestines may not take place at a completely fixed rate. Furthermore, nutrient uptake from the intestines and subsequent feed-back signals from post-absorptive sources may not be a constant factor. Some studies suggest the existence of daily rhythms in the small intestines, e.g. for motility (180;181;201) or enzyme activity (344-349;396); no clear evidence exists for changes in villus height (116). Also intestinal blood flow increases during the cephalic phase of a meal and remains elevated for extended periods; the effects on intestinal nutrient absorption are not completely clear, but probably limited, since only the absorption of highly diffusible substances appears to be strongly blood flow-dependent (124).

Light stimulation can increase sympathetic and decrease parasympathetic outflow, via activation of the retino-hypothalamic tract and subsequent involvement of the suprachiasmatic nucleus. Lesioning of the SCN abolishes this effect (298;299). Modulation of efferent neural connections to the GI tract by the SCN could impose circadian rhythmicity on gastrointestinal function: e.g. this could imply that over the day

fluctuations may occur in the influx of nutrients into the bloodstream and in the amount of nutrients remaining in the intestines, thus potentially leading to a variable exposure of intestinal receptors and a variable inhibitory feed-back from intestinal or post-absorptive sources.

2.3 Factors modulating food intake

The fact that there must be some direct correlation between long-term food intake and rate of gastric emptying could be considered trivial: since the capacity of the stomach for food is limited, logically any amount of food that is ingested will need to be emptied at some point in time to make room for freshly arriving meals. Measured over longer periods the average rate of gastric emptying will need to match the average food intake over that period. However, on a meal-to-meal basis this direct correlation is not necessarily maintained; the following pages will give an overview of some of the factors that can modulate food intake and explore some of the correlations with gastric emptying.

2.3.1 Behavioral factors

Food intake consists of a repeated sequence of meal initiation, feeding (although not necessarily with a fixed rate of intake) and meal termination, followed by an inter-meal interval. Each of these stages can be regulated according to the present state and needs of the animal. Regulation of meal size and meal intervals over the day will eventually determine total daily food intake. Regulation of average daily food intake over extended periods will, together with the average daily energy expenditure over that period, eventually determine body weight gain.

Feeding behavior in general can be modulated on several psychological and physiological levels. First of all it has to be established via specific tests that an animal did

not develop an aversive reaction to the treatment, causing it to stop eating prematurely. Also the behavior of the animal after a meal can be monitored for the occurrence of the "behavioral satiety sequence (140) characterized by eating, grooming and sleeping in the post-ingestive period. It is therefore important to discriminate between satiety and an absence of the motivation to eat. Food intake is a behavior, and is not regulated by physiological mechanisms alone: there are continuously interactions with other behavioral systems and the outcome of this conflict can be a shift in priorities that will determine whether food intake will occur at all and/or to what extent; higher brain functions can modulate the satiating effects of food and/or body weight set points, according to the animals environment, its internal biological rhythms and the time of year (137). An animal therefore needs to bring its behavior in balance with its environment.

Gastric emptying can also be influenced by environmental and psychological factors: stress or activation of the sympathetic nervous system inhibits gastric emptying. A number of recent studies show that gastric emptying of caloric meals is delayed in anorexia nervosa, possibly creating prolonged satiety after a meal (84;86;90;151;164;244;331;332;408): a negative correlation was found between body weight and emptying rate (408). Treatment of the eating disorder increases the rate of gastric emptying to normal values (86;331;408); treatment of the motility disorder improved the psychiatric symptoms (90;355). A food deprivation of a few days inhibits gastric emptying (61); also a restricted feeding schedule slows down gastric emptying (333). These observations are interesting, because a down-regulation of gastric emptying in a state of starvation, although useful as a survival strategy, would be the opposite to what would be expected if homeostatic regulation of body weight and/or total body energy contents would be the main regulatory mechanism.

Another obvious example that leads to a dramatic shift in feeding patterns is the light

dark cycle: a nocturnal animal like the rat is inactive during most of the light phase; their activity, including feeding, is concentrated in the dark period. The sleep-wake cycle forces the animal to fulfil most of the energy requirements for the full 24-hour day within a 12-hour period. In its inactive phase it will have to rely on its internal energy stores, created via overeating in the dark. This implies that in the dark phase the satiating effect of food could be modulated by the circadian system, causing the animals to eat more than its actual energy needs in that phase. This is especially of importance toward the end of the night when rats tend to eat a few more meals, in spite of the fact that the stomach is already well-filled, supposedly as a preparation for the light phase (398;401). Alternatively, learning and memory could be involved in this phase, so that an animal learns to ignore satiety signals at certain times of day. Recent evidence shows that the amygdala is directly involved in the regulation of and metabolic response to feeding (62;114;121;138;221;222;265;336). However, although rats do not learn to anticipate a daily food deprivation period during the last two hours of the night, increasing their caloric intake in the light phase instead, they do immediately respond to a shortening of the dark period by adapting their meal pattern (185). Under normal situations rats appear to adapt to periods of greater energy expenditure by increasing their food intake during the dark period only, up to the point where stomach capacity may become a limiting factor (400).

Circadian rhythms allow an animal to display "feed-forward" behavior (based on the predictability of a cyclic changing environment) and replace reactive homeostatic regulation by predictive homeostasis (267) or rheostasis (279). Diurnal endogenous rhythms, that include daily variations in many hormone and receptor levels (174;279;281;445), neural responsiveness in specific nuclei in the CNS such as the lateral hypothalamus (365) as well as activity and feeding rhythms, are controlled by the

hypothalamic suprachiasmatic nucleus, which is located close to the optic chiasma and is synchronized with the environment mainly via the light-dark cycle. Activity rhythms are not primarily driven by an aversion to light, as can be illustrated by the fact that for the major part these rhythms stay intact when the animal is kept under a "skeleton photo period" with only two brief periods of light around dusk and dawn (403), or "free-running" in absolute darkness. Lesioning of the SCN of rats leads to an immediate and dramatic change in the activity rhythms and meal pattern. Although the total daily caloric intake remains the same, the typical concentration of feeding in the night period disappears. The meals are more evenly distributed over the full 24 hour period, with intermittent bouts of sleep. Some authors find smaller, more frequent meals (394), others find no difference (397). It can be hypothesized that SCN-lesioned rats display a more directly homeostasis-based regulation of their meal pattern, compared to intact animals.

2.3.2 Pre-gastric factors

After food intake has been initiated, satiety signals can be generated from various pre-absorptive and post-absorptive sites. Passage of food through the oropharyngeal and oesophageal area can give the animal only a crude impression of the quality and caloric content of the ingested meal, especially if it eats from a novel food source. Still, smell, taste and texture of the meal can influence the amount of food ingested: an animal will eat less from unpalatable food and more from attractive food (206). Giving a choice out of different, palatable food sources ("cafeteria diet") an animal will increase food intake via an increase in meal size: the hedonic aspects can modulate the satiating effects of food, and the animal gains body weight faster than normal. Pre-gastric sources in general do not seem to play an essential role in the control of food intake: animals that where equipped with oesophageal or gastric fistulas will grossly overeat during sham feeding, when food is

prevented from reaching resp. staying in the stomach (64;64;168;266;461;461). The fact that on consecutive tests rats increase their food intake until a plateau level is reached suggests a learning component may be involved in feeding behavior when animals are kept on standard diets(65). Nonetheless, pregastric stimuli do contribute specific signals, in some recent studies expressed as c-fos induction in the rat brain stem (102), that could be integrated in the CNS together with signals from other sources. Sham-feeding can be inhibited only when nutrients are allowed to stimulate the stomach (199), during infusion with CCK (198), or when simultaneously nutrients are infused into the small intestines (31;118;126;127;309;327). The latter response requires an intact vagus nerve to be fully expressed (463). The gastric fistula does not completely prevent some mechanical or even chemical (78) stimulation of the stomach, however, and interpretation of these experiments can even be more complicated when food is not actively prevented from entering the intestines (378). Little attention has been payed in the literature to the question whether sham-feeding can also be effectively suppressed by iv infusion of nutrients (128).

2.3.3 Gastric factors

The stomach has always been one of the most obvious candidates for a role in the generation of satiety signals. Since the major part of a meal is first temporarily stored in the stomach, this would put the stomach in an ideal position to monitor the amount of food ingested in an ongoing meal. The degree of stomach filling could be the determining factor for food intake regulation: satiety signals that terminate a meal would originating from in-series tension receptors in the stomach wall, measuring stomach distention (68;304;382). The dynamics of stomach filling (by ingestion of food) and stomach emptying together would then regulate food intake. Regulation of daily food intake could

be accomplished by regulation of stomach emptying (250): stomach filling below a certain threshold would allow a new meal to be started. Meal size would be regulated via inhibition of feeding behavior after a certain upper threshold of stomach distention has been reached. This model thus can explain both meal initiation and termination by gastric signals and, when combined with intestinal (370;371) and post-absorptive (303) feed-back regulation of stomach emptying, would also allow a decisive role of gastric signals in the regulation of meal intervals and in regulation of daily and long-term food intake.

A number of requirements for a fully operative system, regulating food intake of omnivorous meal-eating animals like the rat, thus seem to be realized in stomach distention-induced satiety. From a behavioral point of view it would be beneficial to the animal if the short-term satiety signal that inhibits further food intake would be generated before the stomach is completely filled to maximum capacity (although such satiety signals can be overruled by other factors that tend to increase meal size, such as partial food deprivation, availability of highly attractive food, etc.). This would increase the chance that the animal takes smaller amounts from different food sources over the whole day (rather than feeding one or two maximum sized meals from a single source), thus increasing the chance that it fulfils its complete nutrient requirements (esp. proteins and minerals). Also, this would allow the animal a higher degree of mobility in the beginning of its active phase. A decrease over the day in the satiating effect of a certain amount of food could be functional in the survival strategy of the animal, allowing it to fill the stomach up to maximum capacity at the end of its active phase (401). That implies that a certain degree of stomach distention that acts as an inhibitory signal for feeding behavior at the beginning of the active phase should not be satiating at a later time point; this could be accomplished by a slow adaptation of stretch receptors in the stomach wall, or by modulatory influences of the circadian system leading to a daily rhythm in the effect of

these signals (401).

Nutrient sensitivity has been proposed for the stomach (80). However, although a small amount of glucose may be absorbed from the stomach, no clear evidence exists for a role of gastric chemoreceptors in satiety. For a functional system regulating food intake would be sufficient calories were being sensed or measured in the intestines. After a non-nutritive meal the stomach contents would be emptied fast since inhibitory feed-back signals from the intestines would be absent, and feeding behavior would not be inhibited for extended periods after such a meal. The temporary satiety caused by the distended stomach would lead to a short cessation of the meal, and could give the animal time to find another food source before feeding behavior could occur again. Contrary, a highly nutritive meal would be emptied slowly, thereby causing a prolonged distention of the stomach and a prolonged feeling of satiety. Adaptation to a change in caloric density within a single meal would not be possible in such a regulatory system; a considerable amount of stomach contents would have to be emptied first. However, a more precise and direct assessment of the quality of the incoming food would require immediate measurement of its caloric value by post-gastric sources. Supporting evidence for such a mechanism could be the fact that the rate of gastric emptying is increased during feeding (177;422).

2.3.4 Control of food intake: metabolic explanations

Many theories have been formulated about the exact nature of signals and mechanisms that determine food intake behavior. It is important here to discriminate between satiety and the motivation to eat. Food intake is a behavior, and is not regulated by physiological mechanisms alone: interaction with other (also behavioral) systems continuously takes place, and the outcome of this conflict determines in the first place whether food intake will occur or not. Examples here are the fact that choice out of different, palatable food

sources ("cafeteria diet") increases food intake, via an increase in meal size : the hedonic aspects can partly modulate the satiating effects of food, and the animal gains body weight faster than normal.

A number of theories assumes that post-absorptive mechanisms secure a constant supply of specific nutrients (or also minerals, vitamins, etc.) to specific organs: a decrease in availability would then induce food intake (163;284). Generally these theories are based on the concept of homeostasis by negative feed-back: the animal tries to maintain the internal level (i.e. in blood, brain or organs) of the regulated substance between certain limits. Regulation of circulating levels of all separate macronutrients is hypothesized, leading to the glucostatic(241), the lipostatic (184) or the aminostatic theory (256) or variations on these.

In vertebrates the main energy source for the central nervous system is glucose. Since severe hypoglycemia could cause irreparable damage to brain tissue, maintenance of a continuous flow of glucose to the brain is of highest importance for the organism. The glucostatic theory hypothesizes that decrease in glucose utilization or decreased intracellular glucose (rather than the absolute glucose level itself) is detected by gluco-sensitive sites in the brain and induces feeding. This would account for meal initiation after a period of starvation, but would fail to give an explanation for the occurrence of meal patterns as they are observed in free feeding rats, or for the effects of insulin injections on blood glucose level and feeding (392). The mechanism could be considered to be involved in an emergency response under extreme circumstances, rather than in the regulation of feeding under normal circumstances (455).

A more recent model hypothesizes that a comparison between arterial and venous blood glucose levels is being made: a large difference would be a sign for high use in the tissues and would inhibit food intake. The advantage of this model is, that the eventual signal to

the CNS is an integration of both nutrient utilization and nutrient delivery to the blood stream (resulting from previous intake). It not only explains initiation of single meals, but also could describe daily intake. A related theory states that feeding is induced by a well defined, transient decline in the blood glucose level (39-41;207;209;225;233). This model only demonstrates a correlation between blood glucose levels and meal initiation; without a further definition of a regulatory mechanism that temporarily decreases the plasma glucose level, this model can not fully account for the further aspects of food intake.

Under normal circumstances (i.e. except in a situation of severe starvation) energy is available for extended periods in the form of fat stores; also glucose (after exhaustion of internal glycogen stores) can be synthesized from amino acids (gluconeogenesis). That means that diet intake of carbohydrate and fat is not essential for the short-term survival of the organism. A minimum amount of protein intake, however, remains necessary. This would make it feasible that some mechanism would exist that keeps track of the amino acid balance in the body. It is noteworthy that intravenous amino acid infusion leads to a more precise compensatory reduction in caloric intake than the other macronutrients (425;426).

Other models for food intake regulation have been proposed that are based on the assumption that the supply of utilizable metabolic fuels can be regulated by the adipose tissue (110). This model is quite similar to a model proposed by Le Magnen for regulation of food intake over the day (207), but reverses his suggestion for a cause-effect relationship by stating that in periods of lipogenesis, food intake will occur, where in a situation of lipolysis food intake will be inhibited. The liver is proposed to be involved in the monitoring of the impact of adipose tissue in the control of food intake, by changing its rate of oxidation of lipid fuels (110).

None of the theories described above that are entirely based on post-absorptive events

appear to give a fully satisfactory explanation for the regulation of short- and long term food intake (i.e. regulation of daily meal patterns and of body weight) simultaneously. Even if a theory can successfully explain meal initiation, the regulation of meal size is generally not well described. Since the ingested food would not be completely digested and absorbed before a meal is terminated, it is unlikely that a mechanism regulating food intake based on post-absorptive events alone would be able to assess the exact caloric content of that meal. Meal size would be only dependent on the time between the initiation of the meal and the delivery of a sufficient amount of nutrients to the bloodstream to raise the level of the regulated factor. Also it would be difficult to explain why a new meal can be started before the gastrointestinal tract has emptied most of its contents. Another argument against food intake regulation based on levels of circulating nutrients alone would be, that infusion of these three types of nutrients in physiological quantities directly into the bloodstream can result in a partial compensation (425), but usually fails to suppress intake completely.

A theory that tried to link all macronutrient changes is the thermostatic theory (32). This model proposed that the generation of heat by metabolic fuels (or by diet-induced thermogenesis) stimulates or inhibits food intake, according to the body's need to maintain a constant temperature. Some evidence exists that the liver may be involved in the generation of a satiety signal based on the measurement of hepatic temperature (56;73). Body and liver temperature would rise during a meal; if the liver temperature would exceed a certain threshold a satiety signal would be generated that would terminate the meal. After termination of a meal, a lower threshold should be reached first before a next meal could be started. Although relevant in preventing temperatures outside the optimal range for liver enzymes, evidence for a physiological role in the regulation of food intake is still weak. Increased meal size during hepatic cooling could be considered as stronger

proof. This mechanism explains regulation of meal size only.

Hepatic metabolism is also mentioned as a possible regulator of food intake (112;341;342). The present theory states that satiety would be the result of portal hyperglycemia, inducing a decreased firing rate of portal and hepatic glucoreceptors with vagal afferents. This theory is attractive since all metabolic fluxes in the body converge on the liver, but can not explain a number of observations, like the absence of effect of vagotomy on meal patterns, or the absence of an increase in food intake after a portocaval shunt (188;193). More recently it has been shown that metabolic events in the liver may influence food intake, but not directly via measurement of glucose in the portal vein (413). Similar to the proposed mechanism for the glucostatic theory, in this model a difference in hepatic portal and arterial blood glucose concentration may be the crucial factor for the generation of a satiety signal. This control mechanism can not be responsible for the complete regulation of food intake: meal initiation could be regulated on hepatic level, but intermeal intervals and meal patterns in the presence of food in the GI tract are not explained.

2.3.5 Neural factors

Higher brain functions are directly involved in the regulation of food intake. Especially the hypothalamic areas have been implicated in the regulatory circuits: studies showed that animals became hyperphagic after lesioning of the ventromedial hypothalamus (VMH), and hypophagic after lesioning of the lateral hypothalamus (LH). The idea of a dual regulatory system by the VMH regulating satiety and the LH regulating hunger has since been abandoned, in favor of explanations that are based on the neural connections between other nuclei that run through these hypothalamic centers; the hypothalamus is now believed to be involved in the integration of a wide variety of information about the

nutritional state of the animal, as well as in the expression of this information via direct control of peripheral mechanisms or via modulation of other efferent neural signals.

2.3.6 Hormonal factors

A popular recent hypothesis states that insulin is an integrating factor in the measurement of the body's energy stores (452-455). Insulin in the CNS reflects (with some time delay) changes in blood plasma insulin concentration, and insulin-sensitive neurons in the CNS would thus be able to monitor metabolic events and nutrient availability in the body. An increase in insulin would then generate a satiety signal, a decrease would be a hunger signal. This model is attractive because of its integrating power: insulin could be involved in body weight regulation: increases in body fat stores would elevate the level of circulating insulin, and thus induce satiety signals in the CNS, and a concomitant inhibition of food intake until the plasma insulin concentration has diminished. It is not clear whether in this model insulin could be involved in the regulation of meal patterns: a satiety signal inducing meal termination would require a detectable rise of insulin levels in the CNS during the meal. Some apparent contradictions with experimental data still need to be resolved: continuous insulin infusion in diabetic rats leads to an increase in food intake (441;442).

Recently a role for body fat stores in the control of food intake and body weight development has become more likely with the identification of OB protein or leptin: a protein that inhibits food intake and increases energy expenditure, and is released into the bloodstream by adipose tissue (21;95;156;205;338;343;386;430;432) as well as the stomach (18), and reaches elevated levels in the obese. Leptin is believed to communicate the state of the body's energy reserves to the CNS (432).

CCK release appears to inhibit short-term food intake, with a synergistic effect with

stomach distention (370;371;376). It has been demonstrated that both CCK and stomach distention have their effect via vagal pathways (99). This would suggest that a major signal for food intake regulation could originate from the stomach, but that it requires the synergistic effect of simultaneous CCK-release to be fully expressed. In a free-feeding situation animals receiving exogenous CCK in association with each meal suppress meal size but maintain daily food intake by increasing the number of meals (60;437). This would be concomitant with a potentiating effect of CCK in the perception of gastric distension as a satiety signal: with CCK infusion a meal would induce satiety at a lower volume, causing a general reduction of meal size. Since this smaller increase in intragastric volume would be reduced sooner via continuing gastric emptying, the satiating effect would wane quickly and a new meal could be taken sooner.

A number of contradictory results concerning the required doses of CCK to exert an effect on food intake (58) could be explained by the recently discovered synergistic interaction with amylin. Since in a normal, free-feeding paradigm a mixed meal would induce release of insulin and amylin via an early cephalic response and (soon after the start of the meal) an increase in plasma glucose levels, and CCK would be simultaneously released via protein or fatty acid stimulation of the upper gut, the effects in these experimental conditions could be expected to be optimal for the generation of satiety signals. In sham-feeding paradigms, only a small amount of amylin would be released via the cephalic response, and its potentiating action on CCK-induced satiety would be absent. Whereas amylin is as potent as CCK in suppressing food intake (45;228;230;275-277), when combined they have found to be about twenty-fold more potent in inhibiting food intake than when used separately (458).

2.4 Nuclear imaging

2.4.1 Basic physics of nuclear medicine

Radioactivity occurs as the result of the spontaneous disintegration of the nucleus of a radioactive atom. The elemental identity of an atom is established by the number of protons in its nucleus, called its atomic number. Different isotopes of the same element are characterized by different numbers of neutrons in the nucleus and have therefore different atomic mass. Furthermore, a given nucleus can exist in different quantum states, each with its own, exactly defined energy level. A number of isotopes have stable configurations and are not radioactive. The unstable, radioactive elements transform into more stable configurations by shedding excess protons or neutrons or via emission of electromagnetic radiation. The difference in mass between initial and final nucleus can be expressed as an energy difference (according to Einstein's equation $E=mc^2$) and this energy, driving the decay, is released in the form of electromagnetic radiation and/or energetic decay particles. Radioactive decay is essentially a random, statistical process: it cannot be determined when a particular atom will undergo decay. However, each specific isotope decays with a specific rate, typical for that isotope. This rate is usually expressed as the half-life of that isotope: the time required for 50% of the nuclei in a sample of radioactive atoms to disintegrate (46;204).

Radioisotopes decay via three main processes: alpha (α), beta (β) and gamma (γ) decay.

1. Alpha decay is the emission of helium nuclei. All created α particles will have the same energy, in the range of 3 to 10 MeV. Alpha emitters are still used in therapy; their use in research and nuclear diagnostics is limited.

2. Beta decay involves nuclei emitting electrons or positrons, generally with energies in the range of 0.1-2 MeV, and can be subdivided in three different processes:

a. Electron emission (β^-) is the most common type of β decay and involves the conversion of a neutron to a proton, with the emission of an electron and an anti-neutrino (a neutral, nearly weightless particle that was first postulated by Pauli to account for the fact that not all emitted electrons have the same energy). The electrons can have a range of energies from 0 eV to the maximum available energy, the remainder of the energy is transferred on to the neutrino.

b. Positron emission(β+): this occurs when a proton is converted to a neutron with the emission of a positron and a neutrino. This type of decay only occurs when there is an energy difference of 1.02 MeV between the parent and daughter nucleus. Again, the emitted positron and the neutrino share the available energy; the positrons have a range of energy from 0 eV to the maximum energy level.

c. Electron capture: here no charged particles are emitted. In this type of decay, the nucleus captures one of its own atomic electrons, and in this process a proton is converted to a neutron and a neutrino is emitted. It occurs when the decay energy is less than 1.02 MeV; when the decay energy is greater than 1.02 MeV, both electron capture and positron emission may occur.

3. Gamma decay is the emission of short wave electromagnetic radiation (with an energy level characteristic for the isomer) when a nucleus rearranges to a more stable, lower energy state. No changes occur in the number of neutrons or protons. For example, Technetium-99m, one of the most commonly used radioisotopes, is a metastable isomer of Tc-99, with an exited energy level of 0.142 MeV and a half-life of 6.02 hr. Decay proceeds primarily to an intermediate isomer with an energy level of 0.140 MeV, immediately followed by further decay to the ground state Tc-99 (which in itself is a radioisotope with a half-life of 212,000 years); the last transition results in the typical emission of a γ-ray of 140 keV (46;203;204).

^{99m}Tc is produced as a daughter isotope via radioactive decay of its parent, Mo-99, an isotope with a halflife of 67 hr that is produced in a nuclear reactor from Mo-98. In a radionuclide generator containing Mo-99, a progressive fraction of the activity will be contributed to ^{99m}Tc, until at some point in time (in this case about 24 hr) an equilibrium is established and the ^{99m}Tc content is at its maximum. Contrary to its parent MoO_4, ^{99m}Tc is not bound to an aluminum (Al_2O_3) column, and can be separated from the parent by eluting it from the column with 2-25 ml of normal saline (46;204). Another radioactive marker, that is commonly used in dual-isotope studies conjointly with ^{99m}Tc, is Indium-111 (^{111}In). This is a medium-level gamma emitter with a half-life of 67.4 hr and two major radiations at energy levels of 0.172, resp. 0.247 keV emitted during its decay. ^{111}In is produced in an accelerator or cyclotron from a ^{111}Cd source.

Gamma-rays easily interact with matter; there are two main interactions which are important for detection of gamma-rays by a scintillation crystal and thus for nuclear imaging:

1. the photoelectric effect, when the photon interacts with an inner-shell electron from an atom and gives up all its energy. The γ-ray then ceases to exist. In result, the electron is ejected and can transfer its energy to other electrons causing further (secondary) ionization events. An electron from a higher shell may fill the created vacancy, thereby producing an X-ray.

2. Compton scattering, when a gamma photon collides with an electron from a higher shell. Here not all energy is transferred fully; the ejected electron can again cause secondary ionization and the scattered photon, now with decreased energy, will be deflected in a different direction (46;203;204).

The probability of a photoelectric interaction increases roughly with the third power of the atomic number. This means that γ-rays have a low photoelectric interaction with body

tissue, which has a low average atomic number. Lead, with a high atomic number, therefore is a good shielding material for γ-rays. Sodium iodine has an intermediate atomic number, but still high enough to give it suitable properties for use as a detector of γ-rays. Compton interactions take place practically independent of atomic number; therefore this is the predominant interaction with body tissue (203;204).

2.4.2 Nuclear medicine imaging: the Anger scintillation camera

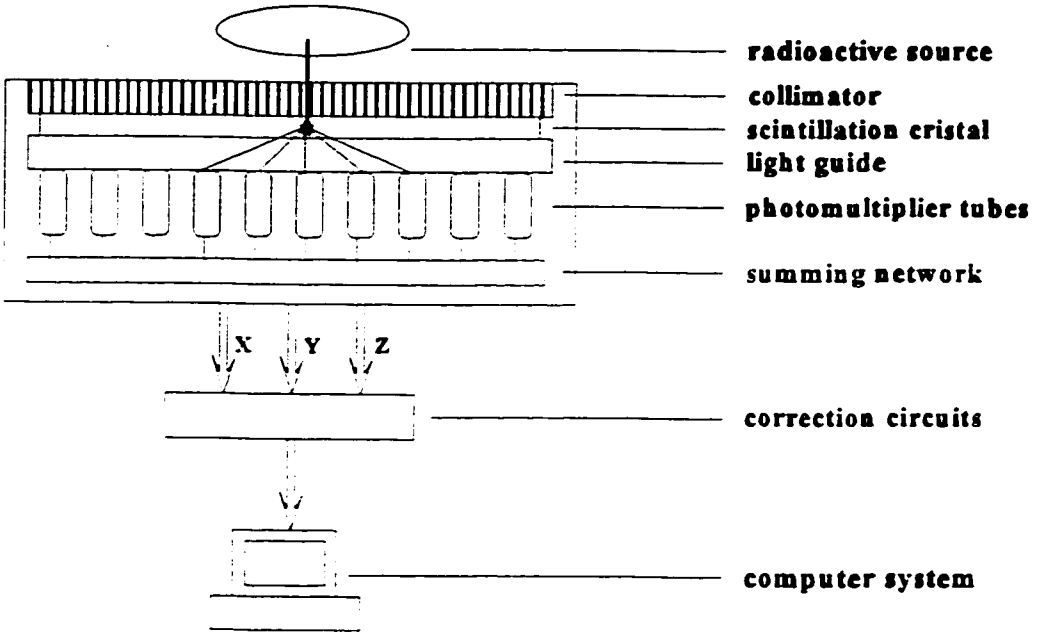


Fig. 2.1 Schematic diagram of a scintillation camera (after {Muehllehner 1988 ID: 30416}).

The Anger scintillation camera (see Fig. 2.1) is designed to create two-dimensional images of the distribution of a gamma emitter in vivo. Since its first introduction in 1958 by Anger at the University of California, the instrument has undergone considerable improvement and is now especially well suited for imaging the typical 140 keV γ-rays

produced by ^{99m}Tc. To create an image, gamma rays that are produced by the source under investigation have to be intercepted and counted, have their energy level measured (allowing incoming radiation with undesirable energy levels to be filtered out) and have spacial coordinates assigned to their point of origin. This is accomplished via scintillation detection of the γ-rays, combined with highly sophisticated analog and digital circuitry and specialized computer software (280).

A collimator is placed between the source and the scintillation detector in order to relate the sites of photon absorption in the crystal to their origin in the source. The most commonly used type, the parallel-hole collimator. This consists of small holes drilled or cast in lead or shaped from lead foils. The lead walls between the holes are called collimator septa. Only gamma rays that travel parallel to the collimator holes will pass the collimator and will be able to interact with the scintillation crystal; all other photons will be absorbed in the collimator's septa. The image created by the parallel gamma rays that have passed the collimator is therefore of the same size as the original radioactive source. Collimators with thicker septa are applied when high-energy radionuclides are used, in order to minimize septal penetration (416).

Scintillation detectors convert the energy of a γ (or β)-ray into a flash of visible light, which then can be converted to an electrical signal and further analyzed. Most gamma cameras are equipped with a large (up to 60 cm in diameter) thallium-activated sodium iodide (NaI(Tl)) crystal. Incoming γ radiation interacts with the crystal via the photoelectric effect or via Compton scattering, so that energy is transferred from the γ-ray via excitation of electrons. The electrons that have left their orbit move through the crystal and use their kinetic energy to excite other electrons in their path. The added thallium atoms in the crystal serve as an "electron trap" where some of the excited electrons lose their excess energy through the release of a light photon; in a pure NaI crystal practically

all of the energy of the γ-rays would be transformed into heat. The mean number of light photons, each with an energy of approximately 3 eV, created by an incoming γ-ray (i.e. the brightness of the light flash produced by the crystal) is approximately proportional to the energy of the γ-ray. The light is emitted from the crystal with a decay time of 240 nsec. The crystal itself is highly transparent for light photons of this energy and the light signal is then passed through a light guide that transmits the light to an array of photo multiplier tubes (PMTs). Photons that are moving towards the space between PMTs are redirected by the light guide to the nearest PMT, thus increasing the efficiency of the camera (302). NaI(Tl) crystals are commonly used in nuclear imaging, because of their high efficiency (relatively high absorption of incoming γ-photons and high amount of light produced per unit of absorbed energy) and short phosphorescent decay time, leading to a low value for its "dead time" (the time that the crystal is unresponsive for newly arriving γ-ray) of only 0.25 μsec.

The crystal receives γ-radiation from three different sources: primary photons, pre-detector scattered photons and background radiation. The primary photons are the γ-rays that have reached the detector directly and in a straight line from their point of origin and will therefore have the specific energy level that is characteristic for the radionuclide in use. Others will have undergone Compton scattering, which means that their energy level will be diminished and their path deflected from the original course. Background radiation can be due to cosmic rays or other radioactive substances in the direct vicinity of the detector, and may have any energy or direction, depending on their origin. This implies that only primary photons give reliable information about their origin; to a large extent they can be singled out based on their energy level. However, a certain amount of noise in the data is generated by incoming background radiation with energy near the primary photons, or by Compton-scattered photons that lost little of their energy. Also the

interactions between the incoming γ-rays and the crystal are a source of noise: first of all not all γ-rays will lose their energy completely to the crystal. Some will go undetected by the detector by passing through without any interaction. Others will escape after one or more Compton interactions, only transferring part of their energy to the crystal. In this case a lower energy level gets assigned to the γ-ray other than its actual value and the origin of the γ-ray is not recognized accurately. In a crystal of sufficient thickness, however, most γ-rays will lose all of their energy to the crystal via a photoelectric interaction, immediately or after some initial Compton interactions. Thicker crystals have better absorption of γ-rays, but also have increased Compton scatter and a greater tendency to diffuse the light that is used for detection (302).

The light emitted after a scintillation event spreads in all directions; at the back of the crystal it is transmitted by a light guide to an array of PMTs. Each scintillation event is registered by several PMTs, with the PMTs closer to the event receiving more light. Each individual PMT converts the light signal into an electronic signal, proportional to the amount of incident light, that can be amplified and further analyzed. The amplified electrical signals are first processed in a summation network that calculates the X,Y spatial coordinates of the source of the radiation (based on the response of the array of PMTs), and are also summed to form a separate energy signal, Z, that contains the information about the total energy of the incoming γ-ray; the Z signal is thus proportional to the energy that was deposited in the crystal by the γ-ray. The Z signal is subsequently processed in a pulse-height analyzer (PHA). This circuit serves as a filter: it classifies the incoming signals according to preset energy limits (a generally narrow "energy window"), set to include only the typical photopeak (or peaks) of the radionuclide in question. Events that do not fall into this energy window are rejected. Ideally the output signal from the PHA would include only primary photons, so that all Compton scattered photons and

background radiation would be rejected and accurate, high-resolution images would be generated. However, photopeaks, theoretically narrow bands, are generally measured by a gamma camera as more or less bell-shaped signals, partly due to the statistical character of the processes involved and partly due to imperfections in the electronic equipment, so that photons of the same energy level are producing pulses of varying height. In a practical setting therefore a compromise has to be found by setting the energy window sufficiently wide to "capture" a sufficient part of the photopeak and achieve adequate sensitivity of the camera. A wider energy window, however, also accepts more events (and assigns incorrect X,Y coordinates to them) that are caused by deflected γ-rays that still have most of their original energy, as well as any background radiation that reaches the crystal with an energy level similar to the radionuclide (280).

Since most scintillation cameras can provide 3 or 4 independent energy windows, the PHA can also be applied to discriminate between separate photopeaks in studies where two different radionuclides are used simultaneously, e.g. in gastric emptying studies where different radioactive markers are used to identify the emptying patterns of the solid as well as the liquid phase of a mixed meal. In this case partial absorption in the crystal of photons from the radionuclide with the higher energy can interfere with the photopeak of the emitter with the lower energy level. Generally dual isotope studies employ radionuclides that have their main photopeaks relatively far apart, thus minimizing the effects of "crosstalk" (or "downscatter") of one radioisotope on the other. By measuring the energy pulses in separate energy windows, each set for a narrow range surrounding the main photopeaks of the radionuclide in question, this spillover of the higher-energy spectrum into the lower-energy window (which can cause errors of up to 20 to 30%) can be reduced and, after application of a correction factor, reasonably accurate images can be distilled for the different markers separately (280;302).

The most commonly used radioactive marker in nuclear imaging is ^{99m}Tc, thanks to its excellent properties: its essential monoenergetic γ-rays of 142 keV are ideal for detection with NaI(Tl) scintillation crystals and it is a relatively "safe" radioisotope with a low penetration power and a short halflife of only 6.02 hours. Nowadays for gastric emptying studies a variety of different compounds is available that are manufactured via integration of radionuclides in a nonabsorbable complex, such as ^{99m}Tc sulphur colloid or Indium-111 diethylene triamine penta-acetic acid (^{111}In DTPA) (113;410;456;142).

2.4.3 Scintigraphic measurement of gastric emptying

Introduced by Griffith in 1966 (130) and further developed by Meyer (261) and Heading (143;142), scintigraphic measurement has become the method of choice for measurement of gastric emptying in a clinical setting. One of the main achievements of this technique is that it is possible to use normal, physiological meals and follow the time course of the emptying of liquids and solids separately and simultaneously by using specific markers. In comparison, one of the limitations of intubation techniques is that this only allows the use of liquid food, and for accurate results a substantial meal size is required. Via standardization of scintigraphic technique, comparison between different studies is possible.

Radionuclide studies are easy to perform, noninvasive, and quantitative, and they deliver relatively low radiation doses. Several basic points are critical for the accuracy of gastric emptying studies (49):

1. Radionuclide markers, whether liquid, semi-solid, or solid, must have a high labeling efficiency and be stable throughout the duration of the procedure.
2. Meal size and composition must be standardized for all studies.
3. A standard patient position and posture should be maintained during imaging.

4. Correction techniques should be applied when needed to compensate for radionuclide decay multiple radionuclide interference, geometry changes, septal penetration, and scatter from high-energy gamma rays.

Major advantages of radionuclide methods are that they do not interfere with the normal physiologic mechanisms in the stomach, they are simple and noninvasive and, therefore, highly acceptable' to patients, and they permit the simultaneous measurement of solid and liquid components of a meal. For the measurement of gastric emptying, radionuclide markers are incorporated into liquid, solid or mixed solid and liquid meals (15-24). The ideal radiopharmaceutical for the measurement of gastric emptying should be nontoxic and nonabsorbable and should not alter the osmolality of the contents of the stomach. In addition, it must be homogeneously distributed in the meal and have a particle size comparable with that of normal food, yet it must be tightly bound to prevent any interaction with other particles of food or the gastric mucosa.

All tests of emptying assume that the gastric emptying of a nuclide represents the behavior of the test meal. Since the liquid and solid phases of a mixed solid and liquid meal empty at different rates (13, 22), the precise identification of each phase is necessary for the accurate definition of the emptying of either phase or of the total meal. Liquid markers are often in a nonabsorbable chelated form, such as Tc-DTPA, or In-DTPA.

Typically, scintigraphic measurement of gastric emptying is performed as follows: a certain amount of a non-absorbable radioactive marker (or two different markers for studies that measure a combination of the emptying patterns of the solid, liquid or lipid phase of a mixed meal simultaneously) is incorporated into a meal and ingested by the subject. The decay process of the marker is continuously monitored with a gamma camera, interfaced with a computer. The image on the computer screen reflects the regions of the GI tract that contain the marker. The stomach can be defined on the display, according to

its shape and localization, and the total radioactivity

within the boundaries of the stomach's image is measured at regular intervals. The radioactivity is directly correlated with the amount of isotope and therefore with the amount of associated food. The activity in the stomach therefore correlates directly with the gastric retention of the labeled food or meal component, and prolonged measurement over time generates a gastric retention curve (Fig. 2.2). Based on the retention curve, the time that was required to empty 50 % of the meal can be calculated. This value is often used to assess emptying disorders or to determine the effect of an experimental treatment.

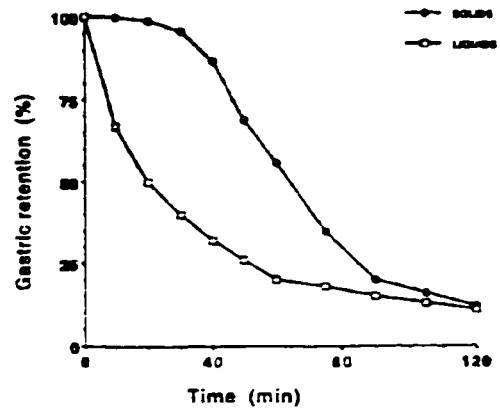


Fig. 2.2 Typical emptying pattern of the solid and liquid phase of a mixed meal.

Chapter 3: Material and Methods

To make comparison between different studies possible, the general setup was held constant throughout the experiments described in this dissertation. Specific modifications that were applied for certain studies will be outlined in a short method section in the description of each experiment separately; the basic methods were as follows:

3.1 Subjects

Parabiotic male Lewis rats (Sprague Dawley, Indianapolis), weighing 700-1050 gram per pair at the time of the various experiments were used. The animals were kept on a restricted feeding schedule with food access between 16.00 and 09.00, with lights off between 18:00 and 06:00. Tap water was available ad lib; in most circumstances, the water content of the liquid diet was sufficient for the needs of the rats, so that no significant additional water intake took place. The food deprivation occurred in a phase of the circadian cycle when the activity and food intake of rats is normally at a minimum (185;398;401;447). The rats were adapted for several weeks to the moderately restrained conditions that were used during the experiments. On non-experimental days, they were kept free-moving in larger home cages on a bedding of saw dust during the seven hours of food deprivation; this allowed time for grooming and undisturbed sleep during the middle of the light phase. The night before each of the experiments the animals had been mildly restricted in their food access to only 80 to 90 percent of their average diet intake of the previous three days. This restriction was done to prevent an unusually high food intake that could leave some unlabeled nutrients in their stomach at the start of a study and also might diminish their intake of labeled food during the gamma camera measurements.

Since gamma camera scintigraphy requires a stationary position of the subject for the full

duration of the measurement period, non-anaesthetized animals have to be restrained to limit their freedom of movement during the measurement period. We chose to work with parabiotic rats because single rats could easily twist and turn around in their cage, unless severe restraint was applied. Even with severe restraint, rotation around the longitudinal axis could hardly be prevented. For that reason parabiotic rats were used as the animal model in all the experiments for this dissertation. During the measurements the animals were kept in specially designed restraining cages. With this preparation the rats could be held in place without having to undergo extreme restriction of movement which could have interfered with gastrointestinal motility. The parabiotic union limited the animals' freedom of forward movement while also preventing them from rotating around their longitudinal axis during the experiment. The parabiotic union was also essential for measurements made on rats that had their intestines crossed

3.2 Restraining cage

The restraining cages that were designed for use in the experiments allowed the nearly continuous measurement of individual cumulative food intake as well as the distribution of the labeled diet within the gastrointestinal tract, while keeping the rats in a relatively stable position with respect to the gamma camera (Genesys, ADAC Laboratories, Milpitas, CA, USA). The cages consisted of two connected compartments of 51 x 5.5 x 7.5 cm (l,w,h) which were separated by a longitudinal barrier and had additional perforated barriers 24 cm from the front of the cage. These barriers restricted the backward movement of the animal while allowing space for the tail to stick through (see Fig. 3.1). A padded semicircular opening (diameter 5 cm) in between the longitudinal barrier allowed the connective tissue of the parabiosis to pass through the longitudinal barrier. A perforated lid on top of the cage which did not cover the front head region of the animals, kept the

animals in place and allowed circulation of air and body heat dissipation. During experiments the cages were placed on a small tray with a thin layer of saw dust; small openings in the bottom of the cage allowed urine and feces to be emptied from the animals' compartments into a tray underneath with a thin layer of saw dust.

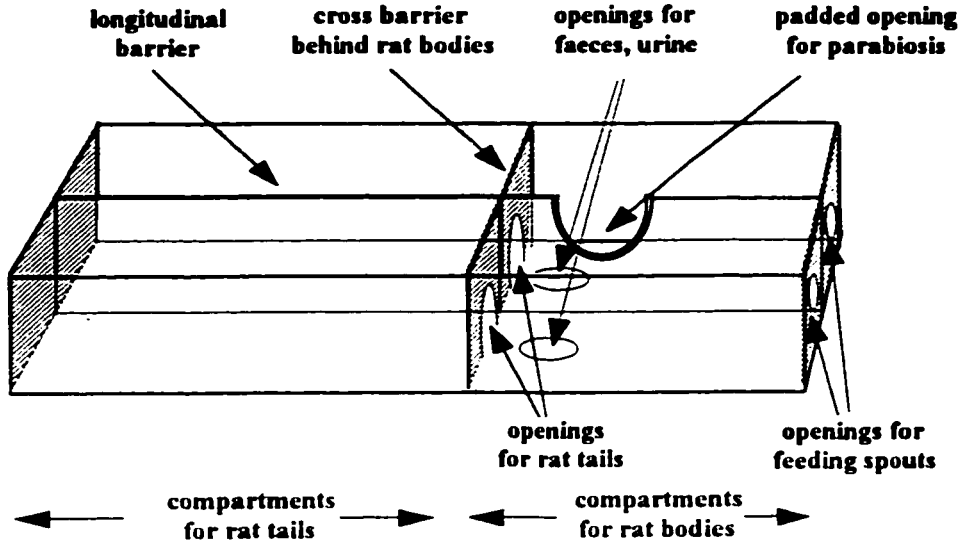


Fig. 3.1. The restraining cage that was used in all experiments.

3.3 Effects of restraint

The animals appeared healthy under the experimental conditions, and ate as a pair the same amount of food as two single, free-moving rats of the same body weight. Although stress is known to inhibit gastric emptying, and therefore could have affected the results, the effects of the relatively minor restraint that was used in our experiments appeared to be limited. There was no strong behavioral response to the experimental conditions: after the animals were exposed to the restraint for a number of days they could be readily positioned in their cages without attempts from their side to avoid the stressor, furthermore they did not show stress signs in the form of secretions from the nose or eyes.

It has been demonstrated that rats show habituation to repeated or continuous mild stress (122;283;383;384). The rats had been accustomed to the experimental conditions on a daily base for several weeks before being used in the actual experiments. No clear long-term effects of the restraint were found: even after studies lasting several months there are no signs of formation of gastric ulcers (195), which would have been an indication for sustained high stress levels.

3.4 Surgery

Although the details of the surgical preparations that were used in the present experiments are well covered elsewhere (195), for the sake of completeness they are briefly described here again.

3.4.1. One-way crossed-intestines preparation.

This surgical preparation is characterized by the isolation of a 30 cm segment of small intestine from one rat, beginning halfway down the duodenum and ending in the lower jejunum. This segment is connected into the mid-duodenum of its partner while maintaining its neural and vascular connections with the original owner. The surgeries were performed in two separate stages as follows: two rats of similar size and weight were anesthetized with ether and shaved along the left and right flank, respectively. After sterilizing the exposed skin with alcohol, an oval of skin of about 8-10 cm by 4-5 cm was cut out from the opposing flanks of the two animals between the fore- and hind legs. The animals were then laid side by side, and the first stage of the parabiosis was performed by connecting the skin of the two partners along the ventral side of the edges of the skin with a continuous 3-0 silk suture. Then a circle of 3 cm in diameter was cut from the abdominal wall muscle of the opposing flanks of the rats, between the rib cage and the rear legs, and

the edges of the wounds were reconnected with a continuous suture with 3-0 cat gut, thus creating a common peritoneal cavity between the two partners. Thereafter the rostral half of the skin of both rats was connected with a continuous 3-0 silk suture. Two stay sutures of 3-0 silk were placed in the intact skin on the frontal side of the parabiotic union and one at the caudal end. These stay sutures prevented the animals from tearing apart. The animals were allowed 10 to 12 days to recover from the surgery; thereafter the silk sutures were removed and the animals were allowed 2-3 weeks of recovery before the second stage of the surgery was performed. The rats were again anesthetized with ether, and an incision was made along the abdominal midline of both partners. The upper duodenum from both animals was cleared from the attached mesenteries and the ligament of Treitz. Small blood vessels were ligated and the duodenum was transected about 2 cm below the common bile and pancreatic duct. Also the jejunum from the right animal was transected at a point 30 cm below the first cut (see fig. 3.2: 1). All further blood vessels and nerves still connecting the 30 cm segment with its original owner were left intact. The two ends of the isolated segment were pulled through the opening in the abdominal wall between the two partners and these ends were reattached to the two ends of the transected duodenum of the left rat while maintaining the original proximal-distal orientation of the crossed segment (see fig. 3.2: 2). Finally, a reanastomosis was performed of the remaining stretch of duodenum from the right rat with the remaining stretch of its jejunum, in essence leaving the left rat with an elongated and the right rat with a shortened intestinal tract (see fig. 3.2: 3).

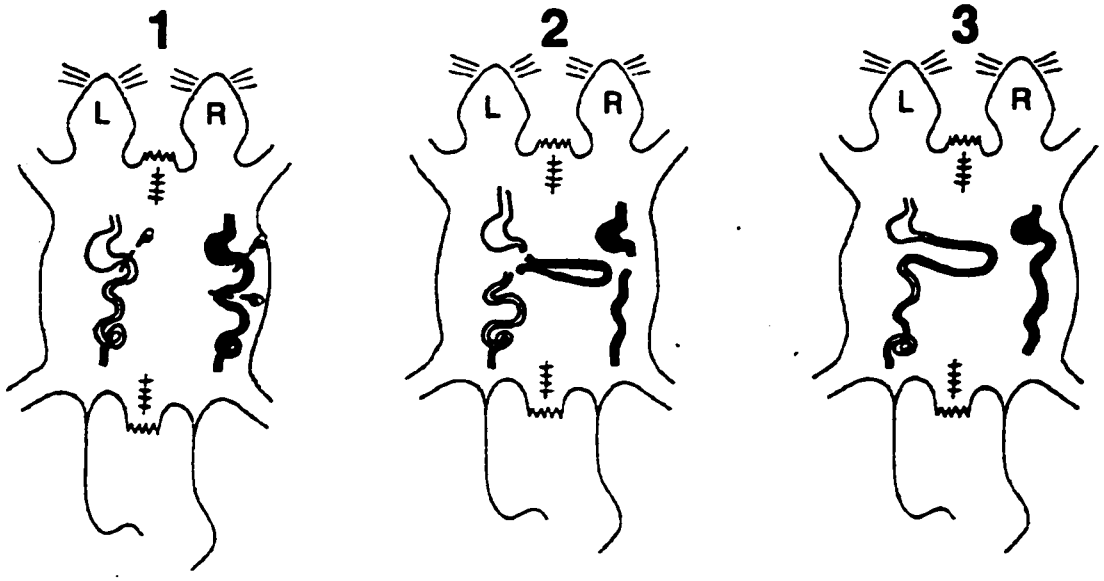


Fig. 3.2. The different stages of the one-way crossed intestines surgery. The rats are viewed from their back sides.

After the incisions along the abdominal wall were closed with 3-0 silk sutures, the animals were kept in restraining cages for most of the time for two consecutive days to prevent forces generated by animal movement from pulling apart the fresh intestinal sutures. The animals were only allowed to drink water or a sugar solution during the first day in order to minimize the risk that the accumulation of small intra-intestinal blood clots and food particles would cause an intestinal blockage. The resulting one-way cross preparation allowed food to stimulate the intestinal tract of the two partners in a physiological way since all food would be processed by the stomach and mixed with gastric, biliary and pancreatic juices in a normal manner.

3.4.2. Control rats.

The parabiosis was essentially performed as described above, with one important modification that was added for the experiments described in Chapters 5 and 9. The

creation of a shared peritoneal cavity (by connecting the circular holes in the abdominal wall of the rats) was needed to perform the crossed intestines surgery but had one major disadvantage: the small intestines of rats have much freedom of movement throughout the peritoneal cavity and therefore stretches of intestine of one rat are often temporarily situated within the boundaries of the body of its partner. This makes the analysis of scintigraphic images considerably more complicated, albeit not impossible, because no simple, direct distinction can be made between the intestinal contents of one rat or the other (see the following pages for a description of the scintigraphic analysis). A different method was therefore developed to create the union: two parallel, longitudinal incisions of about 5 cm were made in the upper and lower portions of the muscle of the exposed abdominal wall of both animals, leaving the blood vessels of the abdominal wall as intact as possible. The sides of the left and right partner were then attached by connecting the (four) corresponding edges of the muscle incisions together with a continuous 2-0 cat gut suture. This method leaves the two partners with completely separated peritoneal cavities.

3.4.3. Sham-operated rats.

Four pairs of rats were sham-operated on, via transection of the intestinal tract as described under Ch. 3.4.1, followed by a reanastomosis the restored the original connections (i.e. without insertion of a crossed segment in the intestinal tract of the partner rat).

3.5 Diet and radioactive labeling

For all experiments the commercially available liquid diet, Ensure plus (Ross Laboratories, St. Laurent, Quebec) was used. This diet consisted for 76.8 % of water, had a caloric density of 1.5 kcal per ml and an energy distribution of 61% carbohydrate, 15%

protein and 24% fat.

Since the experiments were performed using the non-absorbable solid-phase marker, ^{99m}Tc, this relatively dense diet was chosen, based on a lesser tendency to separate in a liquid and a semi-solid phase in an acidic environment, compared to two other standard liquid diets: Ensure (Ross Laboratories, St. Laurent, Quebec) and Sustacal (MeadJohnson, Belleville, Ontario). A quick quantitative comparison was made by adding concentrated hydrochloric acid to a volume of the three diets to a pH of 2 (which would be a common value for the gastric environment) and leaving equal volumes of the mixtures overnight in burets. Compared to the total height of the columns of 60 cm, Ensure plus formed only a modest liquid phase of 1.2 cm, whereas Ensure and Sustacal formed liquid phases with a height of resp. 2.9 and 3.1 cm. The different phases mixed easily after the burets were gently inverted. Considering the fact that the rat's stomach is not immobile, it is conceivable that the diet would remain in a more homogenized form in the rats stomach than was the case in the burets.

For the long-term measurements the labeled diet would remain in stationary burets for more than 24 hours. Since settling of the label over the duration of the experiment (leading to changes in concentration of the radioactive marker for earlier vs. later meals) would affect the results, the tendency of the marker to stay evenly distributed in an homogenized solution was tested by leaving a buret filled with labeled food for 24 hours and testing the radioactivity within equal volumes (as determined by gamma camera scintigraphy via application of ROIs) from a region near the top of the buret, compared to a region near the bottom. No significant changes in activity could be detected.

^{99m}Tc sulphur colloid was chosen as the radioactive marker for all experiments. Presently, it is the preferred choice for most clinical and diagnostic studies measuring gastric emptying of solids (49;50;113;153;263;410). It is the most frequently used marker

in the Department of Nuclear Medicine of Foothills Hospital (where all studies were performed); the fact that all equipment there was well prepared and tested for the application of this radioactive label, plus its ready availability, made ^{99m}Tc sulphur colloid a highly practical choice for the present set of studies. The extensive experience over the last 30 years with this label in clinical studies confirms that it meets the requirements of a high labeling efficiency and stability throughout the duration of the procedure. Also its characteristics in the intestinal milieu are favorable for emptying studies: although ^{99m}Tc sulphur colloid has a tendency to disintegrate in an alkaline environment (157), this does not cause any measurable absorption of the label from the intestinal lumen into the bloodstream (263). Whereas the labeling efficiency of ^{99m}Tc sulphur colloid is high, it does show a small degree (1% to 5 %, measured after 1 or 3 hours resp.) of dissociation from the solid phase into the liquid phase in the stomach of dogs in vivo (410); however, it is unlikely that this would have affected the results in a significant way. First of all, rats will eat a number of meals over the day, so that gastric contents that had been in contact with gastric juices are being emptied and replaced with fresh, labeled food on an ongoing basis; therefore not all labeled food will be exposed for prolonged periods to the acid so that a high percentage of dissociation would not be likely for the major part of the studies. It is not clear whether dissociated label reattaches to food particles or whether free particles can attach to the stomach wall (263). Furthermore some of the nutrient contents of the diet will dissolve into the liquid phase, which will therefore be carrying some of the total caloric load; a certain amount of free label that is emptied with liquids will therefore still represent "real" calories. The use of a liquid diet has advantages over the use of more "solid" food sources, since the emptying pattern of a semi-solid diet (created after curdling of the diet takes place following contact with acid in the stomach) is somewhat in-between the solid and liquid emptying patterns, without the lag-phase that is characteristic for the

solid pattern. When mixed meals of "real" solid food and low- or non-caloric liquids are used, generally most of the liquid has emptied before the end of the lag phase (324). Therefore, even if a small amount of the label would come off from the food and would be emptied separate from solids, the relative effects on the measured rate of emptying would be less pronounced than would have been the case when a typical mixed solid/liquid meal had been used.

Nonetheless, in order to further assess the suitability of diet and marker, a general evaluation study was performed using a dual-isotope technique, measuring the emptying pattern of the solid and liquid phase simultaneously (see Chapter 4).

3.6 Experimental Design

All experiments began at 16.00 (the normal time for refeeding of the animals) and ran for 6.7 hr or 400 min (i.e., from 16.00 until 22.40) for the short-term experiments, or for 26 hours (i.e. from 16.00 on day 1 until 18.00 on day 2) for the long-term studies. Lights were on between 16.00 and 18.00. The rats were equipped with small radioactive markers on their shoulders, to allow easier detection of movements during the study and were put in their restraining cages one hour prior to the start of the study. They were positioned in the visual field of the gamma camera (Fig. 3.3) at least 15 min before the start of the experiment; no further handling of the animals occurred until after termination of the study.

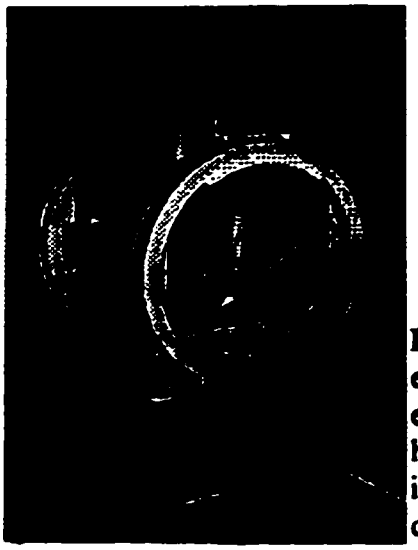


Fig. 3.3 The ADAC gamma camera that was employed for most of the present experiments. Normally it is used mainly for human diagnostic purposes. The arrow indicates the plateau on which the restraining cage is positioned.

The radioactive, non-digestible, marker ^{99m}Tc sulphur colloid was added to the liquid diet Ensure plus (1.0 mCi per 100 ml food for short-term studies, 2.0 mCi per 100 ml for long-term studies), stirred, the weil mixed labeled diet was transferred to graded burettes, and the rats were allowed free access to the food at 16.00. All experiments were performed using this single concentration of the standard diet. Lights were dimmed to a low level after 18.00. During the dark period the rats were further sheltered against the light via application of isolating curtains around the cages and gamma camera. Cumulative food intake during the experiment was measured (by direct readings from the burets) at regular intervals for each animal individually.

3.7 Data Acquisition

In most of the experiments continuous data collection took place in 2-min. intervals, using a dynamic planar protocol, with a resolution of the gamma camera of 128 x 128 x 16 pixels. An acquisition rate with two minutes between frames was chosen because it was generating images with a sufficiently high signal/noise ratio, while maintaining a good

accuracy for the data analysis. Faster acquisition rates could have achieved a further improvement of the accuracy of the analysis, but would also have created an impractically high demand for available resources, especially data storage space and subsequent analysis time. Also higher amounts of radioactive label in the diet would be required to maintain sufficient clarity and resolution of the single images. For periods of rapid gastric emptying, a frame rate of one image per minute has been recommended (5). After termination of the experiment, a first analysis of the data was performed on a Pegasys computer system (ADAC Laboratories, Milpitas, CA, USA). In this process a sequence of computer images was created, showing the distribution of radioactivity within the G.I. tract of both animals over the full duration of the experiment. A summation of the images generated a composite picture that was used to make a first assessment of the study by drawing regions of interest (ROI's) around the desired gastrointestinal structures. Total radioactivity in the different ROI's was then obtained for each consecutive (1- or 2-min.) image and combined, resulting in total curves representing radioactive contents over time. Thereafter, these data set were corrected for radioactive decay (^{99m}Tc has a half life of 6.02 hr). Since no more radioactivity had been added to the food since the start of the experiment, this calculation applied equally to all labeled contents throughout the gastrointestinal tract, originating from all various meals, as well as to any eventually produced fecal material that contained radioactivity.

The decay-corrected data sets were saved as ASCII textfiles. Since the Pegasys system is based on a (Sun) UNIX operating system, the UNIX formatted files had to be converted to standard PC format first; thereafter the ASCII textfiles were parsed into a standard spreadsheet format (Quattro Pro 7, Corel) and analyzed. No mathematical smoothing of the curves was performed, so as to avoid loss of accuracy of the data set. All values for the different curves were recalculated from radioactive counts/2 min into kcal/2 min

contained within the various ROI's by comparing the total food intake at the end of the experiment (as derived from the graded burets) with the total amount of radioactivity contained in the animals (plus eventually produced feces). This not only allowed to interpret the data in a more physiologically relevant context, but also prevented the occurrence of a possible source of error in the data via variation between experiments in the concentration of the radioactive marker in the diet.

Although especially for 24-hour experiments the different gastrointestinal structures are not easy to discriminate on the composite images (due to motion artifacts and to the summation of noise caused by background radiation and Compton scatter adjacent to the GI tract), they could usually be easily and accurately determined on the separate images (see fig 3.4).

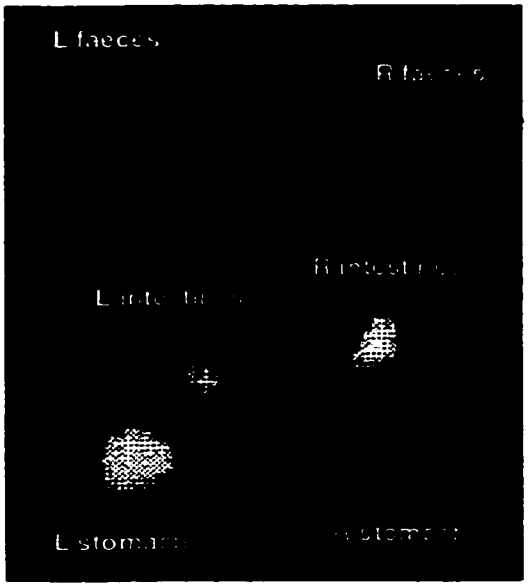


Fig. 3.4. A typical gamma-camera image, taken five hours after the first meal. Different shades of grey represent different levels of radioactivity (measured as radioactive counts per 2 min per pixel). The image is vertically mirrored, so that the stomach of the left rat is shown in the lower left corner.

The use of small animals for scintigraphic studies has certain advantages over conventional, large-bodied subjects; since the total body size of the rats did not exceed the measurement area of the gamma camera, it was possible to monitor the cumulative increase over time of radioactivity in the whole body (plus the eventually produced feces)

of the animal (Fig. 3.5, ROI 1). This produced a cumulative food intake curve that could be used directly as an on-line measurement of both meal size and timing.

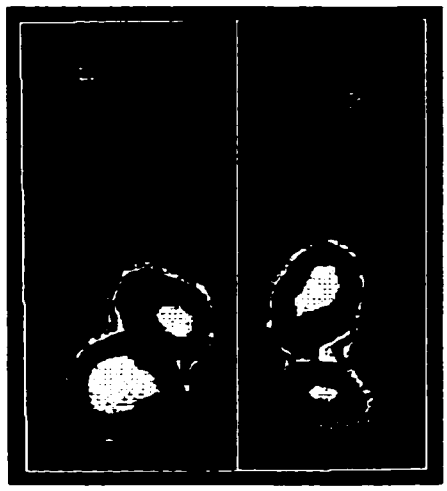


Fig. 3.5. ROIs around the whole body of each separate animal (including eventual produced feces), and around the two rats as a pair ("ROI 1").

Gastric contents over time were directly derived from ROIs around the left and right animal's stomach (Fig. 3.6, ROI 2). This is the standard technique to measure gastric emptying, via the creation of a retention curve after one meal, eventually allowing calculation of half-emptying time of the meal. Measurement of gastric emptying during feeding is not reliable using this method, since gastric contents during this phase are a combination of food entering the stomach via intake, and of food leaving the stomach via gastric emptying.

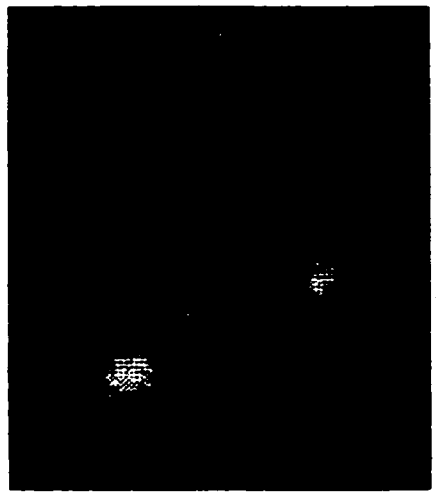


Fig. 3.6. ROIs around the stomach of each rat ("ROI 2")

Instead gastric emptying can be measured directly and accurately by measuring the total post-gastric increase in radioactivity over time, i.e. the cumulative radioactive contents in the small and large intestines plus all activity in the feces that the rat releases (Fig. 3.7, ROI 3). Contrary to the standard method, changes in emptying rate during meals will be accurately measured via analysis of ROI 3, thus also allowing measurement of a number of successive meals.

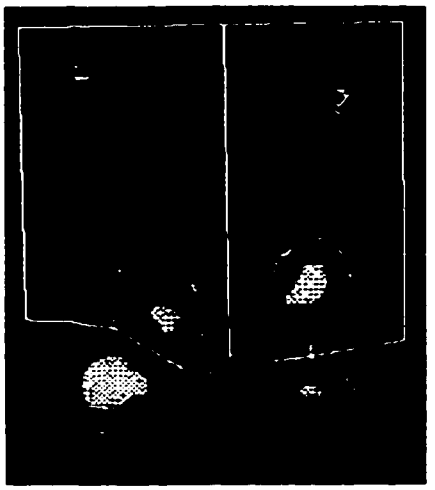


Fig. 3.7. ROIs around the areas containing both the small and large intestines as well as the feces of each rat ("ROI 3").

3.8 Motion correction

The choice for mild restraint during the experiments was deemed necessary to limit the stress that the rats experienced. However, this allowed them a relatively high freedom to move for short distances. Unfortunately even minor movements forward or backward created motion artifacts in the data that were collected after application of a single ROI. Since these errors could not automatically be corrected (due to a lack of effective motion correction programs), a highly labor-intensive correction method had to be applied by

comparison of the defined location of the ROI with the actual location of the animal on all successive frames. Motion artifacts where then corrected by hand by redefinition of the location of the ROIs for the appropriate frames, in effect establishing ROIs for every image separately. For each of the different ROIs (maximally 15 for some studies) new curves were created via application of the modified ROIs and subsequently a final curve was generated via manual recombination of all data points from the different curves. Occasionally, the rats made a significant movement in their cage during the data collection, and stomach and intestines could not clearly be identified on the resulting image. In such cases, interpolated values between the previous and the subsequent image had to be calculated.

3.9 Accuracy of image analysis

The assignment of ROIs is based on a subjective decision process. Some experience is required to accurately recognize the boundaries of stomach and intestines. The use of computer-generated color maps, that assign different colors or color intensities to different levels of radioactivity for each pixel of the image was highly beneficial in the identification of the different gastrointestinal structures. Also the "window" of the color maps (i.e. the range of counts per pixel that get a certain color assigned to it) can be adjusted manually, effectively changing the discriminatory power of the analysis. It was found to be of major importance to choose these settings carefully and assess critical or obscure images repeatedly by using various color maps and settings and by repeatedly comparing defined ROIs for a given image with previous as well as with subsequent images. For instance, in many situations a stretch of duodenum was found to be situated close to the stomach, but this could only be identified correctly by choosing the proper color map settings: certain settings that are effective to separate low count values but fail to discriminate between

higher values would lead to a virtual fusion of these two GI structures on the image. The resulting combination would erroneously have been associated with the stomach alone which would subsequently have led to a misinterpretation of the distribution of the label in the GI tract and an underestimation of the rate of gastric emptying for such a frame.

A certain amount of noise in scintigraphic data is caused by small movements by the animal during the acquisition time of an image: if the change in location is large enough to re-position stomach or intestines outside the ROI that was selected for analysis of that particular frame, the result is a decrease of the radioactive count-value for that ROI (and probably an increase for another ROI, for instance if a backward motion leads to the new location of the stomach being partially situated in ROI 3, instead of ROI 2). The effect of such movements on the end results are limited: first of all the information in the image that is used to draw each ROI is always based on more than 50 % of the acquisition time (movements at the very beginning or end of the acquisition period contribute relatively little information to the image). Also, large movements are relatively rare; the rats appear to have only a few favored positions. They often move forward to start a feeding bout or backwards to return to their resting position. Small movements cause relatively little noise, since the distribution of radioactive label in the GI tract is such that most of the contents are situated in clearly separated locations (see Fig. 3.4). Large, obvious motion artifacts can be detected easily via inspection of the resulting curves and manually corrected by calculating the average of previous and subsequent images after verification of the movement by comparing the original images, as described above. Finally, movements can be assumed to be distributed randomly over the group of experimental animals (maybe the only exception being the period after the very first meal, when most animals will move back from the spout after they finish feeding) so that the effect of their movements on the end results will be averaged out to a large extent.

Chapter 4: Validation of diet choice and radioactive labeling via dual-isotope measurement of solid and liquid emptying.

4.1 Introduction.

The use of the liquid diet, Ensure plus, for all the studies described in this dissertation had several practical advantages, such as consistency of texture and caloric composition throughout all the experiments, convenience of preparation of the diet and labeling with the radioactive marker and acceptance by the rats as their standard food source during as well as between experiments. Also the diet had a consistency that was somewhat between a solid- and a liquid-type meal. This consistency would avoid the disadvantages of typical liquids and solids (444). The diet would therefore comply with all the main criteria that have to be considered for labeled meals in gastric emptying studies (213). Also the diet contained all macronutrients (61 % carbohydrates, 15 % proteins, 24 % fat by caloric value) and was comparable with the standard North-American diet, making the results more widely applicable, compared to studies using a single macronutrient like glucose.

However, for the diet to be an effective vehicle for the solid-phase marker Technetium-99m (^{99m}Tc) sulphur colloid, a few issues needed to be resolved: it still had to be established that the diet would behave in the stomach as a (semi-)solid food source rather than a liquid one, and that the radioactive label would remain associated with the nutrient content of the diet,\ instead of being emptied with the liquid phase containing ingested fluids and gastric secretions. Although many gastric emptying studies use liquid diets such as glucose solutions, the emptying characteristics of such meals can not be fully representative of the emptying of normal meals.

To explore further the issue of the markers attachment to the solid nutritive contents of

the diet, a dual-isotope technique was applied: the animals were given access to the diet after it was labeled simultaneously with the two radioactive markers, ^{99m}Tc sulphur colloid and Indium-111 diethylene triamine penta-acetic acid (^{111}In DTPA) and the distribution throughout the GI tract of each of the two labels was measured over time. The two markers have a different energy spectrum: ^{99m}Tc has a single energy peak at 142 keV, whereas ^{111}In has two main peaks at 173 keV and 247 keV. By collecting data in different energy windows and application of pulse-height analysis the information for the different markers can be separated and further analyzed. ^{111}In DTPA is considered to be one of the most satisfactory agents for specific measurement of liquid emptying (113;456), whereas dissociated ^{99m}Tc sulphur colloid will also be emptied associated with the liquid phase (^{99m}Tc sulphur colloid has been used in some scintigraphic studies as a liquid phase marker (113)). Since liquids generally empty faster from the stomach than solids, the suitability of both the diet and the ^{99m}Tc sulphur colloid label could be assessed by comparing the emptying patterns of both radioactive markers at one time. If both markers would empty from the stomach with similar rates, this would imply that the diet would have the emptying characteristics of a liquid and/or the ^{99m}Tc sulphur colloid label did not attach well to the solid particles of the diet. In the latter case, the experimental setup would have to be questioned, and more information would have to be acquired about the labeling efficiency of the ^{99m}Tc sulphur colloid marker when used in combination with the Ensure plus diet. If, on the other hand, a clear difference would be found between the emptying patterns of the two markers, the assumption could be made that both requirements (solid or solid-like emptying characteristics of the diet, and efficient labeling with the solid-phase marker) would be met when Ensure plus is used as the standard diet, in combination with ^{99m}Tc sulphur colloid as a single marker.

4.2 Methods

4.2.1 Experimental Design

The experiments began at 16.00 (the normal time for refeeding of the animals) and ran for 6.5 hours (i.e. from 16.00 until 22.30), with lights on between 06.00 and 18.00. The rats were put in their cages one hour prior to the start of the study, and were positioned above the gamma camera before 15.45. The radioactive, non-digestible, markers ^{99m}Tc sulphur colloid and ^{111}In DTPA were added to the liquid diet Ensure plus (1.0 mCi ^{99m}Tc sulphur colloid and 0.25 mCi ^{111}In DTPA per 100 ml food). More ^{99m}Tc than ^{111}In was used to diminish the contribution of down-scatter of the ^{111}In-label into the ^{99m}Tc energy window. The well mixed labeled diet was transferred to graded burettes for each individual animal, and the rats were allowed access to the food at 16.00. For the purpose of further correction for down-scatter a small vial containing some of the labeled diet was placed in the field of the camera, well separated from the animals. Lights were dimmed to a low level after 18.00. During that period the rats were further protected against the light by application of curtains around the gamma camera. Food intake during the whole experiment was measured at regular intervals for each animal individually throughout the 6.5 hour measurement period.

4.2.2 Data Acquisition

Continuous data collection took place in 2-min. intervals, using a dynamic planar protocol, with a resolution of the gamma camera of 128 x 128 x 16 pixels. Data collection took place in three separate energy windows: 140 keV (20 %) for the ^{99m}Tc marker, and 174 keV (15%) and 250 keV (20%) for the ^{111}In label. After termination of the experiment, the data derived from the two ^{111}In energy windows were combined and a first

analysis of the data for ^{99m}Tc and ^{111}In separately was performed on a Pegasys computer system (ADAC Laboratories, Milpitas, CA, USA). In this process a sequence of computer images was created, showing the distribution of radioactivity within the G.I. tract of both animals in 2-min intervals for the full duration of the experiment. For each separate image, three regions of interest (ROI's) were defined around the whole body plus feces of each animal, around the stomach, and around the intestines plus feces (see also Chapter 3: Methods), as well as around the separate vial containing a fixed amount of diet. The total radioactivity in the different ROI's was then obtained for each consecutive 2-min. image and combined, resulting in 6 ½-hr curves.

An estimation was made for the amount of "down-scatter" from ^{111}In into the ^{99m}Tc window by comparing the decay-corrected values over time for the vial: since the amount of labeled food contained in the vial was constant, a constant value for both markers should have been found. A certain percentage of scattered γ-rays emitted from the ^{111}In label, however, will have an energy level similar to γ-rays coming from the ^{99m}Tc label and will be counted as such by the gamma camera, and since ^{99m}Tc has a half life of 6.02 hr and ^{111}In of 67.4 hr, the relative contribution of scattered ^{111}In γ-rays to the total counts in the ^{99m}Tc window will increase over time. Therefore, whereas the decay-corrected curve for the vial contents measured for ^{111}In remained on a constant level (Fig. 4.1, lower curve), the value for ^{99m}Tc slowly increased over time (Fig. 4.1, upper curve). A deduction factor of 20 % of the ^{111}In counts from the ^{99m}Tc counts (*before* decay correction) corrected this problem (Fig. 4.1, middle curve), and was therefore applied to all further curves collected in this experiment.

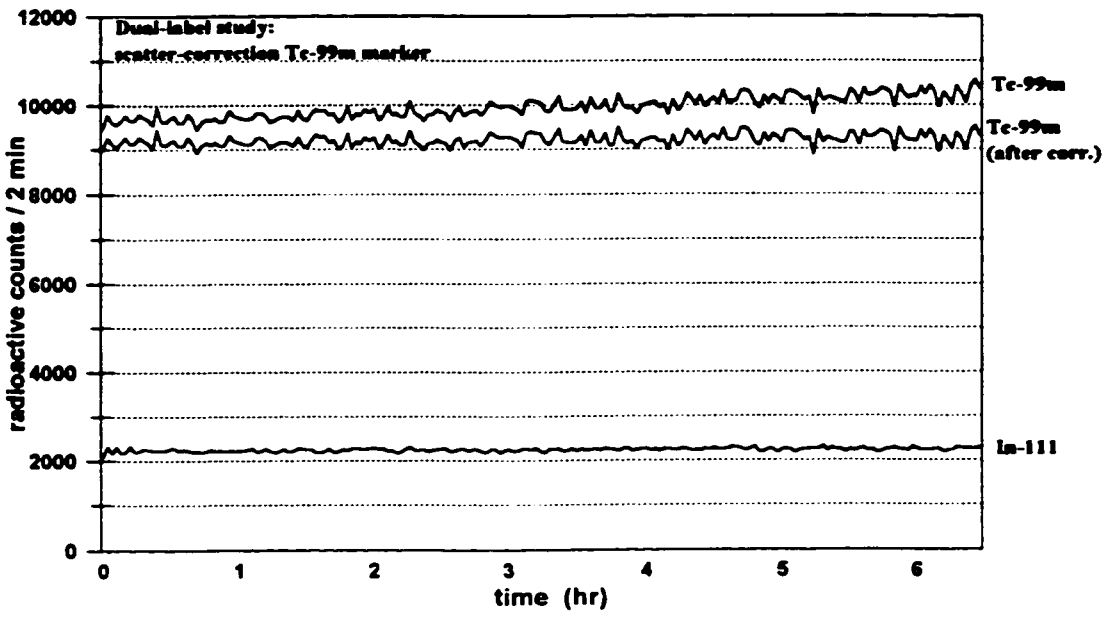


Fig. 4.1. Correction of down-scatter for the ^{99m}Tc window: decay-corrected curves for the ^{99m}Tc marker before ("Tc-99m") and after ("Tc-99m after corr.") scatter correction, as well as the ^{111}In marker ("In-111").

Finally, all data sets were corrected for radioactive decay. Since no more radioactivity had been added to the food since the start of the experiment, this calculation applied equally to all labeled contents throughout the gastrointestinal tract, originating from various meals, as well as to any fecal material that also contained radioactivity. After a manual motion-correction (see Chapter 3: Methods) in which the various time points of the decay-corrected data sets were combined by hand into a final 6½-hour data set, all values were converted from counts/2 min into kcal/2 min (the total amount of radioactivity in the GI-tract of the animals, as measured via the gamma-camera, was correlated with their actual food intake as measured from the burets), and the curves were analyzed using a standard spreadsheet (Quattro Pro 7, Corel) on an IBM-clone personal computer. No mathematical smoothing of the curves was performed, so as to avoid loss of accuracy of

the data set. Statistical analysis was performed via Anova and t-tests.

4.3 Results

The following two graphs show food intake, gastric emptying and gastric contents of one individual rat, measured via application of the ^{99m}Tc sulphur colloid (fig. 4.2) and. the ^{111}In DTPA label (Fig. 4.3).

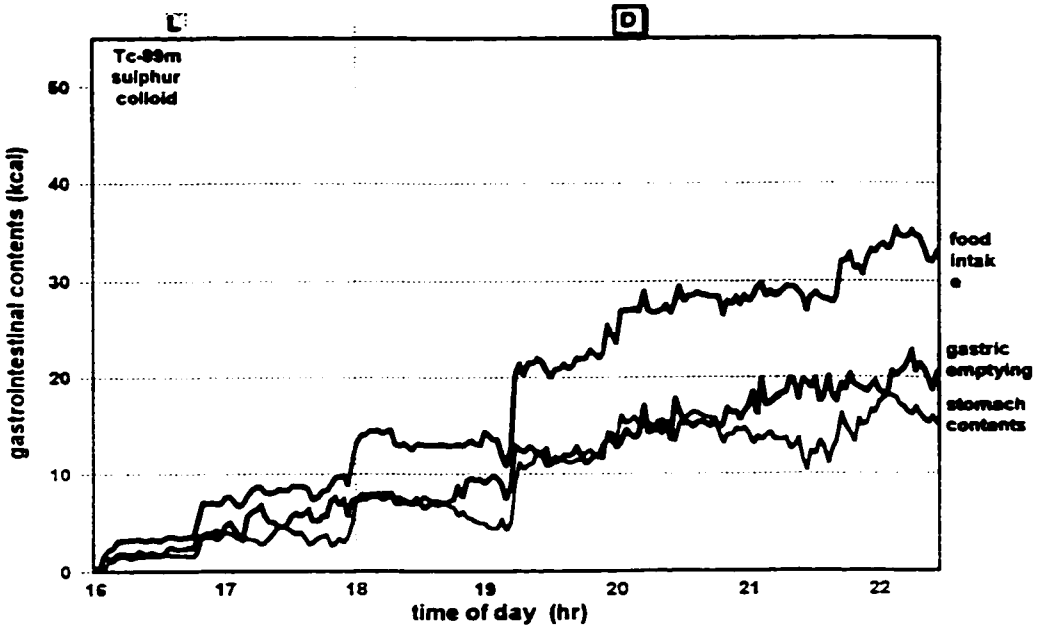


Fig 4.2 Food intake, stomach contents and emptying pattern of the solid phase of the ingested diet, measured in a single animal via application of the solid-phase marker ^{99m}Tc sulphur colloid.

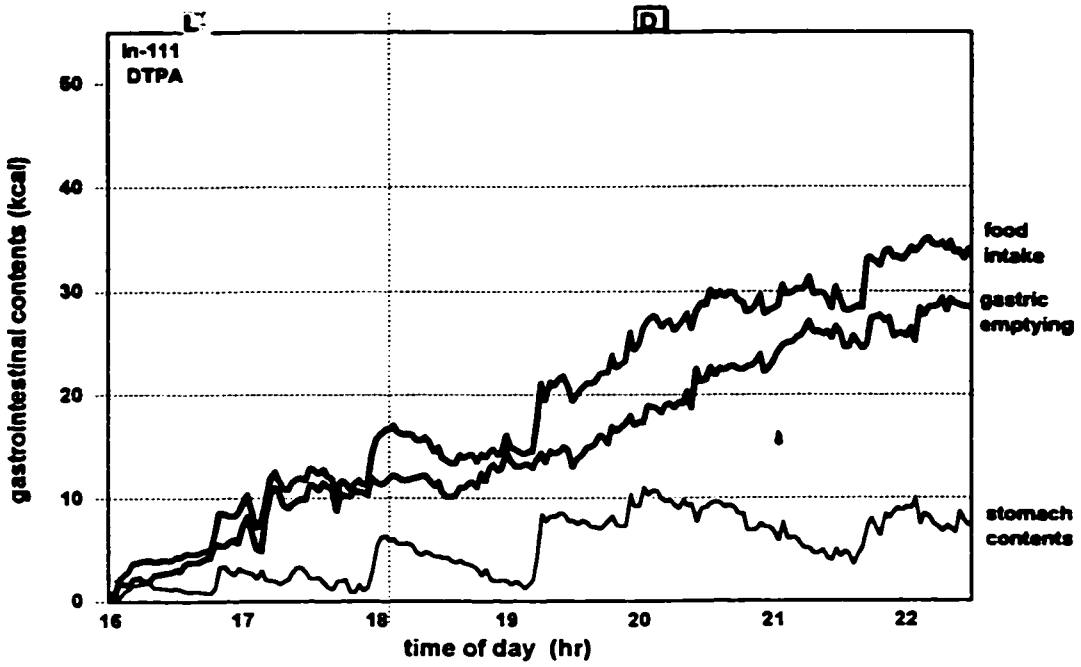


Fig. 4.3. Food intake, stomach contents and emptying pattern of the liquid phase of the ingested diet, measured via application of the liquid-phase marker ^{111}In DTPA

When the comparison was made for the average emptying curves of all eight rats, no statistical difference was found for food intake as measured via the two different radioactive markers; this is not surprising, since all curves had been calibrated for total food intake at the end of the study. However, the average emptying curves, representing the solid and liquid phase of the diet, were very different for the ^{99m}Tc sulphur colloid and the ^{111}In DTPA markers ($p<0.000001$, $F=26.5$), as were the curves for gastric contents over time ($p<0.00000001$, $F=125.1$). The ^{99m}Tc sulphur colloid label showed a relatively slow emptying pattern, resulting in a gradual accumulation of label in the stomach during the 6½ hour duration of the experiment (Fig. 4.4).

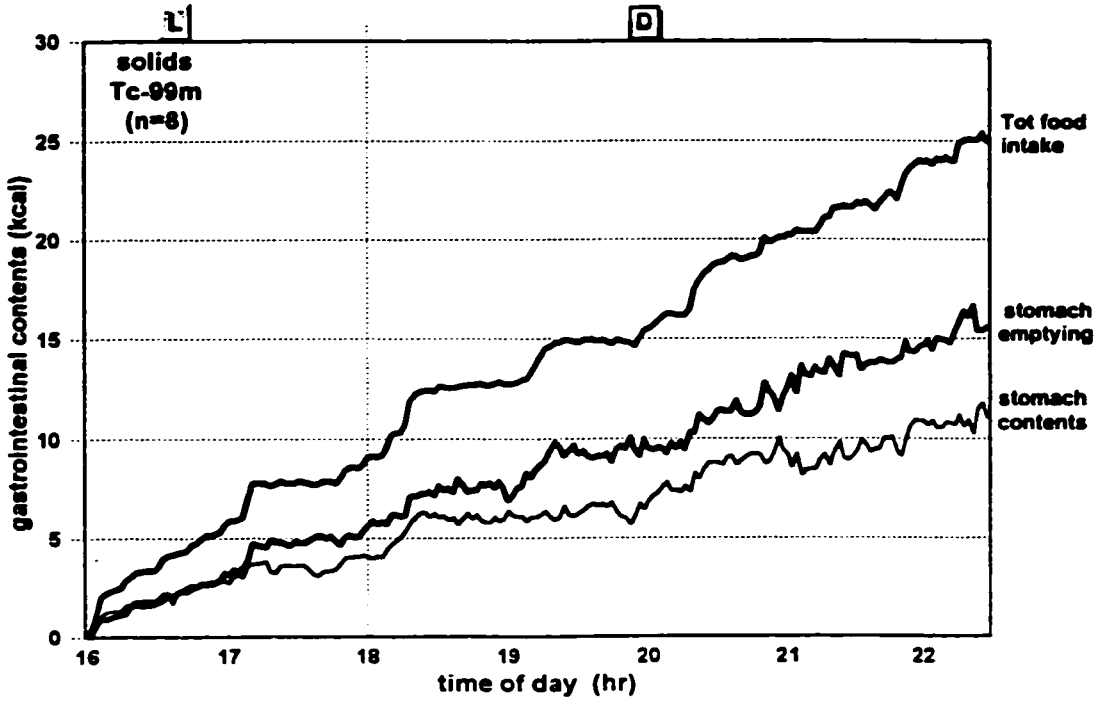


Fig. 4.4. Average food intake, stomach contents and emptying pattern of the solid phase of the ingested diet, measured via application of the solid-phase marker ^{99m}Tc sulphur colloid (n=8 pairs).

The ^{111}In DTPA label, that leaves the stomach associated with the liquid phase, showed a more rapid emptying pattern and hardly any further increase in stomach contents after the first 2½ hours of the experiment (Fig. 4.5).

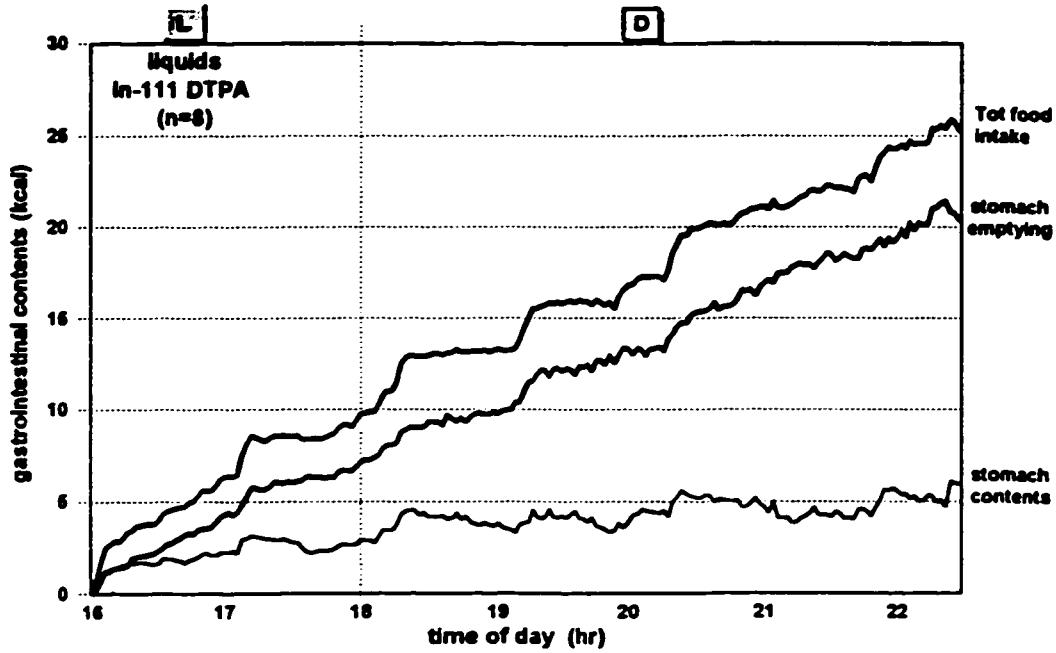


Fig. 4.5. Average food intake, stomach contents and emptying pattern of the liquid phase of the ingested diet, measured in via application of the liquid-phase marker ^{111}In DTPA (n=8 pairs).

This was also illustrated via a regression analysis of the emptying curves of both markers: although a high correlation was found with the food intake curves measured by the same marker ($p<0.0001$), the correlation for the ^{111}In DTPA label appeared to be stronger ($F=39973.7$) than for the ^{99m}Tc sulphur colloid label ($F=28102.7$).

All values of the 2-min images were expressed as kcal in order to allow an assessment whether the emptying data were physiologically realistic; extrapolation of the 6½-hour emptying curves based on the ^{99m}Tc sulphur colloid label would suggest a 24-hour emptying rate of about 60 kcal. This is not significantly different from the average 24-hour energy intake of the rats in the week preceding the experiment.

4.4 Discussion

The results from the dual-isotope experiment suggest that the emptying characteristics

of the diet Ensure plus make it a satisfactory choice for the present studies, and that it can be efficiently labeled with the solid marker ^{99m}Tc sulphur colloid. The fact that the emptying curves acquired via application of this label are very different from the emptying curves based on the liquid marker ^{111}In DTPA suggests that the ^{99m}Tc sulphur colloid attaches well to the diet, and also that the Ensure plus diet does not have the emptying characteristics of a liquid. A comparison in a single animal between the ^{99m}Tc sulphur colloid label (Fig. 4.2) and the ^{111}In DTPA label (Fig. 4.3) suggests that most of the liquids are being emptied relatively quickly, in a pattern not directly associated with the solid phase, whereas the solids are retained longer. This is fully in accordance with the results from many other gastric emptying studies using a variety of techniques, including scintigraphic measurement with various food sources and radioactive labels, as well as intubation and other techniques. The amount of liquid marker contained in the stomach dropped to a low level within an hour after a meal (Fig. 4.3), a detail that is partly obscured when the average contents for several animals are displayed (Fig. 4.5), due to meal intake at different times for different animals. If a major amount of the ^{99m}Tc sulphur colloid would have become dissociated from the solid phase, a similar fast emptying pattern would have been expected, instead of the accumulation of ^{99m}Tc sulphur colloid in the stomach that was observed during the 6½ hours of the experiment. The fact that the behavior of the ^{111}In DTPA label did not reflect the gradual build-up of food stored in the stomach that is observed in rats under normal conditions also suggests that ^{111}In DTPA is not a suitable marker to measure stomach emptying when using Ensure plus as a diet.

It is quite likely that the diet separates in the stomach into a liquid phase and a solid phase, as was already to some extent the case in the *in vitro* experiment (with added acid) that was used to test different diets (see Chapter 3.5). In a standing buret the solid particles remain in a suspension within the liquid phase, while in the mechanically and chemically more volatile gastric environment, especially during the process of trituration in

the distal stomach, a separation between solids and liquids probably would occur. If a substantial part of the diet would be emptied quickly in the liquid phase, then this could have direct implications for the interpretation of some of the results, especially for the description of nutrient contents in the stomach over time, and its possible correlation with gastric distension. If the ingested food were to separate into a solid and a liquid phase, then the liquids would empty relatively quickly, which would imply that the correlation between stomach contents and caloric contents would then be consistent throughout the study, but the direct correlation between ingested volume and stomach volume would be altered. In other words, the solid phase of the nutrients in a certain ingested volume of Ensure plus (with a caloric value of 1.5 kcal/ml), after losing some of its fluid content, would take up less space in the stomach after ingestion. It is likely that the solid phase in the stomach remains associated with some liquids, so that a correlation (albeit at a lower level) between gastric nutrient contents and gastric volume is upheld. After some time an equilibrium may be established between gastric secretion and liquid emptying (236;237). This issue is further addressed in Chapter 5: 24-hour gastric emptying in control rats.

A related issue that gets surprisingly little attention in the literature is the question of how far the emptying of the ^{111}In DTPA label really represents the emptying of the liquid phase of the meal, especially when measured for extended periods after feeding. Contrary to the solid phase, the liquids contained in the stomach are not part of a "closed system" that is only driven by food intake and gastric emptying, but are also affected by the ongoing process of gastric secretion. Since gastric secretion would dilute any label retained in the liquid phase and this dilution would increase more and more over time, the measurements of label in the liquid phase after some period of time would no longer accurately represent the emptying of the liquid components of the meal: an artificial decrease in the measured emptying rate could be the result. Since gastric secretion in humans is at its peak between 30 and 60 minutes after a meal (237)and many gastric

emptying studies measure over time periods up to 2 hours, this would be likely to influence the estimates for the emptying rate later into the study, and could easily exaggerate the exponential emptying curve found for non-nutritive liquids. In the experimental design of the present study this potential effect would be somewhat diminished by the fact that the rats eat several meals over the 6.5 hours of the study, which partly and irregularly restores the balance between the amount of label and the liquid volume in the stomach.

Further support for a successful labeling of the diet could be found in the fact that extrapolation of the curve for the 6½ hour data generated realistic values for 24-hour emptying. Extrapolation of the gastric emptying curves of single meals often leads to a considerable underestimation of the daily food intake of the animal (30;177). Under the present free-feeding experimental conditions, where emptying of several separate meals is measured over time, a significant difference between 24 hour food intake and estimated 24 hour gastric emptying would have cast doubt on the reliability of the measurements.

Chapter 5: Measurement of gastric emptying during and between meal intake.

5.1 Introduction

Many studies during the last few decades have focused on the specific details of gastric emptying of a fixed, chemically specified meal after a long period of food deprivation, usually overnight. However, detailed information about the movement of food in the gastrointestinal tract under natural, free-feeding conditions is rarely available, mainly due to a lack of availability of appropriate, non-invasive measurement techniques. As a result, many methodological issues in the study of gastrointestinal transit have not yet been fully resolved and a wide variety of techniques are employed that by themselves could directly affect the measurements. For example, many studies make use of catheters that are temporarily or permanently placed into the stomach and/or small intestines (30;175;177;248;249;322;406). However, gastrointestinal intubation has been shown to influence the rate of stomach emptying and gastrointestinal transit time, likely via direct stimulation of mechanoreceptors (322). Also, in most studies, only the emptying rate of a single meal is measured, usually after a period of food deprivation. The food must be eaten quickly within a specified time period or can even be instilled directly into the stomach as a bolus injection. Measurement of gastric emptying often begins only after meal termination and, sometimes, the animals have to be manually handled just before the measurements begin. It is possible that under such circumstances an abnormal emptying rate will be found. First of all, the possible effects of food deprivation have to be taken into account: after an extended deprivation period the gastrointestinal tract will be mainly empty, so that inhibitory feedback signals on stomach emptying arising from the gut will be on a lower level than in a fed animal. Under natural circumstances, the stomach and intestines of a free-feeding rat always have food present (14;419). Also, direct instillation of food into the stomach, albeit allowing for standardization of meal composition and size, will bypass

the oropharyngeal cavity and the oesophagus. The act of swallowing, however, evokes reflex mechanisms that are involved in the regulation of intragastric pressure, a process known as "receptive relaxation". Infusion of food into the stomach could lead to a greater difference in fundic-duodenal pressure and an increased rate of gastric emptying. Furthermore, manipulation of the animal could exert mechanical pressure over the abdominal wall on stomach or intestines, again altering the pressure balance between stomach and duodenum and inducing artifacts in the measurements. Finally, eating or infusing food, especially at a higher than natural intake rate, could also conceivably exceed the capacity of the fundus of the stomach to relax as a reaction to an increase in intragastric pressure: "adaptive relaxation" (2;170;462), which again may increase the rate of gastric emptying. Indeed, various studies (30;175;177;249) indicate that during a meal the rate of gastric emptying may be temporarily increased. Failure to include this period in the measurement session will result in an underestimation of the total amount of food emptied from the stomach since meal onset, and will change the calculated rate of gastric emptying. An estimation of the total amount of calories emptied from the stomach over a 24-hour period, based on data that do not include the feeding period, will greatly underestimate the actual daily caloric intake of the animal (30).

The aim of the present study was to investigate the stomach emptying pattern under natural, free-feeding conditions by applying a new technique of gamma scintigraphic measurement that we modified for use in small animals such as the rat. A major advantage of scintigraphy is that it is a non-invasive technique that allows for normal gastrointestinal function. The movements of gastrointestinal (G.I.) contents, expressed in a two-dimensional plane, can be monitored continuously (in one or two minute bins), without further handling of the animals, which makes this method well suited for measurement of the rate of gastric emptying. Traditionally this technique is used only to acquire a gastric retention curve after intake of a single meal; however, emptying patterns during the ingestion of

food can not be measured as reliably if only the stomach is visualized. In contrast, the modifications allow continuous acquisition of information about variations in the pattern of gastric emptying during and between several consecutive meals, while simultaneously measuring cumulative food intake and the radioactively labeled nutrient content retained in the stomach.

5.2 Material and Methods

The general procedures have been described in Chapter 3 (Methods); only details specific for the present experiment will be outlined here.

5.2.1. Procedure

Eight pairs of male Lewis rats (Sprague Dawley, Indianapolis, IN) weighing an average of 786 ± 13 g (SEM) were fed the liquid diet Ensure Plus. The animals were kept on a restricted feeding schedule with food access between 16.00 and 09.00, with lights off between 18:00 and 06:00. Before the actual experiments the animals had been deprived of food for an additional day, effectively subjecting them to a total deprivation period of 31 hours. The rats had been adapted for several weeks to the moderately restrained conditions that were used during the experiments..

5.2.2. Experimental Design

The experiments began at 16.00 and ran for 400 min (i.e., from 16.00 until 22.40), with lights on between 16.00 and 18.00. The radioactive, non-digestible, marker Tc-99m sulphur colloid was added to the liquid diet Ensure plus (1.0 mCi per 100 ml food), the well mixed labeled diet was transferred to graded burettes, and the rats were allowed free access to the food at 16.00. All experiments were performed using this single

concentration of the standard diet.

5.2.3. Data Acquisition

Continuous data collection took place in 1-min. intervals, using a dynamic planar protocol, with a resolution of the gamma camera of 128 x 128 x 16 pixels.

5.2.4. Meal criteria and data analysis

Although the general gastrointestinal filling pattern for each animal can be graphically represented via the curves generated by the various ROI's , a more detailed analysis was performed on the rate of emptying that occurred during and between meals. For these calculations, only meals were included that fulfilled the arbitrary criteria of a minimum size of 1.0 kcal and a minimal post-meal interval of 10 min.

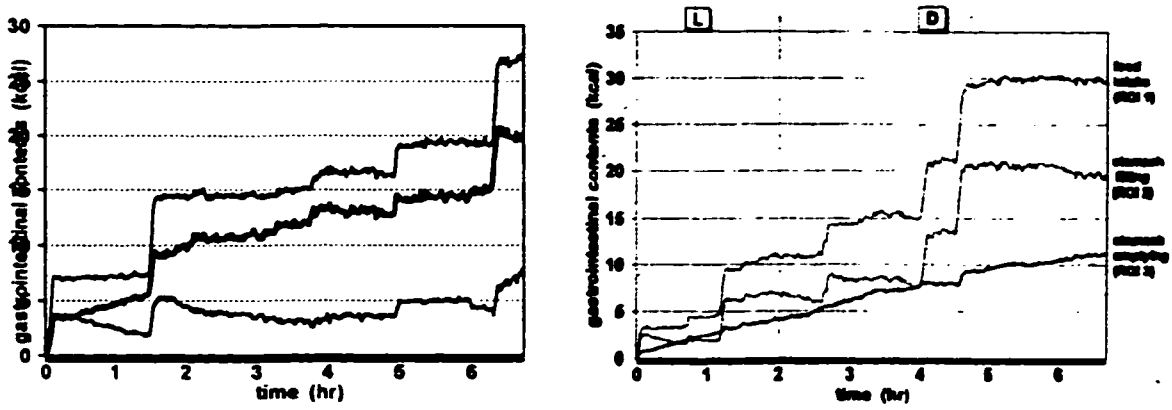
The total amount of nutrients emptied during the meal could be calculated via determination of the exact size and timing of the separate meals (ROI 1) and the change in radioactivity in the gastric emptying curve (ROI 3) during the concomitant period. A direct division of the two values gives the percentage of the meal that was emptied during feeding. These values could be compared with the average emptying rate for each animal over the entire experiment, the average emptying rate in the first 10 min of each post-meal interval, or the average emptying rate in-between meals (calculated by dividing the total number of calories emptied during periods without food intake by the total time spent without feeding activity during the entire experiment). Also the average rate of caloric intake was calculated for each animal by dividing total food intake by total meal duration.

The data of one animal had to be discarded due to a spillage of labeled food into the rat's restraining cage.

5.3 Results

Typical curves of two single animals are shown in Figs. 5.1 and 5.2.

Fig. 5.1, 5.2 Two individual examples of the curves generated by application of the different regions of interest to the series of successive 1-min gamma camera images.



Curve 1 (top curve, ROI 1): cumulative food intake (kcal/min) over the full 6 ⅔ hour of the experiment.

Curve 2 (regular line, ROI 2): gastric nutrient filling (kcal/min).

Curve 3 (heavy line, ROI 3): cumulative gastric emptying (kcal/min).

L=light phase, D=dark phase (lights off 2 hours after start of experiment)

The Y-axis represents gastrointestinal nutrient contents, expressed as kcals over time. All curves were originally obtained as decay-corrected cumulative radioactive counts per minute within the various ROI's. This data set was transformed into gastrointestinal caloric contents over time by comparing the radioactivity within the whole animal (ROI 1) with the actual food intake of the animals in ml, measured by taking regular readings from the graded burets. Meal size and timing were directly and accurately derived from the cumulative food intake data over the full duration of the experiment: meals are represented by an increase of radioactivity within the animal (plus radioactive feces). The inter-meal

intervals are periods without food intake and are expressed as a fixed plateau phase between rises (see top curve). The heavy solid line represents cumulative gastric emptying, as derived directly from ROI 3.The third curve (ROI 2) shows gastric nutrient filling over time, illustrating the amount of radioactively labeled food present in the stomach, which is increased by meals and followed by a steady decrease of gastric nutritive contents via the emptying process.

At the end of the 400 min period of the experiment, the average number of calories emptied from the stomach was 16.4 ± 1.2 (SEM). Under the assumption that gastric emptying takes place at a relatively constant rate over the day (see Chapter 04), extrapolation of the average emptying rate of 2.46 ± 0.17 kcal/hr that was observed in the present study would have resulted in a total amount of 59.1 (±4.2) kcal being emptied over a 24-hour period. This was not significantly different from the rat's average daily intake of 68.5 (±4.3) kcal/day in the week preceding the experiment ($p>0.05$, 2-tailed t-test). On average, the maximum amount of food found in the rat's stomachs during the course of the study was 15.1 ± 2.0 kcal, which equals 10.0 ± 1.3 ml of the diet. During the 6 ⅔-hr measurement period, the rats ate an average of 29.6 ± 1.9 kcal. Based on the defined meal criteria, the animals took an average number of 6.0 ± 0.37 meals during the experiment, with an average meal size of 4.3 ± 0.27 kcal and an average duration of 7.4 ± 0.44 min.

The total of 90 meals taken by the animals during the experiment were subjected to a more detailed regression analysis. A strong correlation was found between meal size and meal duration ($P<0.0001$, $F=53.2$). During intake of larger meals, a higher number of calories were emptied from the stomach ($P<0.0001$, $F=47.0$); however, the percentage of the meal that was emptied during feeding was less with larger meal size ($P<0.025$, $F=5.5$). Similarly, meals of longer duration caused a significantly higher number of calories to be emptied from the stomach ($P<0.0001$, $F=27.4$), but no effect on the percentage of the

meal being emptied was found ($p>0.38$). A higher rate of food intake during the meal caused a higher number of calories to be emptied from the stomach during the feeding bout ($P<0.01$, $F=8.0$), but had only a non-significant negative effect on the percentage of that meal that was emptied during feeding ($P>0.14$). Meal size and intake rate were positively correlated ($p<0.0001$, $F=3.4$); the average intake rate tended to diminish during longer meals ($p>0.06$).

Since all animals had been food deprived for 31 hours before the start of the experiment, it could be assumed that early in the experiment each animal would have a significantly lower amount of food in its intestines than in the later stages of data collection. Inhibitory feed-back signals arising from the gut could therefore be expected to be on a lower level during the first meals, potentially leading to higher emptying rates in the early stages of the experiment. To explore this issue, four different meals were arbitrarily selected: the first, second and last meal, and a meal taken halfway, i.e. more than 3½ hours into the experiment (see Table 5.1).

	First meal	Second meal	Meal > 3.5 hr.	Last meal
time since start of experiment (min)	9.7 ± 5.0	64.9 ± 10.7	229.9 ± 10.1	316.1 ± 14.0
meal size (kcal):	5.0 ± 0.9	3.4 ± 0.5	4.8 ± 0.5	4.6 ± 0.8
meal duration (min):	8.1 ± 1.4	5.7 ± 0.7	6.9 ± 0.6	8.1 ± 1.1
amount emptied during meal (kcal):	1.1 ± 0.3	0.8 ± 0.1	1.4 ± 0.3	1.0 ± 0.2
% emptied during meal:	22.1 ± 2.8	25.6 ± 2.9	27.2 ± 3.8	26.4 ± 4.2
rate of caloric intake (kcal/min):	0.64 ± 0.07	0.61 ± 0.05	0.71 ± 0.07	0.59 ± 0.08
emptied 10 min post-meal (kcal)	0.19 ± 0.02	0.20 ± 0.05	0.08 ± 0.03	0.14 ± 0.02

Table 5.1. Meal parameters and gastric emptying rates for the first, second and last

voluntary meal and post-meal interval. Values are means ± SEM. No significant differences were found between the four conditions, except for the emptying rate during the 10 min after a meal ($p<0.0001$, $F=43.7$, $n=15$).

No significant differences were found between the four meals in meal size ($P>0.36$, $n=15$), meal duration ($P>0.28$) or rate of caloric intake ($P>0.62$). Therefore, a direct comparison of the gastric emptying characteristics could be made between the four meals. There were no significant differences in the number of calories ($P>0.40$) or percentage of the meal ($P>0.73$) emptied during feeding. The emptying rate during the meal was more rapid, compared with the average gastric emptying rate, with the average emptying rate in-between the meals, or with the average emptying rate during the first 10 minutes of the post-meal interval, showing highly significant differences between the four conditions (Table 5.1, $P<0.0001$, $F=43.7$, $n=15$).

	emptying rate (kcal/hr)	p values vs. average emptying rate	p values vs. emptying in-between meals
average emptying rate	2.46 ± 0.18	*n. a.*	$p < 0.025$
emptying rate during meals	8.88 ± 0.84	$p < 0.0001$	$p < 0.00001$
in-between meals	1.68 ± 0.12	$p < 0.025$	*n. a.*
during first 10 min post-meal	0.96 ± 0.12	$p < 0.0001$	$p <0.001$
during last 10 min pre-meal	1.71 ± 0.09	$P < 0.025$	$p >0.48$

Table 5.2. Average gastric emptying rates during and between meals and post-meal intervals. Values are means ± SEM ($n=15$). Values for last 10 min pre-meal calculated for meals > 30 minutes apart.

A direct comparison between the different conditions (t-test, two-tailed, assuming unequal variance) showed a significantly faster emptying rate during meals compared to the average emptying rate ($P<0.0001$). In the first 10 minutes after the meal, however, the gastric emptying rate was significantly diminished when compared both to the average emptying rate ($P<0.0001$) and to the average emptying rate in-between meals ($P<0.001$). To evaluate the accuracy of measurements based on a 10 minutes interval, also the emptying rate was measured during the last 10 minutes preceding each meal. Since measurements taken shortly after a preceding meal (when stomach emptying could be inhibited) could lead to an artificially diminished value, meals were excluded that were taken less than 30 min apart. Of course, no pre-meal information was available for the first meals, when the stomachs of the rats were still empty. For the remaining meals no significant differences between the 10 min pre-meal interval and the average intermeal rate of gastric emptying could be established ($p>0.48$, $n=50$).

A regression analysis was performed on the total of all 90 meals taken over the time course of the experiment, exploring the effects of time of day and of different levels of gastrointestinal filling. The timing of the meals had no significant negative effects on meal size ($P>0.74$), meal duration ($P>0.83$) or intake rate ($P>0.65$), nor had it effects on the amount ($P>0.80$) or percentage ($P>0.26$) of kcals emptied during feeding. The amount of calories emptied during the 10 min post-meal interval, however, significantly decreased over the time course of the experiment ($P<0.025$, $F=5.4$).

5.4 Discussion

This study presents for the first time, data of food intake, gastric caloric filling and gastric emptying measured simultaneously over several hours in conscious animals with free access to a standard liquid diet. Gamma scintigraphy is currently considered the gold standard for gastric emptying studies, but for practical reasons, it is still mainly used in

human studies for the determination of a gastric retention curve following a single meal. A main limitation of the standard gastric retention method is that this approach is unsuitable to accurately measure changes in gastrointestinal contents during the ingestion of food: gastric contents during this period are the resultant of food moving into and out of the stomach simultaneously. If only a gastric retention curve is measured, changes in the rate of gastric emptying during this period may remain largely undetected by this method. The adaptation of this noninvasive technique, measuring the cumulative amount of radioactive label in the intestines and faeces, allowed the measurement of gastric emptying both during and between several meals taken voluntarily over 6-7 hours. The rats could be left relatively undisturbed throughout the experiment and data could be collected over extended time periods without repeatedly handling the animals. This procedure minimizes disruption of the feeding pattern and stress-related inhibition of gastric emptying.

The most striking result of the present study was the finding that the rate of gastric emptying, which is fairly constant when measured over longer time periods, appears to vary considerably in periods when feeding occurs. Three different phases could be identified: during food intake there was an elevated rate of gastric emptying, which was followed by an attenuation of the emptying rate directly after meal termination. Thereafter, stomach emptying continues at a fairly constant intermediate rate.

The major part of the incoming food, in the present study on average 73.1 ± 2.7% of total meal size, still remained stored in the stomach at the end of the meals. Pregastric and gastric reflex mechanisms that inhibit gastric motility and decrease intragastric pressure are thought to be involved in this process: swallowing food induces a vagal reflex that induces relaxation of the proximal stomach (1;42;72;170;240)which counteracts the increase of intragastric pressure that would normally result from gastric distension due to the arrival of new food in the stomach. An increase of intragastric pressure in itself activates another reflex mechanism (2;170;462), resulting in a further relaxation of the

gastric fundus. Also post-gastric feedback mechanisms arising from intestinal osmo-, chemo- and mechanoreceptors are thought to be involved in the inhibition of gastric motility and may induce gastri rela ation, lowering the rate of gastr c emptyin . However, these inhibitory me hanisms appear to be less effective in controlling gastric emptying during meals: the present data confirm and extend recent findings of Kaplan et al. and Mc Hugh & Moran (177;249), showing that in the rat and rhesus monkey the emptying rate of the stomach is increased during food intake. Kaplan et al. have already demonstrated that this elevated emptying rate is maintained for the full duration of a meal (177) and the present data show that this still holds true for multiple meals ingested at various levels of gastrointestinal filling. Similar to previous results of Kaplan et al (177), the present study found a direct correlation between ingestion rate and the number of calories emptied during meals. However, the percentage of the meal that was emptied during feeding was not affected by the ingestion rate. In accordance with the "volume differential hypothesis" proposed by Kaplan et al. (177), the arrival of fresh food in the stomach and the resulting dynamic changes in intragastric pressure appear to be the major driving force behind the described phase of rapid gastric emptying.

The amount and the percentage of ingested food that emptied from the stomach during each meal did not significantly differ during the first, second, middle and last meal of the 6.7 hr experimental period (see Table 5.1). This result is quite interesting since there are two major changes that occur in the gastrointestinal tract, especially during the early phase of the experimental period which started after a food deprivation period of 31 hours. The stomach becomes more and more distended with food and the food that is emptied from the stomach moves down the gut, spreads over a larger and larger surface area and is digested and absorbed along the way. Apparently, these changes had no major effect on the amount or percentage of ingested food that emptied from the stomach during each meal. In particular, the increase in stomach emptying that normally occurs during a meal

was not affected by the level of intragastric meal volume, since the present data show that the intragastric food contents were quite different during each of these meals yet similar amounts and percentages of ingested food were released during all the meals. In addition, the amount emptied during a meal did not seem to be controlled by the amount of food that was present in or absorbed from the small intestine. Again, similar amounts and percentages of ingested food left the stomach during each of these meals, but, especially during the first one or two meals after the deprivation period, one may assume that the amount of food that was already present in the small intestine (and quite probably the length of small intestine that was in contact with and stimulated by food) was smaller than during the later meals. The most likely explanation for the consistent pattern of emptying during the meals is that the increase in gastric distension and intragastric pressure during the meal allows a certain percentage of the gastric contents to pass on immediately into the digestive tract.

The percentage of the meal that was emptied during feeding (27% in the present study) is quite comparable to the values that can be derived from the study of Kaplan et al. (ca 35%, (177)), thus validating results that were acquired via two entirely different techniques. In their carefully designed study, glucose solutions were infused directly into the stomach at rates similar to normal ingestion rates for the rat, whereas in the present experiment, the rats were allowed normal oral intake of the meals, thus allowing the activation of the receptive relaxation reflex.It should be kept in mind that all experiments described in this dissertation were carried out using one single concentration of the diet. Different concentrations would not necessarily have induced exactly the same percentage of the meals to be emptied from the stomach.

Another basic result of this study was the decrease in gastric emptying rate that occurred during the 10 min after a meal as compared to the average emptying rate during the intermeal intervals. The average emptying rate between meals was 1.68 kcal/hr while the

emptying rate in the 10 min following a meal was 0.96 kcal/hr or 57% of the average intermeal rate. These data show that emptying slows down just after the meal-related delivery of food to the upper small intestine. This effect is relatively short-lived in nature, as illustrated by the fact that the emptying rate during the 10 min pre-meal intervals was not different from the average emptying rate in-between meals when calculated for meals more than 30 minutes apart. An important conclusion that can be drawn from the emptying rate during the 10 min pre-meal interval is that meal initiation apparently is not caused by lack of nutrient availability on the level of the gastrointestinal tract. This casts some doubt on one current theory that states that meal initiation is triggered by a temporarily small decrease in the blood glucose level (39-41;207;209;225;233). Although the pattern in itself has been well documented, the underlying mechanism is not known. The present data suggest that this drop in plasma glucose levels may be a secondary effect of some other factor, that is somehow associated with the preparation of the rat for an imminent meal, rather than a reduction in nutrient availability in the gastrointestinal tract and a consequential decrease in glucose influx into the bloodstream.

The results suggests that, when stomach contents are at a stable level, the presence of food in the small intestine inhibits stomach emptying, as has been hypothesized for many years (16;30;159;160;214;216;217;248;252). Also the present data show that this inhibition of gastric emptying just after a meal is greater in the later meals than in the earlier meals ($p<0.025$, see Table 5.1). The decrease in gastric emptying rate immediately following a meal is most likely to be caused by increased stimulation of the small intestine. It could be hypothesized that, after the initial fast and during the further course of the experiment, more and more food had been released from the stomach and had spread over a larger surface area of the small intestine, supposedly until a balance had been reached between the rate of gastric emptying and the absorptive capacity of the gut. A faster rate of gastric emptying, as occurs during a meal, would then lead to an increase in

nutrient availability in the intestines. Thus the signals that were generated by the presence of food in the small intestine could intensify and lead to a greater reduction in the rate of gastric emptying in the 10 min post-meal interval (214).

Taken together, the data suggest that, during and after meals, changes do occur in the level and expression of inhibitory signals on gastric emptying. During a meal, a larger amount of nutrients is emptied into the intestines (177), compared to the average rate of emptying over the full day. This initial phase of rapid emptying, which, to a large extent, escapes intestinal feedback regulation and is possibly driven by increases in intragastric pressure, would then generate a higher level of receptor stimulation in the gut, inducing stronger inhibitory feed-back signals on gastric emptying via relaxation of the gastric fundus and increased resistance in the pyloric-duodenal region (16;240;252;262). In the later phase of the meal, some of the intestinal contents will also be absorbed into the bloodstream (391), possibly activating post-absorptive inhibitory mechanisms (239;353). Thus, in this phase, the stomach may already receive a higher level of inhibitory signals. The existence of a certain level of inhibitory feedback even during the meal is demonstrated by the finding that emptying during feeding is faster after gastric branch vagotomy (176). The arrival of fresh food would still induce changes in intragastric pressure, causing relatively rapid emptying. After meal termination, however, with the level of stomach filling stabilized, a more precisely regulated intestinal phase of gastric emptying would take place: a higher level of stimulation of intestinal receptors, and/or stimulation of a longer stretch of intestine, would evoke stronger inhibitory signals on gastric emptying, A phase of slower stomach emptying would then be established, until the excess of food was fully absorbed from the gut. Thereafter, stomach emptying would resume at its standard intermediate rate.

The stomach is often considered as a main source for the generation of satiety signals (177;249;406). The present data, showing faster gastric emptying during feeding, do not

exclude that under natural feeding conditions both gastric and postgastric factors may be involved in meal termination: not only is there an increased distension of the stomach, but also a substantial part of the meal is already leaving the stomach during the meal, thus providing a possible intestinal or postabsorptive signal for the inhibition of food intake.

Another important conclusion is that the absolute level of gastric distension by itself does not control the termination of food intake. The first meal is terminated at a level of intragastric meal volume that is insufficient to terminate the second or third meal (see Fig. 5.1 and 5.2). Successive meals are started and terminated at increased levels of gastric filling, showing that the feeding control system must use distension along with other cues to terminate a meal or must receive a gastric distension signal modified by endogenous circadian rhythms. Five hours into the study, the two individual animals depicted in Figs. 5.2 and 5.3 held 4.5 resp. 21 kcal of food in their stomachs. In how far these values can be converted in stomach volume is questionable: it should be kept in mind that the measurements of gastric nutrient contents were based on the intake of radioactively labeled food particles. Any (unlabeled) secretion of gastric juices into the lumen of the stomach would add to the total gastric volume whereas emptying of unlabeled liquids would decrease gastric volume; this would remain undetected by the gamma camera,

An interesting result from the present study is that food is emptied from the stomach at a rate that is fairly constant throughout the 6.7 hr observation period. Although there is an increase in the rate of stomach emptying during each meal and a slowing of gastric emptying just after the meal is complete, a look at the cumulative curves in Figs 5.2 and 5.3 shows that gastric emptying continues at a fairly steady rate between meals throughout the study period. This is somewhat different from many scintigraphic studies showing an exponentially declining emptying rate which may be explained by the fact that most of these studies measure only gastric emptying after one single meal, sometimes including at least part of the feeding period itself. Since gastric emptying is faster during the meal,

while the last traces of the radioactive marker will not empty easily from an almost-empty stomach, the best-fitting curve would suggest an emptying rate with an exponential decay. The present experiment avoids these problems by allowing the rats to eat several meals, thus measuring gastric emptyin from a generally well-filled stomach.

Food intake patterns and gastrointestinal transit can be altered under the influence of stress (438). An attempt was made to minimize these effects in the present studies: the rats were used to the restraint of parabiosis and they had undergone several weeks of adaptation to the experimental conditions. Although restraint is commonly described as a potent stressor, these effects are thought to be stronger during a single exposure and appear to decrease during repeated restraint (283). Furthermore, the animals were kept in relatively loose restraint, compared to most restraining studies. On a behavioral level, no clear aversion to the conditions was found: the animals could be readily positioned in their restraining cages after a few days of training, and did not display obvious visual signs of discomfort during the experiments. Ideally, the total number of calories emptied from the stomach over a 24-hr period should be similar to the average daily food intake of the animals. The fact that in the present experiment the estimated gastric emptying rate was not significantly different from the average daily food intake of the rats suggests that the data that were generated in this study are in a normal physiological range. These data underscore the importance of inclusion of the ingestion period of a meal in the gastric emptying measurement (30;177).

During the 6 ⅔-hr measurement period the animals ate an average of 29.6 ± 1.9 kcal, leaving 3.6 ml of food intake that were not accounted for after application of the meal criteria. This discrepancy results from small meals of less than 1.0 kcal in size or from occasional drops falling from the feeding spouts that are readily licked up by the rats without inducing further feeding behavior. The total intake covered almost 50% of the animal's daily needs over a time period that included less than five hours of the 12-hour

dark period. Normally about 85% of the daily intake of the rat, a nocturnal animal, takes place in the dark phase (398). Thus, the rats ate an acceptable amount of food during the study compared to their normal intake in this phase of the circadian cycle. Since other meal parameters, such as the relationship between meal size and meal duration, were also well in accordance with behavioral studies , the data suggest that the animals were behaviorally well adjusted to the experimental conditions and that, at least at a behavioral level, the measurements were not affected in a major way by external stress factors.

By using the standard liquid diet, Ensure plus, both during the training period as well as during the study itself, a relatively normal feeding pattern was obtained. The chosen diet is quite comparable in composition with the standard North-American diet, allowing all the macronutrients to stimulate the gut in a normal way. The use of a less natural or less complex food source, such as a single macronutrient like glucose or a non-nutritive substance, may generate a different emptying pattern (177).

The quality and resolution of the images that were acquired via the gamma camera were sufficient to accurately discriminate and outline the desired gastrointestinal structures throughout the study. Contrary to human studies, the small size of rats did not allow for detailed measurement of movements of food within the stomach. However, their small body mass also had the advantage that the effects of tissue attenuation of the gamma radiation were less severe, so that all measurements could be made with a single, anteriorly placed, gamma camera.

In summary, a new technique was described for the measurement of the movement of radiolabeled food through the GI tract while an animal is free-feeding over 6-7 hours. Scintigraphy is a non-invasive technique that avoids the use of tubes and direct handling for the infusion or withdrawal of stomach or gut contents, but has confirmed many earlier results that used these techniques. By defining three regions of interest within the gamma camera image, it was possible to measure continuously and concurrently the time and rate

of spontaneous food intake, the changing nutrient content within the stomach and the minute-by-minute rate of gastric emptying. The major change from current scintigraphic technique is the measurement of radioactivity in the intestines and feces to obtain a more accurate measurement of the rate of gastric emptying. The present results argue against fixed set points for stomach distension as major signals for meal initiation or termination; the continuous availability of nutrients in the intestines and the different levels of stomach filling at the start and end of different meals suggest that meal patterns are not likely to be regulated by signals arising from the gastrointestinal tract alone. The data show that fluctuations in gastric emptying rate, caused mainly by food intake, occur over the 6 ⅔ hr study period, which included the early dark phase of the circadian cycle. During meals, gastric emptying is fast and appears to be influenced by the delivery of new food to the stomach, whereas between meals, gastric emptying seems to be under more accurate feedback regulation by the intestines or other post-absorptive signals. The higher gastric emptying rate during feeding is followed by an inhibition of emptying directly after the meal, which ensures that over longer periods of time a fairly constant emptying rate can be maintained. The fact that later in the experiment, when the gastrointestinal tract was fully stimulated with nutrients, a higher level of immediate post-meal inhibition of gastric emptying was found, suggests that this inhibition of gastric emptying may be affected by the length of intestine stimulated by food.

Chapter 6: Measurement of gastric distention and gastric emptying throughout the 24-hour circadian cycle.

6.1 Introduction

Gastric emptying, digestion and absorption from the intestinal tract form the connecting link between food intake behavior (that is aimed at securing a sufficient supply of nutrients to the body by taking a number of meals at discreet intervals over the day) on one side and the continuous (and continuously changing) nutrient and energy needs of the body on the other. A precise regulation of the energy flow within the body takes place via neural and hormonal pathways, redirecting temporal excess nutrients into the storage tissues or releasing these from the same depots if a relative energy deficit occurs. Since in a well fed animal these nutrient depots, especially the fat stores, are sufficient to cover its energy needs for extended periods, theoretically the fluctuations in the internal energy needs and availability could be completely covered by redirecting these metabolic pathways; under normal free-feeding circumstances food intake does not occur as an immediate response to an immediate energy deficit in any of the body organs, but rather functions to replete the different storage sites after a certain amount of energy or nutrients have been withdrawn. The stomach (although technically speaking located "outside" the body) serves, via its reservoir function, effectively as one of these storage sites. The process of gastric emptying can act as a first level of regulation to smoothen the delivery of nutrients to the tissues and dampen the fluctuations in the energy flux that would otherwise be caused by the periodic intake of meals and periods of non-feeding.

The process of gastric emptying of meals has been extensively studied, using a wide variety of techniques (423). Nonetheless, many questions about the regulation over the day of gastrointestinal motility and the delivery of nutrients to the body are still unresolved, partly due to technical factors: many measurement techniques are invasive and

often do not allow the undisturbed animal to take voluntary meals, also usually the emptying rate of only a single meal is measured, often after termination of feeding. Gastric aspiration only measures liquid emptying; also intubation techniques may directly affect gastric emptying (322). Measurement of the rate of gastric emptying under such circumstances often leads to artificial results: an estimation of the number of calories emptied from the stomach over the full day via extrapolation of single-meal data rarely matches the actual average total nutrient intake of the animal (30;177), most likely because increases in gastric emptying rate during feeding are not accurately measured (177). The modified scintigraphic technique that was applied for the present experiment (see Chapter 3), however, was specifically designed for long-term measurement of gastric emptying over several meals and was applied to determine the rate of gastric emptying over a full day.

The purpose of the present study was to acquire simultaneously information about 24-hour variations in the pattern of food intake, gastric filling, gastric emptying and intestinal transit of free-feeding rats that had access to a radioactive labeled, liquid diet, Ensure plus. Although several earlier studies found circadian rhythms in food intake and gastric filling, data about daily variation in gastric emptying and small intestinal content are conflicting (14;123;415;419). Precise knowledge of possible fluctuations in the degree of gastric filling and the rate of gastric emptying over the day is therefore still lacking, although this would be essential for a better understanding of the regulation of food intake over the day and the links between food intake and metabolism. The measurement of gastric filling over the day was used to test the hypothesis that gastric distension is one of the main satiety signals that terminate meals.

6.2 Material and Methods

6.2.1 Experimental Design

The experiments began at 16.00 (the normal time for refeeding of the animals) and ran for 26 hours (i.e. from 16.00 until 18.00 the next day), with lights on between 06.00 and 18.00. The extension of 2 hours was added to the full 24 hour period because of the possibility that some unlabeled food from the previous day could still remain in the rat's stomach. This could effectively decrease the measured emptying rate during the beginning of the experiment, since the labeled food would be diluted and co-emptied with the unlabeled leftovers. This problem would not occur during the last two hours of the experiment, since after a full day into the experiment all old food in the stomach would have been replaced by fresh, labeled food.

The rats were put in their cages one hour prior to the start of the study and were positioned above the gamma camera before 15.45. The radioactive, non-absorbable marker ^{99m}Tc sulphur colloid was added to the liquid diet Ensure plus (2 mCi per 100 ml food), the well mixed labeled diet was transferred to graded burettes, and the rats were allowed access to the food at 16.00. The food was removed again the next day between 9.00 and 16.00; by maintaining the same food deprivation period as during the maintenance routine, its effects on stomach filling at the start of the feeding period could be assessed. The effects on the normal meal pattern of the rats were relatively small since, during this part of the light phase, activity and food intake are at their lowest level. Lights were dimmed to a low level between 18.00 and 06.00. During that period the rats were further protected against the light by application of isolating curtains around the cages. Food intake during the whole experiment was measured at regular intervals for each animal individually. In the final analysis of the data these data were used to standardize the radioactive count values by correlating the total amount of radioactivity in the GI-tract of

the animals (as measured via the gamma-camera) with their actual food intake.

6.2.2 Data Acquisition

The general protocol for data acquisition was followed as described in Chapter 3.7. Data acquisition took place continuously in 2-min intervals for the full duration of the experiment, resulting in 26-hour curves that were decay-corrected and corrected by hand for motion artifacts (see Chapter 3.8). Also total gastrointestinal transit for the labeled food was established for each animal by identifying the first time point where radioactive feces were excreted; these events were easily recognizable on the series of images. Since often the marker had reached the most distal sites of the GI tract well before defecation, also the first unequivocal appearance in the rectum was determined. However, this value could not be determined completely objectively, since it is influenced by the fact that the gamma camera's view on the full stretch of rectum is partly obscured by more proximal parts of the GI tract overlaying the most distal sites; the location of these stretches of intestine within the peritoneal cavity appears to change often over time, caused by movements of the animal.

An important issue regarding the regulation of food intake is the possible generation of satiety signals by gastric distension. The volume contained in the stomach consists of the amount of ingested food plus the added volume of gastric secretion, minus the amount of material that has been emptied from the stomach over time. Since little information about the relationship between stomach nutrient contents and gastric distension is gained from the application of standard-sized ROI's that are drawn with a wide margin around the stomach (See Fig. 3.5), this question was investigated by drawing ROI's tightly around the boundaries of the stomach on the computer image, determining the number of pixels contained within the ROI, and measuring the number of radioactive counts within the stomach by applying the ROI to that image. One image per hour for the last 24 hours of

the experiment was analyzed for all animals and the correlation between stomach contents (in counts/2 min) and stomach surface area (in pixels) was determined, so that the relationship between gastric nutritive content and two-dimensional outline of the stomach could be examined. This comparison would allow the effects of (unlabeled) gastric secretion on total gastric volume over the day to be assessed. All ROI's were drawn using a single color map and fixed values for the sensitivity (Thal_256 Linear; Background=0; Brightness=2300), so as to standardize the color or intensity (= the level of radioactivity) on the computer screen that indicated the outer boundaries of the stomach.

6.3 Results

Since the pairs of parabiotic rats shared a common peritoneal cavity so that sometimes a part of the intestinal tract of one rat was located in the intestinal ROI of its partner, a considerable amount of noise was created in the individual animals' emptying curves. The stomachs of the animals, however, were always well separated from each other on the images and could accurately be defined. This allowed for a straightforward correction method for this problem by calculating the average food intake, gastric emptying and stomach filling curves per pair, thus eliminating the need for a more labor intensive manual frame-by-frame correction for each animal separately.

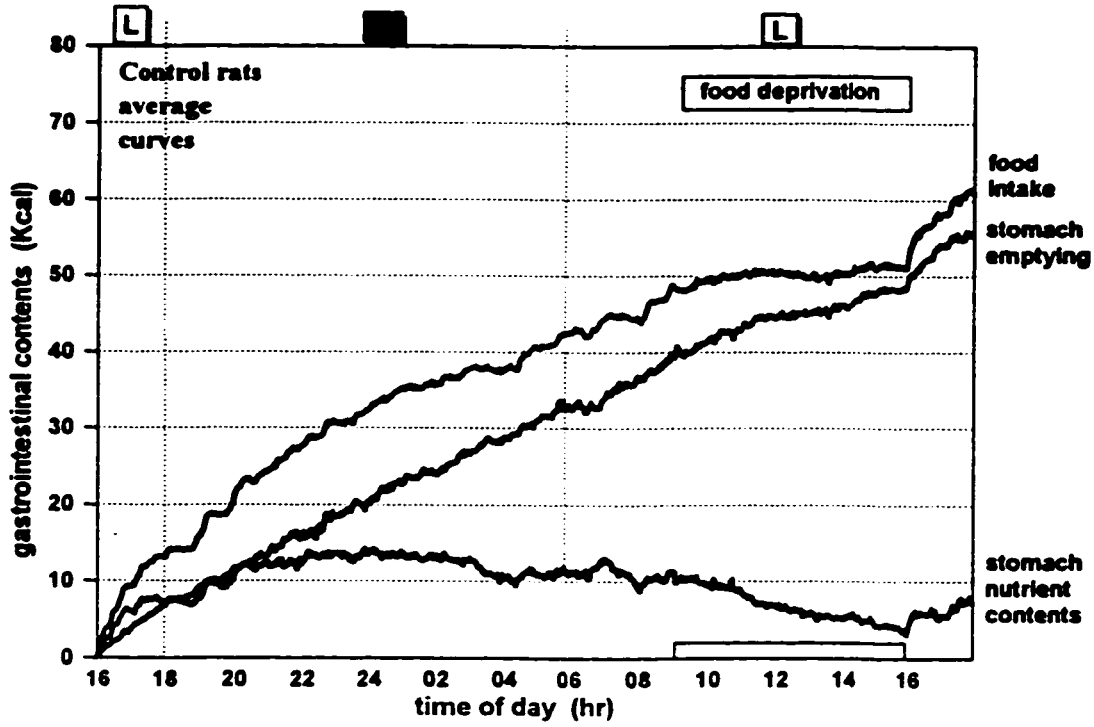


Fig.6.1. Control rats: average 26-hr curves of food intake, stomach emptying and stomach caloric contents (n=6x2). L=light phase, D=dark phase. Food deprivation from 09.00-16.00.

The average cumulative food intake, the average emptying rate and the degree of gastric filling for the six pairs are shown in fig 6.1. The data, that were collected in radioactive counts per 2 min, were converted into kcal / 2 min by comparing the radioactive counts values with the animals' actual food intake in ml at the end of the experiment. On average, gastric emptying appeared to take place at a practically constant rate over night and day, in this study with an average rate of 2.13 ± 0.17 (SEM) kcal/hr. Over the full day considerable changes took place in food intake and in the degree of gastric filling. The average 24-hr emptying rate of 51.1 ± 4.1 kcal/day was not significantly different from the average food intake of the same animals of 60.1 ± 3.6 kcal/day in the week preceding the experiment.

Examination of the "overlapping" period of an extra two hours that had been added to the end of the 24-hour experiment, as a precaution against the possibility that at the beginning of the experiment a certain amount of unlabeled food could still be contained in the stomach, showed that this potential source of error did not affect the results under the experimental conditions. A comparison between the first and the last two hours of the experiment (i.e. the equivalent periods of day 1 and 2) did not show any significant differences for food intake ($p>0.09$), gastric contents ($p>0.4$) or gastric emptying ($p>0.8$).

During the light phase, the activity and food intake of nocturnal animals is on a lower level than in the dark. In combination with the 7-hr food deprivation period (09.00-16.00) clear effects on food intake could be expected: indeed the comparison between the dark phase (18.00-06.00) and the light phase (06.00-18.00) of the last 24 hours of the experiment showed a significant attenuation of total food intake in the light period ($p<0.05$, $F=4.96$). The average level of gastric filling was lower in the light phase, but not on a significant level ($p>0.07$). Interestingly, the average rate of gastric emptying was not affected by the circadian cycle or by the food deprivation period ($p>0.43$). A more detailed analysis (ANOVA) in which the effects of time of day were investigated by comparing 2-hour periods throughout the day gave similar results: a highly significant effect on food intake ($p<0.00001$, $F=5.27$), a highly significant effect on gastric contents ($p<0.0025$, $F=3.12$), and no effect on gastric emptying ($p>0.21$).

In the present experiment, the animals had their highest food intake in the first hours after they had been given access to the food at an average rate of intake that was higher than the average rate of gastric emptying, effectively filling up the stomach to its highest level halfway through the dark phase. For the rest of the dark phase and the first two hours of the light phase, food intake and gastric emptying were more or less in equilibrium, so that the stomach contents remained on a similar level. The rest of the light phase was characterized by a gradual decline in stomach contents. A comparison between

food intake and gastric emptying by t-test (two-tailed, assuming unequal variance) for successive 2-hr periods showed only significant differences between 16.00-18.00 hr, 20.00-22.00 hr, and 10.00-14.00 hr ($p<0.05$).

A look at the individual data reveals again how, in spite of large fluctuations in food intake (Fig. 6.2) and gastric filling (Fig. 6.3) over time and between animals, still quite similar emptying curves emerge (Fig. 6.4). One drawback of the experimental design with free-feeding animals became evident: four of the six pairs have very similar 24-hr emptying rates in the range of 50-60 kcal/24 hr, but the two other pairs had considerably lower emptying rates (in the range of 35-40 kcal/day), and one animal of each of these two pairs also had an unusually low food intake in the period preceding the food deprivation (between 09.00 and 16.00). Since gastric emptying is absent when the stomach contains insufficient labeled food, the attenuated emptying rate during the food deprivation can be attributed to the empty stomachs of the animals in question.

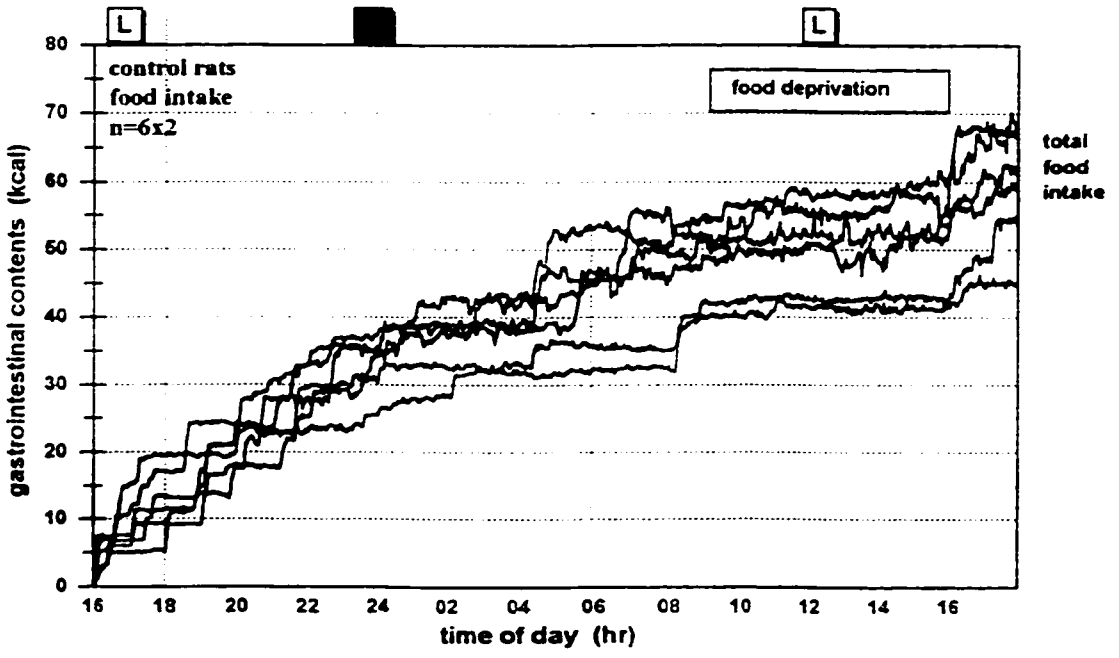


Fig. 6.2. Control rats: cumulative food intake of all animals over the full 26-hour experiment, averaged per pair. L=light phase, D=dark phase. Food deprivation from 09.00-16.00.

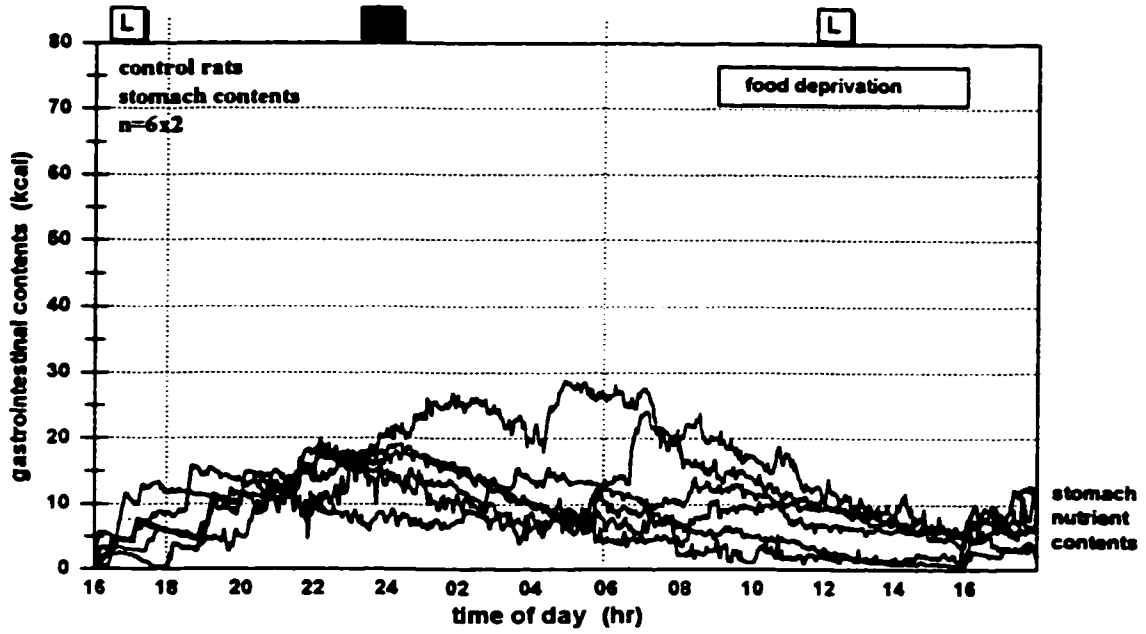


Fig. 6.3. Control rats: stomach contents of all animals over the full 26-hour experiment, averaged per pair. L=light phase, D=dark phase. Food deprivation from 09.00-16.00.

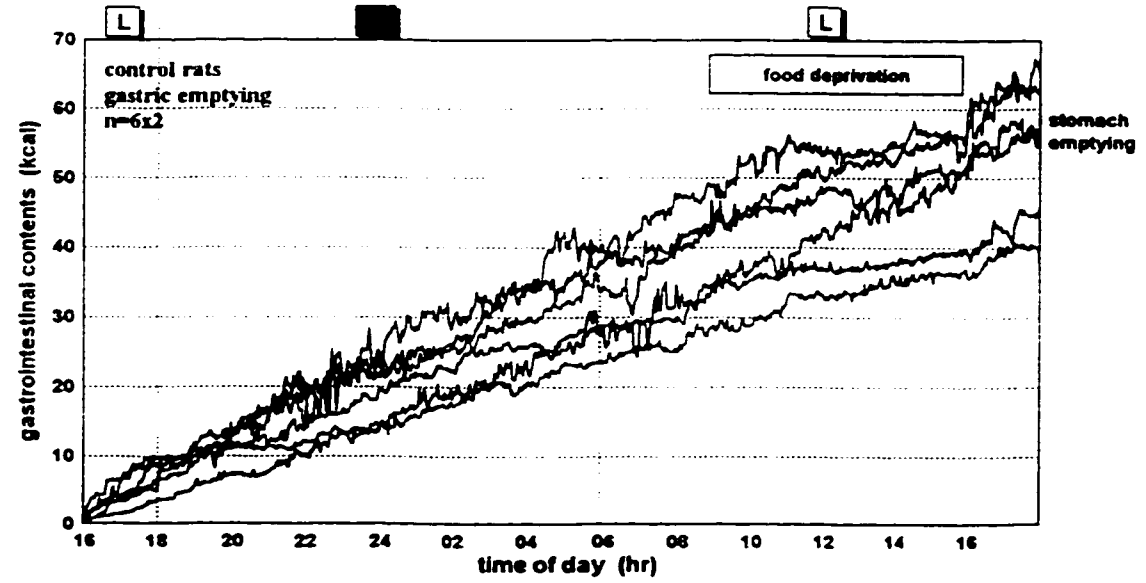


Fig. 6.4. Control rats: gastric emptying of all animals over the full 26-hour experiment, averaged per pair. L=light phase, D=dark phase. Food deprivation from 09.00-16.00.

The results for gastrointestinal transit showed that on average the ^{99m}Tc sulphur colloid label was transported over the full length of the GI tract in 407.8 ± 35.1 (SEM) min. The first unambiguous appearance in the rectum took place after 359.6 ± 28.0 min, which was not significantly different from the value for complete GI transit ($p>0.29$).

The data acquired by drawing ROI's exactly along the outer boundaries of the stomach showed a highly significant correlation between gastric contents (converted in kcal) and stomach surface area over the full 24 hours of the experiment (Fig. 6.5).

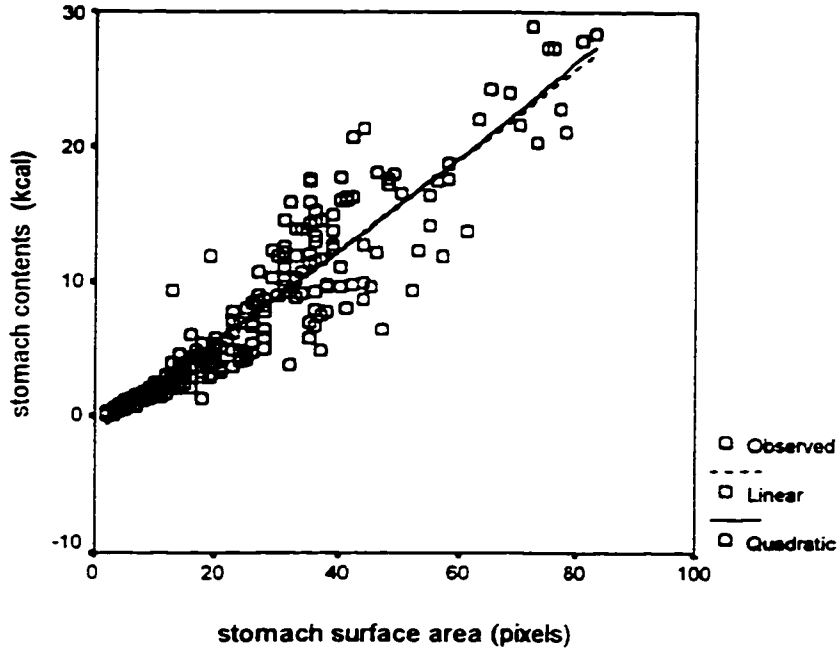


Fig. 6.5. Control rats: relationship between gastric contents and stomach surface over the full day (n=288).

The best fitting linear curve showed a highly significant correlation ($y=ax+b$, $a=0.339 \pm 0.008$, $b= -1.214 \pm 0.219$, $p<00005$, $F=1978.8$, $r=0.935$, $n=288$), also a best fitting quadratic curve showed a highly significant correlation ($y=ax^2+bx+c$: $a=0.0002 \pm 0.0003$, $b=0.323 \pm 0.022$, $c= -1.062 \pm 0.298$, $p<0.00005$, $F=988.2$, $r=0.935$), but it was hardly different from the linear one. Since 50 % of these data were acquired in the light phase

when stomach contents are generally lower, this could have overemphasized values in the lower range. Therefore a second analysis was performed on this data set for the night values only. The results were similar to the 24-hour data (Fig. 6.6): a highly significant correlation for a linear best fitting curve ($a=0.336 \pm 0.012$, $b= -1.555 \pm 0.388$, $p<0.00005$, $F=853.3$, $r=0.922$, $n=144$) as well as for a quadratic one ($a=0.0001 \pm 0.0004$, $b=0.326 \pm 0.037$, $c= -1.435 \pm 0.569$, $p<0.00005$, $r=0.922$, $F=433.3$).

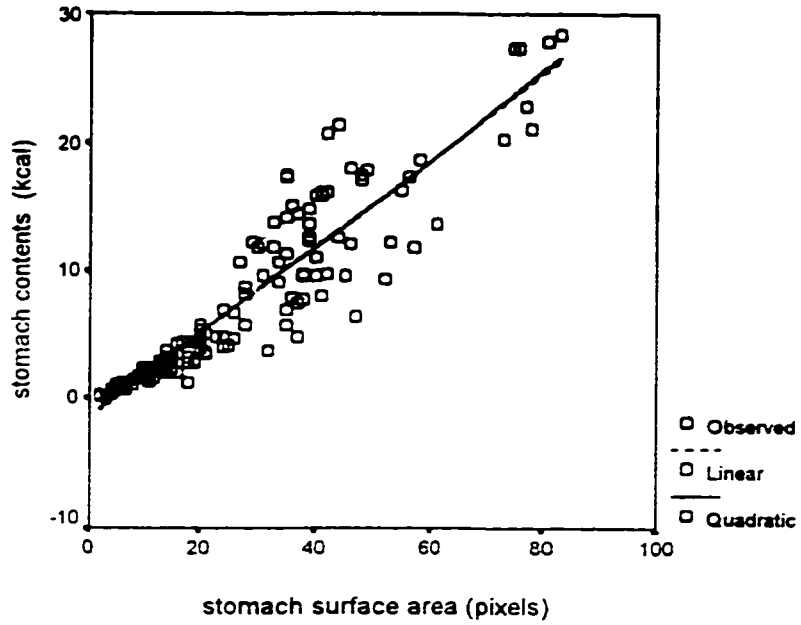


Fig. 6.6. Control rats: relationship between gastric contents and stomach surface during the dark phase (n=144).

6.4 Discussion

The results show that, although minor artifacts due to small animal movements and to the random character of radioactive decay and background radiation still occur, the method of gamma camera scintigraphy appears generally effective for measurement of food intake, gastric nutrient content and gastric emptying simultaneously over periods as

long as a full day. Since the experiment was carried out over a time period of more than four half-lives for the ^{99m}Tc marker, towards the end of the 26-hour period the relative effects of background radiation and stochastic noise in the data had increased by more than 16-fold (after decay-correction of the data). Although an increase in noise on the data is noticeable in the individual curves, the fact that the experiments were carried out with a considerable amount of radioactive label (in this case 2 mCi per 100 ml of food; many short-term human studies use less than 1 mCi) assured that the effects on the results were minimal. Studies that are extended well past the duration of the present experiment, however, may need to use a different radioisotope with a longer half-life than ^{99m}Tc, since over time the quality of the data will deteriorate further, so that at some point in time the signal-noise ratio may become unacceptable. Increasing the concentration of the marker would not be an attractive option because of increased radioactive exposure for the experimenter.

The most striking result of the present experiment is that stomach emptying appears to be a tightly regulated process that, when measured over longer time periods, takes place at a practically constant rate over night and day, in spite of considerable changes in food intake and in the degree of gastric filling over this period. Although there is a considerable variety in the temporal pattern of gastric filling between the animals and a wide fluctuation of gastric contents of each rat over time, this does not translate in an analogous variety in emptying rate. Apparently, the process of gastric emptying takes place relatively independent from the physical effects exerted by incoming food and/or distension of the stomach, and is under continuous control by physiological feed-back mechanisms. The absence of a circadian variation in emptying rate was somewhat surprising, since such an effect was described in earlier studies (123;415;419). These studies, however, applied restricted feeding schedules (123;415), were only measuring on two different times of the day while possible differences in gastrointestinal contents were not completely accounted

for (123) or measured gastrointestinal transport of a synthetic diet {Vachon & Savoie 1987 ID: 2227}, and may not be fully representative for a normal physiological situation. In another study were rats were allowed continuous access to a standard diet no light-dark variation in emptying rate was found (14).

The present data confirm and extend previous findings that stomach emptying of a single meal takes place at a relatively stable rate (160;248;249). The present results show that this relationship remains intact over a wide range of gastric filling and independent of time of day and of food intake. The modified techniques that were used for the present experiments allowed not only monitoring of gastric filling and stomach emptying simultaneously, but also a relatively high resolution involving measurements every 2-min over the full 24-hour period, compared to studies that use stomach catheters to withdraw gastric contents after meal infusion or feeding. Recent reports that indicate that the rate of emptying is temporarily increased during meals (177;248;422) and is directly associated with the dynamic changes in gastric volume caused by the incoming meal (177)could only partly be confirmed by the present experiment: although closer inspection of the individual data revealed fluctuations of gastric emptying rate during and shortly after meals, a data collection in 1-min intervals was thought to be more appropriate to make precise measurements of the relevant parameters (See Chapter 5). The fact that no significant difference was found in the rate of gastric emptying during periods of high feeding activity earlier in the dark phase, compared to periods of low food intake (or food deprivation) in the light period, suggests more complicated changes in the pattern of gastric emptying around meals than just an alternation between a high emptying rate during feeding and a slower rate in-between meals: it would imply that the transient faster emptying rate during a meal must be partly compensated for by an attenuation of the emptying rate immediately after this initial rush of food into the small intestines has taken place.

The fact that on average over the full 24 hour period (Fig. 6.5) or during the dark (Fig.

6.6) a strong correlation is maintained between stomach contents and gastric surface area would suggest that the contribution of gastric secretion to gastric volume is relatively constant over time. A certain noise in the data around the ideal curve is noticeable; this noise may reflect imperfections in the ROI analysis or it still may reflect some inherent variation in 2-dimensional representation of nutritive contents within the stomach (see also Fig. 6.7). This would be in accordance with earlier studies in humans where, after ingestion of a mixed meal, liquids were emptied quickly; thereafter an equilibrium was established between gastric secretion and liquid emptying (236;237). The absolute level of gastric distension in this experiment, however, can probably not be directly derived from the caloric gastric contents (via the original composition of the diet where 1 ml of Ensure plus equals 1.5 kcal); a certain percentage of the food consists of water, and the liquid phase may be emptied relatively quickly from the stomach, so that counts only represent the solid particles from the diet. Furthermore, the two-dimensional area obtained from counting activated pixels will not necessarily accurately represent the three dimensional character of the nutritive contents found within the lumen of the stomach. The J shape of the curve relating stomach contents to stomach surface area (Figs 6.5 & 6.6) suggests that, at low volumes, the nutritive material spreads into the empty lumen and covers a larger area than its 3-dimensional mass would require. However, at higher gastric volumes, the most interesting aspect of this data correlation is that the surface area provides such a good linear representation of the nutritive volume after an area of about 20 pixels has been covered. This result shows that as contents fill the stomach, it expands along its greater curvature, enlarging its two-dimensional outline to a much greater extent than it expands toward the front and back abdominal muscles, which would enhance volume at the expense of an increased outline of gastric area.

The present results have several important implications for current models concerning the mechanisms that regulate food intake. Since all ingested food at some point will have

to be passed on to the intestines by the stomach, measured over several days the average daily food intake must be almost equal to the average amount of food that leaves the stomach over the 24-hour period. On a simplified level, two basic models could be considered: one option would be that food intake, regulated mainly by post-gastric mechanisms, could take place independently from gastric filling and emptying, and since the stomach has a limited capacity for the storage of food, it would quickly fill up to maximum capacity, and from that point onwards gastric contents would be simply pushed out of the stomach by the physical action of incoming fresh meals, thus making gastric emptying for most of the day a direct derivative function of food intake. Since in the rat food intake is high at night and low in daytime, major changes in emptying rate and in nutrient influx into the bloodstream would be expected over the light-dark cycle. Such a model would be supported by the finding that in the rat the night period is associated with lipogenesis, and the light period with lipolysis (210;212). Alternatively, gastric distension could be a major regulatory signal for feeding behavior, and the degree of gastric filling would be directly regulated by a controlled process of gastric emptying over time. In this case, the rate of food intake would be controlled via the rate of gastric emptying. Supporting evidence for this model would be the fact that behavioral or pharmacological manipulation of daily food intake also can change gastrointestinal motility: the appetite suppressing drug fenfluramine has been found to slow the rate of gastric emptying of a test meal (29); also gastric emptying is slower in anorexia nervosa (84;86;90;151;164;244;331;332;408). Several observations derived from the present data set must be considered: 1) the rate of gastric emptying does not seem to be directly correlated with the amount of food in the stomach at any given time of the day (except when the stomach is almost empty), 2) on average the rate of gastric emptying does not appear to change substantially over the light-dark cycle, and 3) the stomach is gradually being filled over the first several hours of the dark phase; meals are being initiated and

terminated at various degrees of gastric filling. Taken together these results would suggest that the rate of stomach emptying is indeed set independently from the rate of food intake. The mechanisms that control the rate of gastric emptying could thus be directly involved in the regulation of daily food intake and body weight.

If gastric distension were a major satiety signal, at least one important issue remains to be resolved: the fact that the satiating effect of a certain degree of gastric distension appears to change over time. Even when the assumption would be made that at the end of the light phase the GI tract is for a major part empty (although this is not supported by literature data or the results of the present experiment), it seems clear that after several hours of gastric emptying with a fairly constant rate this situation would be reversed and satiety signals from all possible pre- and post-absorptive sites, including the hormonal profile that is associated with a fed animal, would be fully expressed. For instance, glycogen stores in the liver increase over the first few hours of the night (211). Nonetheless, new meals are being initiated on a relatively well-filled stomach, whereas meals were terminated early in the study on a relatively empty stomach. Gastric distention, therefore, cannot be a simple, hard-wired signal that terminates a meal as soon as a certain threshold has been reached. A possible modulation of the effects of distension signals by endogenous circadian rhythms can be hypothesized: it is known that lesions to the suprachiasmatic nucleus (SCN) alter the meal pattern of rats dramatically; instead of the typical concentration of feeding in the dark phase, meals are taken at regular intervals over the full light-dark cycle (402). A repeat of the present experiment with SCN-lesioned animals would answer the question if in that case all meals would be terminated at the same level of gastric filling. Alternatively a slow adaptation of gastric mechanoreceptors over several hours could be possible, so that their firing rate slowly declines over time at a certain level of gastric distension.

The finding that gastric emptying takes place at a constant rate over the day raises some

interesting questions about the possible involvement of post-gastric and post-absorptive signals in meal initiation and/or termination. The amount of nutrients that leave the stomach and can be absorbed from the gut appears to be relatively constant over time. Only during feeding does the rate of emptying increase (177;422), but rapid emptying appears to be followed by a temporary inhibition of gastric emptying. The time required for full absorption from the intestines (especially if a non-elemental diet id used) of the relatively small extra amount of calories that are emptied during feeding on top of the normal ongoing gastric emptying, would further blunt the effects on plasma nutrient levels. A post-absorptive mechanism that would meter fluctuations in the plasma nutrient level therefore will need to be highly sensitive for even small changes. This, however, can not be completely excluded; a number of experiments have suggested that rats are able to recognize a specific pattern of small decreases in blood glucose level that may be somehow involved in meal initiation (39-41;207;225). Based on the present data, showing ongoing gastric emptying over the full day, it seems unlikely that fluctuations in nutrient availability in the gut initiate or terminate a single meal. It is difficult to conceive how a post-gastric satiety signal could be evoked by single meals, unless they are generated by the more rapid gastric emptying during meals. The present findings seem to be more supportive for theories that suggest an important role for the stomach in the regulation of food intake (77;79-81;83;250;251).

The present results do not appear to support the finding that a daily cycle of lipogenesis at night and lipolysis in the day is created by changes over the 24-hr period in the rate of gastric emptying. One possible explanation may be found in the quality of the food source that is being used in the various experiments. Since Ensure plus is a concentrated liquid diet that does not contain "bulk" material such as cellulose fibers, it is possible that a rat can cover most of its energy needs during the day with the nutrients that are stored in the stomach at the end of the dark phase. A solid food source of lower caloric value may not

be sufficient in that respect, so that the animal will need to use some of its internal caloric sources, especially the extensive amount of energy stored in its fat tissue.

In summary, scintigraphy has been successfully used to measure food intake, gastric filling and gastric emptying in rats throughout a 26-hour period. Gastric emptying rate is fairly constant throughout the day-night period, while the level of food intake and the degree of gastric filling varies greatly. The data suggest that gastric distention may be a major signal for ending a meal, but the necessary amount of gastric distention varies with the time of day. They also suggest that the rate of gastric emptying may be important in controlling daily food intake.

Chapter 7: Measurement of gastric distention and gastric emptying throughout the 24-hour circadian cycle in one-way crossed-intestines rats.

7.1 Introduction

Although great progress has been made in this century to unravel the different mechanisms that are involved in the regulation of food intake, the exact nature of the signals that evoke satiety is to a large extent still an enigma. Many different approaches have been tried to tackle this problem; in order to isolate the specific physiological factors under investigation, often some type of invasive treatment or surgery is required. In many cases the experimental design involves stimulation of specific sites in the organism, bypassing a number of other types of internal signals that may also be involved in the integrated response to food intake. This implies that in many cases a stimulus is applied that is non-physiological, that is, out of the range that the target of the stimulus would encounter in any normal situation. For instance, many studies involve the infusion of nutrients into the gastrointestinal system or into the bloodstream, but often the physical/chemical composition, the dosage and/or the concentration of the infusion are not in a normal physiological range at the infusion site. Although such experiments can generate important information regarding the factors that may contribute to the regulation of feeding behavior, the results also have to be evaluated with some caution, since the reaction to the stimulus may have limited relevance to normal physiology and may instead reflect some type of escape-mechanism from extreme situations.

The majority of experiments try to establish a reduction of food intake as a reaction to the treatment. This is a very reasonable approach when the research subject under investigation is the generation of satiety, but it has at the same time the considerable drawback that many treatments that will lead to discomfort to the animal generally will

also lead to a reduction in food intake. Although sensitive testing methods have been developed that deal with this issue, such as conditioned taste aversion (80;82;282), or the generation of the "behavioral satiety sequence", a pattern of behavior of the rat that includes grooming and sleep and that is part of the response to food intake under natural circumstances (9;89;139;247;264;443). Relatively few experimental conditions lead to an increase in food intake, which would be a unequivocal indication that the treatment has specifically influenced the expression of satiety signals.

An interesting surgical preparation that generates both an increase and a decrease of daily food intake in the two partners of a parabiotic pair is the one-way crossed intestines rat model (Fig. 7.1) that has extensively been studied in our lab (194;197;428;429).

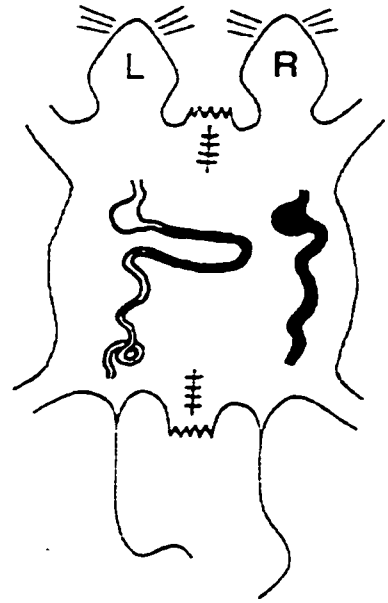


Fig. 7.1 Diagram of the one-way crossed intestines rat model. The isolated segment of 30 cm of upper gut which was connected into the left rat's (L) intestinal tract (grey color-coded) still retains all of the major blood vessels and nerves of its original owner (R). See Chapter 3.4.1 for a more extensive description of the preparation.

In this model, nutrients ingested by the left rat (in which food travels a longer distance before reaching the ileocecal valve) are partly absorbed from the inserted segment into the right rat's bloodstream. The food has to travel through the full 30 cm segment before the

left rat can absorb nutrients into its own bloodstream. Food ingested by the right rat stays in its own shortened digestive tract and is absorbed only into its own bloodstream. Neural feed-back signals generated by nutrient stimulation of intestinal receptors in the transplanted segment exert their effect in the right rat. The effects on food intake of the rats are dramatic: the left rat (that feeds itself, as well as -partly- its partner via the continuous loss of nutrients from the transplanted segment into the bloodstream of the right animal) increases its food intake by ca 50 %, while its partner decreases its intake by approximately the same amount. As a result, the left rat eats about three times as much as the right animal, a difference that is maintained for the rest of their lives. The pair as a whole eats the same amount of food and increases its body weight at the same rate as control parabiotic animals, although the right rat in a crossed intestines pair generally gains slightly more weight than its partner (194;197). The main advantages of this model over many other preparations is that the two partners have their digestive system stimulated by different amounts of nutrients, yet all ingested food is being processed in a normal physiological way via mixing with saliva and enzymes in the oral cavity and further mixing with acid and enzymes in the stomach. Also delivery to the intestines occurs via gastric emptying, a more physiological route than via direct infusion. The fact that a relatively small surgical interference generates such a robust effect on food intake may hide some important clues about the generation of hunger and satiety signals.

The effects on daily food intake that characterize this model have been explained mainly via pre-absorptive intestinal signals or post-absorptive nutrient monitoring: the fact that the right animal of the pair covers a major part of its daily energy requirements via the continuous absorption from the crossed intestinal segment would decrease its need for food intake, whereas the left rat need to overeat to make up for the caloric losses by the same process. Food intake would thus be mainly controlled by the amount of nutrients that

are being absorbed into the bloodstream, although the exact mechanism how nutrients levels in the general circulation or energy depots (such as glycogen stores or fat) feed back to the brain to control food intake are not fully elucidated. A number of experiments have shown that mimicking the enhanced nutrient influx that the right animal experiences by direct nutrient infusion into the bloodstream indeed decreases daily food intake, but that the compensation for the infused calories is incomplete (424;425).

Based on these experiments, it seems likely that for a full expression of satiety a pre-absorptive signal is required that is bypassed by intravenous infusion. This missing link may be found in the satiating effects of gastric distension: in accordance with the theory that underlies the present dissertation, it can be hypothesized that the regulation of daily food intake in the one-way crossed intestines rats takes place al least partly via regulation of the rate of gastric emptying. This explanation could be as follows: the left rat, by overeating 50 % compared to control rats, will cause a high level of nutrient stimulation of the transplanted segment. Via stimulation of intestinal receptors a strong feed-back signal is generated, causing an inhibition of gastric emptying in the right rat (with all neural connections to the 30 cm segment still intact and hormonal signals generated in the segment released into the bloodstream of the right rat). The right animal therefore would be characterized by a low rate of gastric emptying, and therefore a slow decrease of gastric contents; only a few meals would be causing a long-term distension of its stomach. On the other hand, for any nutrients to evoke feed-back signals from the left rat's intestinal tract, food will have to travel through the transplanted segment first (although a high level of stimulation of its upper duodenum still takes place). Since probably about one-third of the nutrients emptied from the left rat's stomach will be lost to its partner (the right rat grows slightly faster than the left rat, while eating only 50 % of its pre-surgery food intake), the left rat will need to empty food rapidly and in sufficiently high quantities

meal patterns, and the rate of gastric emptying would regulate daily food intake.

3. The left rat's stomach will empty rapidly and may or may not expand above values seen in control animals; the stomach of the right rat empties more slowly and its contents remain below control values. This would suggest that gastric distension plays a role in controlling daily food intake but that other factors play a role in food intake regulation as well: a level of distension that appears to be below the value that inhibits food intake in a control parabiotic rat, stops the right rat from eating. In that case gastric distension can not be an absolute inhibitory signal for the control of daily food intake and modulation of its effectiveness with other co-factors would be quite likely.

7.2 Methods

7.2.1 Subjects

The surgery leading to the one-way crossed intestines preparation has been fully described in Chapter 3.4.1 (Methods). Eight pairs of one-way crossed-intestines male Lewis rats (approximately 850 g. per pair) were fed the liquid diet Ensure Plus (Ross Laboratories, St. Laurent, Quebec). They were kept on a restricted feeding schedule with food access between 16.00 and 09.00, with lights on between 06.00 and 18.00; tap water was available ad lib.

7.2.2 Experimental Design

The experiments began at 16.00 and ran for 26 hours (i.e. from 16.00 until 18.00 the next day), with lights on between 06.00 and 18.00. The rats were put in their cages one hour prior to the start of the study, and were positioned above the gamma camera before 15.45. The radioactive, non-digestible, marker ^{99m}Tc sulphur colloid was added to the

liquid diet Ensure plus (2 mCi per 100 ml food), the well mixed labeled diet was transferred to graded burettes, and the rats were allowed access to the food at 16.00. The food was removed again the next day between 9.00 and 16.00; by maintaining the same food deprivation period as during the maintenance routine its effects on stomach filling at the start of the feeding period could be assessed. Lights were dimmed to a low level between 18.00 and 06.00. Food intake during the whole experiment was measured at regular intervals for each animal individually.

7.2.3 Data Acquisition

The general protocol for data acquisition was followed as described in Chapter 3.7. Data acquisition took place continuously in 2-min intervals for the full duration of the experiment, resulting in 26-hour curves that were decay-corrected and corrected by hand for motion artifacts and for translocation of sections of the intestinal tract from one rat into the peritoneal cavity of its partner (see Chapter 3.8). Also total gastrointestinal transit for the labeled food was established for each animal by identifying the first time point where radioactive feces were excreted.

7.3 Results

The average food intake, gastric filling and gastric emptying of the left and the right rats are shown for the full experiment in Fig. 7.2., and for the first 4 hours in Fig. 7.3.

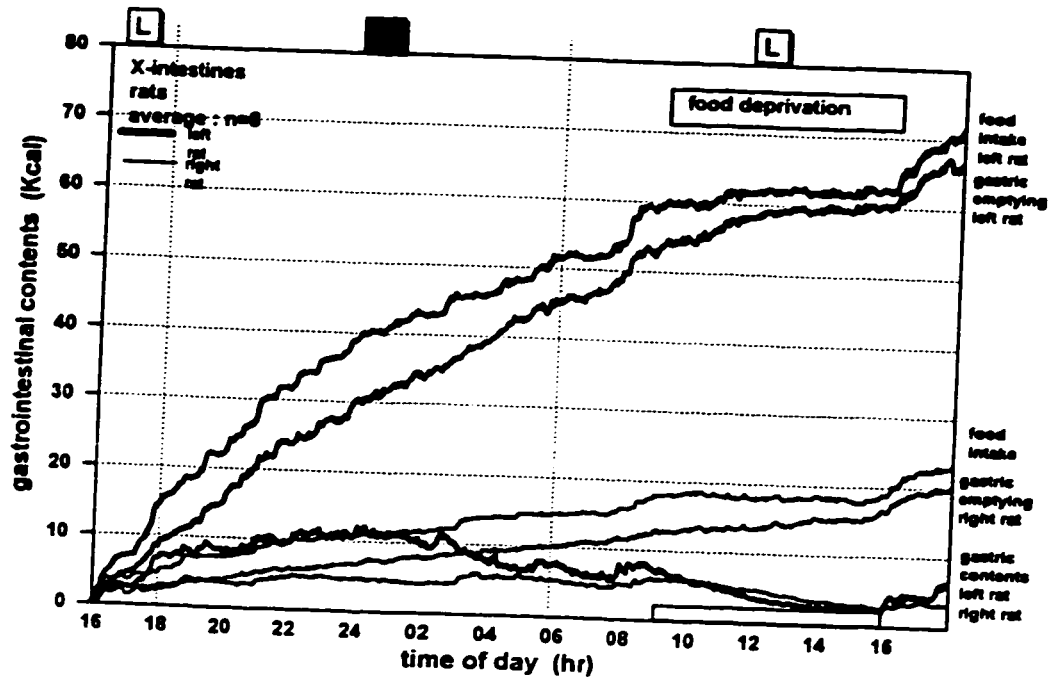


Fig. 7.2. Average 26-hour curves of the left and right rat in the one-way crossed intestines preparation.

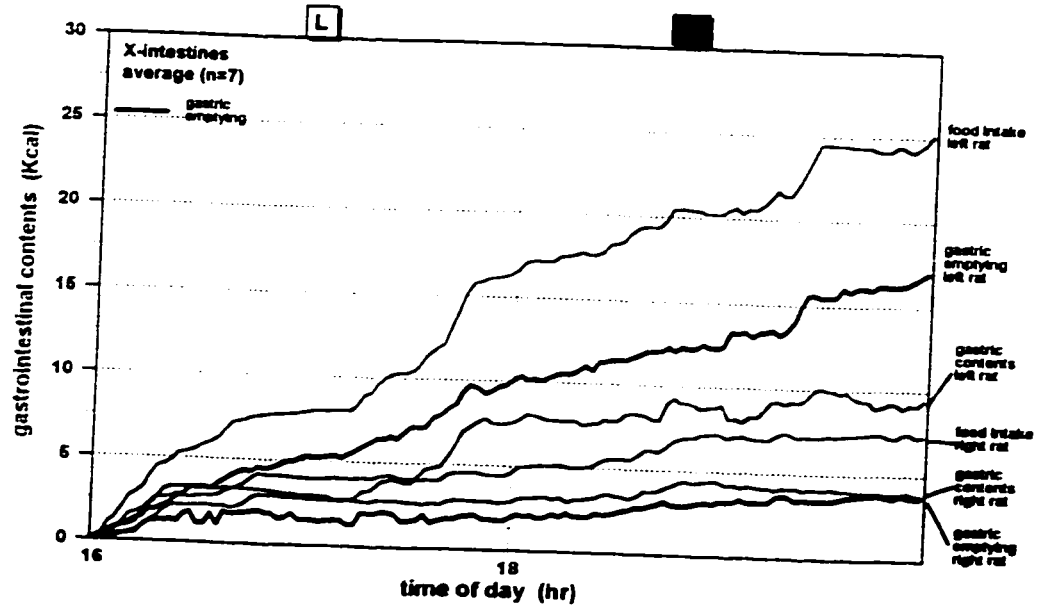


Fig. 7.3 Average curves of left and right crossed-intestines rats over the first four hours of the experiment. The heavy solid lines represent the gastric emptying curves.

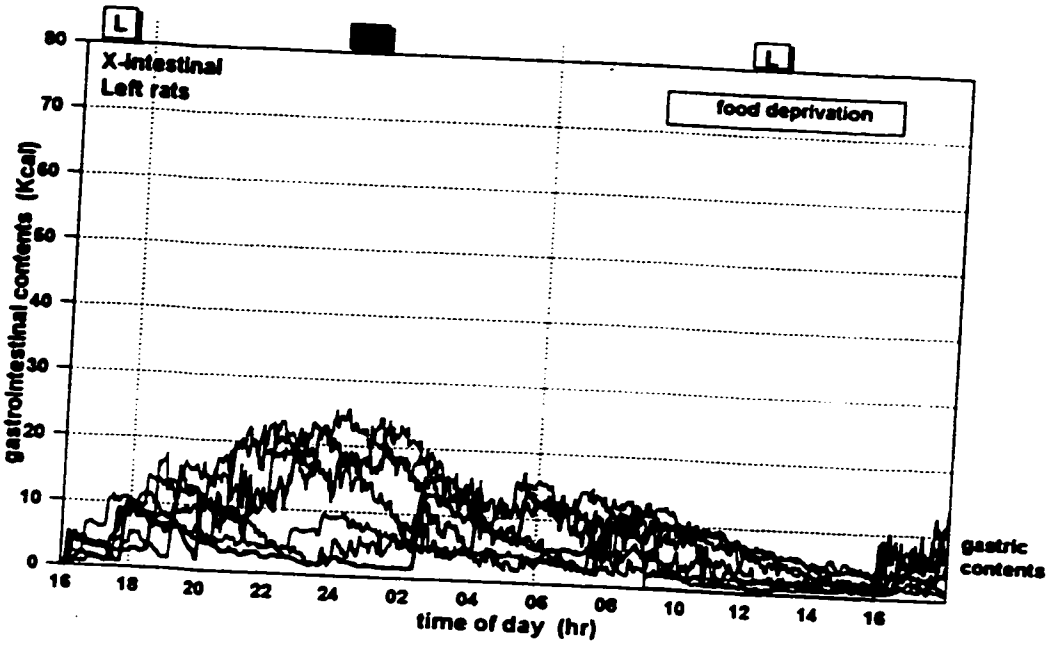


Fig. 7.4 Individual curves for stomach contents of the left rats of one-way crossed intestines rats.

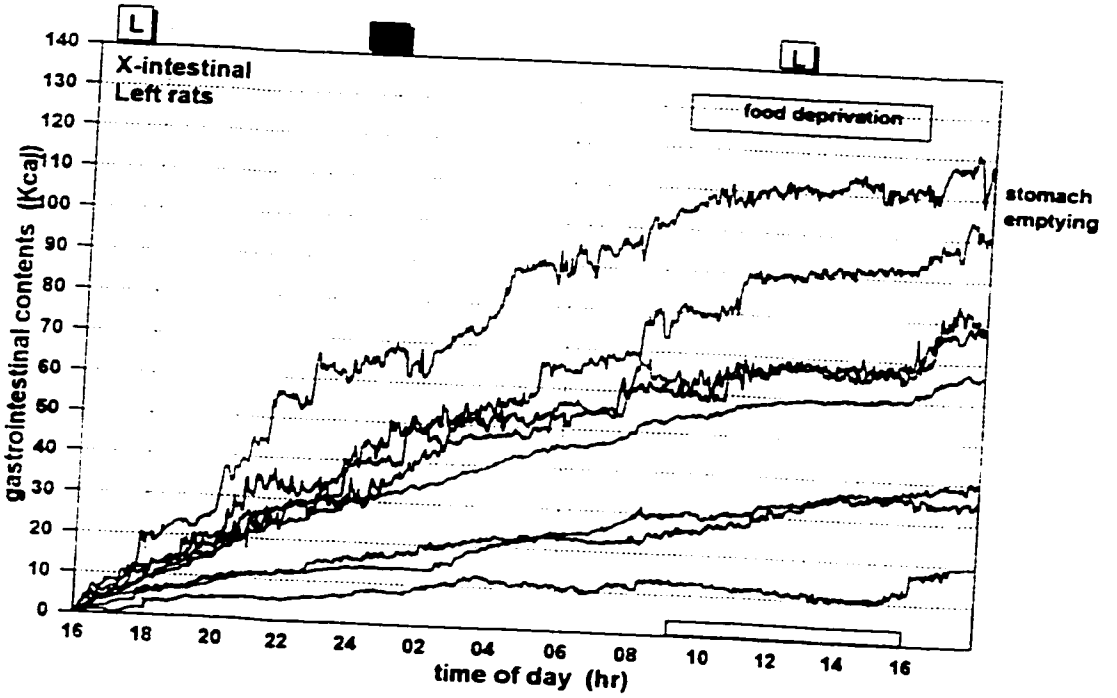


Fig. 7.5 Individual gastric emptying curves of the left rats of one-way crossed intestines rats.

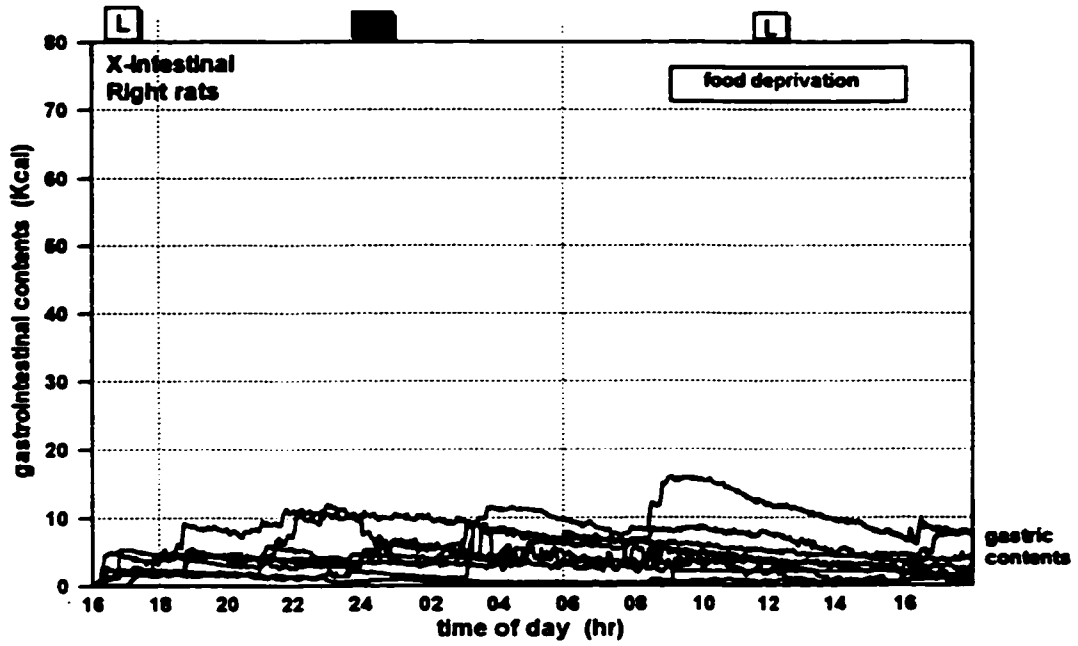


Fig. 7.6 Individual gastric contents of the right rats of one-way crossed intestines rats.

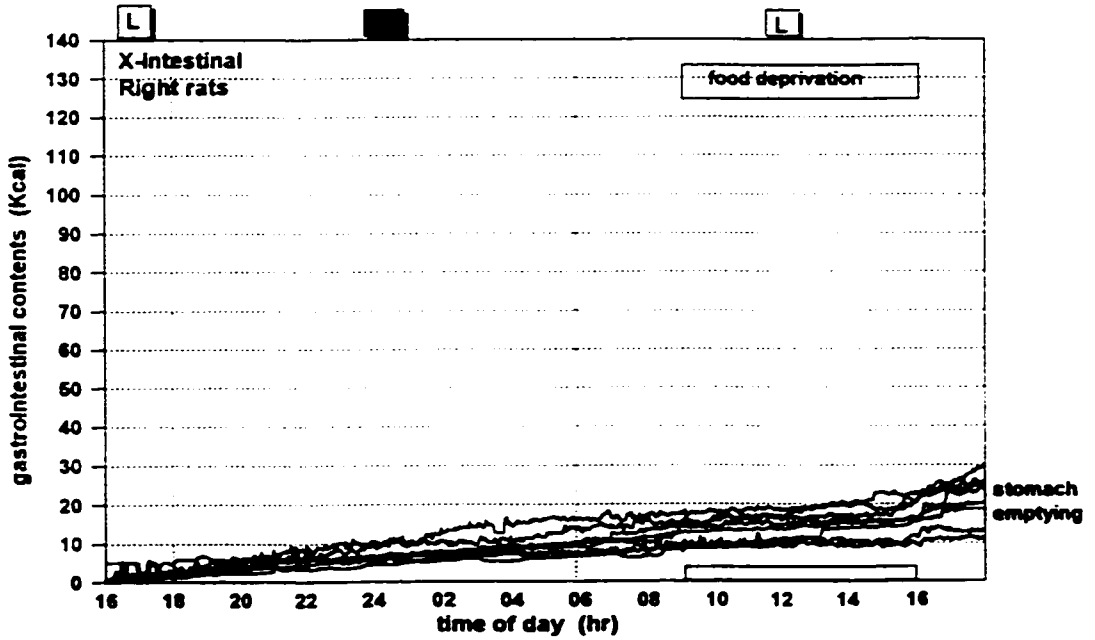


Fig. 7.7 Individual gastric emptying curves of right rats of one-way crossed intestines rats.

The direct relationship in time of food intake, gastric filling and gastric emptying was shown more precisely in Fig. 7.3: since not all animals started feeding at the first minute of food access, the data were now presented synchronized for the starting point of the first meal and only for pairs that starting feeding within 2 minutes apart from each other. Only one pair did not meet this criterion and was not included in this analysis. Individual data are presented in Figs. 7.4, 7.5, 7.6 and 7.7.

The most striking result of the experiment is, that the emptying rate of the left and right rat in the one-way cross preparation are dramatically different, for major parts of the experiment the left animal empties almost four times as fast as the right animal; towards the end of the day the differences are similar to the differences in food intake (Fig. 7.2). In itself, this result could be considered trivial, since over a full day the total amount of food eaten, especially for the left rat, by far exceeds the maximum capacity of the stomach, so that a direct relationship between the total amount of food that enters the stomach and the amount of food that is emptied over the day must be found. However, a closer examination of the first four hours of the 26-hr experiment (Fig. 7.3) reveals that the difference in gastric emptying rate begins almost from the very start of the experiment. The difference in food intake is apparent from the curve at about 4 minutes, but reaches significance after 20 minutes into the study ($p<0.05$, $F=4.4$), whereas the difference in gastric emptying is also apparent at the same time but reaches significant levels after 24 minutes ($p<0.05$, $F=4.5$). Also quite remarkable is the wide variation in emptying rate of the individual left rats (Fig. 7.6), especially when compared to the results of the 26-hr experiment with control animals (Fig. 6.2), even although the gastric contents (Fig. 7.4) do not appear to be any higher than the in the control rats (Fig. 6.3).

A comparison between the food intake data based on 2-hour intervals revealed a significant difference between left and right rat ($p<0.005$, t-test, one-tailed, assuming

unequal variance); compared to the data collected from control animals (Ch. 5). There was no difference between food intake of the left rats and controls ($p>0.14$), but a significantly lower food intake for the right rat ($p<0.005$). A highly significant difference existed for the emptying rate of the crossed-intestines partners ($p<0.00005$), the emptying rate of controls was significantly lower than the emptying rate of the left rats ($p<0.025$), and higher than the emptying rate of the right rats ($p<0.00001$). Interestingly (Fig. 7.8), the stomach of the left rat contained on average over the 26 hours of the study more food than the right rat ($p<0.001$, two-tailed t-test assuming unequal variance), but somewhat less than in controls, although no significant difference was found ($p>0.63$). The difference in gastric filling between right rats and controls was highly significant ($p<0.000025$).

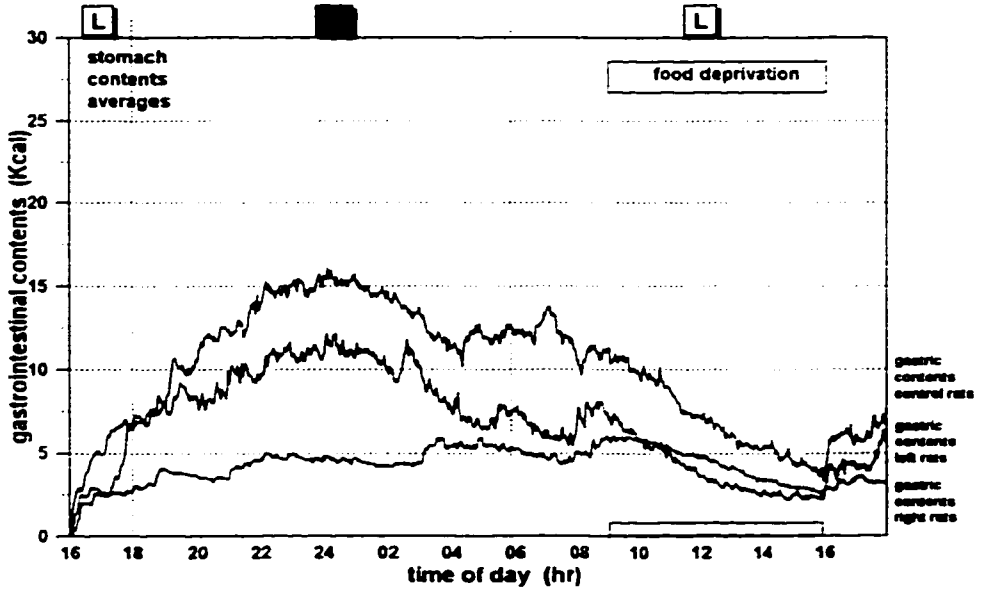


Fig. 7.8 Average 26-hour curves of stomach contents for crossed-intestines and control rats.

The day-night comparison between the various curves showed that food intake was significantly higher during the night for the left ($p<0.01$, $F=10.57$) as well as for the right ($p<0.01$, $F=9.25$) rat. The stomach contents were not significantly different ($p>0.11$ resp.

$p>0.56$), but the rate of gastric emptying was higher at night for the left ($p<0.05$, $F=2.15$) but not for the right rat ($p>0.54$). A more detailed analysis of successive 2-hour intervals showed clear variations in food intake over time ($p<0.001$, $F=3.30$, resp. $p<0.05$, $F=1.91$ for left and right rat), as well as for gastric contents ($p<0.0025$, $F=3.17$) and gastric emptying ($p<0.01$, $F=2.59$) for the left rat, but not for the right rat ($p>0.44$, resp. $p>0.45$).

The measurement of gastrointestinal transit generated some surprising results. The appearance of radioactive label in the feces was delayed for both the overfeeding left (777.2 ± 125.1, SEM, $p<0.025$, $F=8.09$) and underfeeding right rat (1072.6 ± 146.7, $p<0.0005$, $F=19.42$) of the one-way cross pair, compared to the transit time of 407.8 ± 35.1 of the control rats (Chapter 6). Although there was a large difference in transit time between the left and right rat, this did not reach significant levels ($p>0.14$). No effect of the surgery was found on these measurements: the transit time for sham animals (601.8 ± 77.0) was not different between the left sham-operated and right-operated sham rat ($p>0.99$), nor different from control rats ($p>0.49$). A similar measurement for the first identifiable appearance in the rectum did not change any of these conclusions: average times for left (711.8 ± 129.9) and right (1062.0 ± 149.5) cross-intestines rats did not differ from each other ($p>0.09$), but were significantly longer than for controls (359.6 ± 28.0, $p<0.025$, $F=7.03$, resp. $p<0.00025$, $F=21.31$). Sham rats (476.8 ± 42.8) did not differ from controls ($p>0.78$).

7.4 Discussion

A few important conclusions can be derived from the present experiment. First of all the possibilities that gastric distension alone can regulate the feeding pattern of the two partners of the one-way crossed intestine preparation can be ruled out. The data clearly show that gastric distension can not be solely responsible for the generation of satiety

signals: the right rat has a lower level of gastric contents than its overfeeding partner or control rats (Fig. 7.8), and therefore would not experience a high level of inhibitory signals that would be present if gastric distension were the main signal. Instead, it eats fewer meals over the day than its partner or than control rats. Also the left rat, in spite of a higher level of gastric distension, does not show a noticeable inhibition of food intake compared to its partner, nor a similar or reduced food intake compared to control animals. This implies that for gastric distension to be a major satiety signal in a normal situation, at least some co-factor must be involved that has different levels of expression in the different animals.

Food intake and the rate of gastric emptying are functionally connected. However, the left cross-intestines rat has a higher food intake than control rats, but not a higher level of gastric contents; this demonstrates that in these animals food is not merely emptied by force from a full stomach by incoming fresh food so that gastric emptying must be regulated independently. The individual variation in emptying rates (Fig. 7.5) clearly shows that, at least for most of the animals, the maximum possible rate of gastric emptying has not been reached. Especially during the first few hours of the experiment the rate of food intake exceeds the rate of gastric emptying, so that the stomach of the left rat fills up, although not to maximum levels. From that point of until the end of the dark phase the animal eats at a slower and slower pace, while gastric emptying continues with a fairly constant rate throughout the day and night. The theoretical possibility that in these animals gastric emptying is completely being driven by food intake can therefore be abandoned.

This means that some of the results of the present experiment are inconclusive, in so far that gastric distension can not be singled out as the main factor that can be held responsible for the generation of meal patterns and daily food intake. However, they do supply a strong argument for the involvement of regulation of gastric emptying as an

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important factor in the regulation of food intake. Both in control rats and in the right rats of the crossed-intestines preparation (Fig. 7.7) the rate of gastric emptying is set independently from food intake, and takes place at a relatively constant level throughout the day and night. Only the left crossed-intestines rats appear to escape this tight control to some extent; although the rate in itself is still relatively constant as long as there is enough food in the stomach, the absolute levels vary widely between individual rats (Fig. 7.5). In these rats, gastric emptying follows food intake closer (Fig. 7.2). This could partly be caused by the fact that gastric emptying is faster during feeding (177;422). The high level of food intake in these animals would therefore be associated with a higher effect on the emptying rate, while a compensatory reduction in emptying rate after meal termination does not appear to occur, possibly because of the rapid succession of meals or possibly because of an insufficient level of immediate intestinal inhibitory feed-back signals. Again, in the left rat this would lead to a closer association between food intake and gastric emptying, whereas in the right rat and in controls the opposite would be the case. An inspection of Fig. 7.2 shows that, while average food intake between the left and right rat differs by a factor of about 3.5 for most of the experiment, the differences in emptying rate between the two partners is more than fourfold.

The main alternative explanation for the feeding behavior in the rat model would be the hypothesis that it is the level of nutrient absorption and subsequent feedback from nutrient levels in the circulation or from storage sites that would set the level of food intake in these animals (197). In that case, however, it would be difficult to explain why such great differences occur in food intake, while (considering their similar body weight gain development) the total nutrient absorption by each of the two partner rats does not differ greatly, and also why the rat does not compensate fully for nutrient infusion into the bloodstream by an appropriate reduction in its food intake (424;425). The most likely

explanation for the present results may be a combination of the two factors, so that a regulation of gastric emptying by intestinal and post-absorptive feed-back signals takes place, while food intake may be affected via gastric distension (regulated by gastric emptying), as well as by other mechanisms.

A number of other factors, however, can not be excluded: a few differences between the two partners in the one-way cross preparation have to be considered. One potential difference that could contribute to the imbalance in food intake and gastric emptying between the two partners may be found in the different manner of stimulation of the upper gut; the duodenum is sensitive to a variety of stimuli and is believed to be an important site for generation of inhibitory feedback signals (375). Since the crossed 30 cm segment includes the lower duodenum of the right rat and is inserted into the left rat's mid-duodenum, this will affect the level of stimulation of the different sites considerably. Whereas the upper duodenum of the right rat is receiving a low level of nutrient stimulation due to its attenuated gastric emptying rate, the first half of the duodenum of the left rat is stimulated with a high level of nutrients; this apparently does not inhibit its gastric emptying rate in a sufficient manner to bring this down to normal control values. The lower duodenum shows the opposite pattern of stimulation: the right rat will receive a high level of stimulation, whereas that of the left rat will only come in contact with chyme that has traveled through the full transplanted segment and therefore has lost a substantial amount of its nutrient contents. It would be possible that differences in neural response from duodenal receptors, or a different pattern of hormonal or paracrine responses from this site contribute to the observed changes in feeding and gastric emptying. This explanation is not very likely, however: another surgical rat model, the doubly-stimulated lower gut preparation (196)junction of its right parabiotic partner; the distal part of the left rat's intestinal tract is closed off. This model also shows an increase in food intake of the

left rat, and a decrease in feeding in the right rat. Since more food travels through the left animal's duodenum and jejunum in this preparation than through the upper gut of its partner, this should cause a counteracting signal reducing its food intake if duodenal feedback was the most important regulatory signal. Since this is not the case, it seems unlikely that duodenal signals are responsible for the differences in the one-way cross preparation. Again, however, a synergistic effect together with other factors can not be completely excluded.

Another possible explanation for the observed differences between the two partners would be a difference in the profile of nutrient absorption of the two rats. Since glucose is absorbed rather quickly from the gut, the right rat may absorb a higher amount from the 30 cm segment that was inserted into the left rat's mid-duodenum than its partner. Since an elevation in glucose levels would trigger pancreatic release of not only insulin, but also amylase, this would open up the possibility that via the synergistic effects of CCK and amylin, possibly by modulation of neural regulatory mechanisms in the brainstem via a hormonal action on the area postrema (231;339;457), both gastric emptying and food intake would be inhibited in the right rat. This could be potentiating the effects of gastric distension in that animal, so that a relatively low level of gastric distension could still contribute to satiety. Amylin also decreases the rate of gastric emptying as well as of intestinal transit (55), whereas no clear relationship appears to exist between the rate of gastric emptying and of intestinal transit itself (323). Measurements of blood glucose and insulin levels in the one-way cross preparation have been made at three different time points over the day (197), one during a food deprivation, one during feeding in the dark, and one in the light period when food intake is at a low level. The results showed significant differences in insulin (as well as lactate) but not in glucose concentration between the two partners. However, since the glucose samples were taken from the

general circulation, this does not completely excludes the possibility that a higher level of glucose absorption takes place in the right animal. The increased insulin levels in the right rat would likely have caused a higher disappearance of glucose into the tissues. Also the elevated insulin level in the right rat would be associated with a higher release of amylin, which would be in accordance with the present hypothesis. Measurement in the two parabiotic partners of circulating levels of amylin and CCK, of glucose levels in the general circulation directly after a meal eaten by the left rat, and of glucose levels in the portal vein, could help clarify this issue.

The results of the gastrointestinal transit measurement showed a significant increase in transit time for both partners of the pair, compared to control or sham-operated rats, this in spite of the fact that the combined length of the intestinal tract is not different between the different parabiotic preparations. The extended transit time is not surprising in the case of the left rat, since food has to travel through its completely intact "own" digestive tract, as well as through the inserted 30 cm segment that still receives neural input from the right animal. Since the left rat shows a normal growth rate, a similar amount of food must travel through its "own" GI tract as would be the case in control rats. Gastrointestinal motility in the right rat was considerably impaired. This could have been a reflection of the occurrence of intestinal adaptation in the right animal to the shortening of its gut. Adaptation generally takes place via two processes: increased surface area (through villus hyperplasia and intestinal dilatation) or decreased transit time, increasing mucosal contact time for nutrient and fluid absorption (439). In the one-way cross preparation, however, no significant changes were found in intestinal weight between the two parabiotic partners, suggesting that hyperplasia had not occurred (194). In the more pronounced situation of short-bowel syndrome, where generally more than 75 % of the small intestine has been resected (287;288;379), the effects on gastrointestinal motility are not consistent:

unaltered gastric emptying and postprandial motor activity (compared to healthy controls) has been described (328), but also decrease of intestinal motility (314). The present data, with the striking inhibition of gastric emptying and gastrointestinal transit time in the right animal, could have been the result of such an adaptation. However, since in the one-way cross preparation this large effect was created via the disconnection of only a relatively short segment consisting of less than 30 % of the total length of small intestine from the right rat's GI tract, a strong inhibitory signal that is generated via the high level of stimulation of the crossed jejunal section and via nutrient uptake from that segment into the bloodstream of the right rat, appears to provide a more likely explanation for the observed changes. An alternative explanation for the slower transit time in the right rat would be that, since the right rat eats less than control rats and empties food slower from its stomach, its intestinal tract contains less food, so that the initiation of peristaltic reflexes by the contents of the lumen (via stimulation of intestinal mechano-and chemoreceptors) will be on a lower level than in the parabiotic controls.

Chapter 8: Regulation of gastric emptying in one-way crossed intestines rats.

8.1 Introduction

The one-way crossed intestines model is characterized by large differences in food intake for the two parabiotic partners (Fig. 8.1). Measurement of the gastric emptying pattern of the two partners demonstrated that there are similar differences in their emptying rate, and that the rate of gastrointestinal transit is attenuated in both animals but more strikingly in the underfeeding right rat. The decrease in gastric emptying rate and increase of transit time in the right rat of the pair could be the result of adaptation to its shortened intestinal tract. Adaptation generally takes place via two processes: an increase in intestinal surface area (through villus hyperplasia and intestinal dilatation) or a decrease in transit time, thus increasing the contact time between intestinal contents and the mucosa, resulting in improved nutrient and fluid absorption (439).

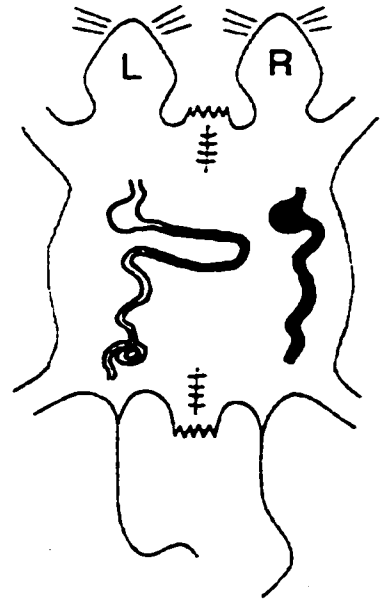

Fig. 8.1 Diagram of the one-way crossed-intestines preparation. For further details of surgery: see Chapter 3.

Adaptation is not the only possible explanation for the observed changes in transit rate. The lower food amount of food contained in the right animals small intestine (compared to control parabiotic rats) would cause less stimulation of intestinal receptors and a lower level of initiation of peristaltic reflexes. An alternative explanation would be that because of the high rate of gastric emptying and high food intake of the left rat, a high stimulation of intestinal receptors would occur in the crossed 30 cm segment (which remains connected via all major blood vessels and nerves to the right rat). This could be accompanied by intestinal secretion of cholecystokinin (CCK) and gastric inhibitory peptide (GIP) , as well as an increased uptake of glucose into the bloodstream of the right rat, causing a release of the pancreatic peptides insulin and amylin; the synergistic effects of CCK and amylin would then cause a potent inhibition of gastrointestinal motility and food intake (55;228;232;276;278;458;460).

To test the hypothesis that the decrease in the rate of gastric emptying in the right rat is not caused by an intestinal adaptation mechanism that is related to its shortened gut (see also Chapter 7), but indeed via stimulation of and/or increased absorption from the transplanted segment, the following experiment was designed: in a six-hour study only the right rat of a one-way crossed intestines pair was allowed access to the radioactively labeled diet for the first three hours, while its rate of gastric emptying was measured continuously via gamma camera scintigraphy. After this period the left rat was also allowed to feed for another 3.5 hours.

If the attenuated gastrointestinal motility in the right rat that had been observed in the free-feeding situation (Chapter 7) was caused by a general intestinal adaptation that persisted over days or weeks due to long-term adaptation, no major influences by the feeding condition of its partner would be expected, and similar emptying rates should be found during the first and the second 3-hr periods. If on the other hand stimulation of the

transplanted jejunal segment and/or nutrient absorption from that segment were to be the main regulatory mechanism, more significant differences would be anticipated: in the first 3-hr period a significantly higher emptying rate would be anticipated compared to the right rats of crossed-intestines pairs that were allowed to feed freely, whereas in the second 3-hr period a return to lower emptying values would take place.

8.2 Methods

8.2.1 Subjects

The surgery leading to the one-way crossed intestines preparation has been fully described in Chapter 3.4.1 (Methods). Eight pairs of one-way crossed-intestines male Lewis rats (approximately 875 g. per pair) were fed the liquid diet Ensure Plus (Ross Laboratories, St. Laurent, Quebec). Between experiments they were kept on a restricted feeding schedule with food access between 16.00 and 09.00, with lights on between 06.00 and 18.00; tap water was available ad lib. The animals were adapted for several weeks to the moderately restrained conditions that were used during the experiments.

8.2.2 Experimental Design

The experiments began at 16.00 and ran for 6½ hours (i.e. from 16.00 until 22.30), with lights on between 16.00 and 18.00. The rats were put in their cages one hour prior to the start of the study, and were positioned above the gamma camera before 15.45. The radio-active, non-digestible, marker ^{99m}Tc sulphur colloid was added to the liquid diet Ensure plus (1 mCi per 100 ml food), the well mixed labeled diet was transferred to graded burettes, and the right rat of each pair was allowed access to the food at 16.00. The barrier that separated the left rat from its food was removed three hours later, at 19.00,

and both animals were allowed to eat freely until the end of the experiment. Only the first six hours after the first initiation of feeding activity by the right rat were used for the final analysis.

8.2.3 Data Acquisition

The general protocol for data acquisition was followed as described in Chapter 3.7. Data acquisition took place continuously in 2-min intervals for the full duration of the experiment, resulting in 6½-hour curves that were decay-corrected and corrected by hand for motion artifacts and for translocation of sections of the intestinal tract from one rat into the peritoneal cavity of its partner (see Chapter 3.8). The data were compared with 6½ hour-data from one-way crossed-intestines rats (without the food deprivation of the left animal) and with parabiotic control rats (left rat deprived, as well as free-feeding).

8.3 Results

The cumulative food intake and gastric emptying rate of the one-way crossed intestines rats and of the control animals when the left rat of each pair is deprived of food for the first three hours are shown in Fig. 8.2., resp. Fig 8.3.

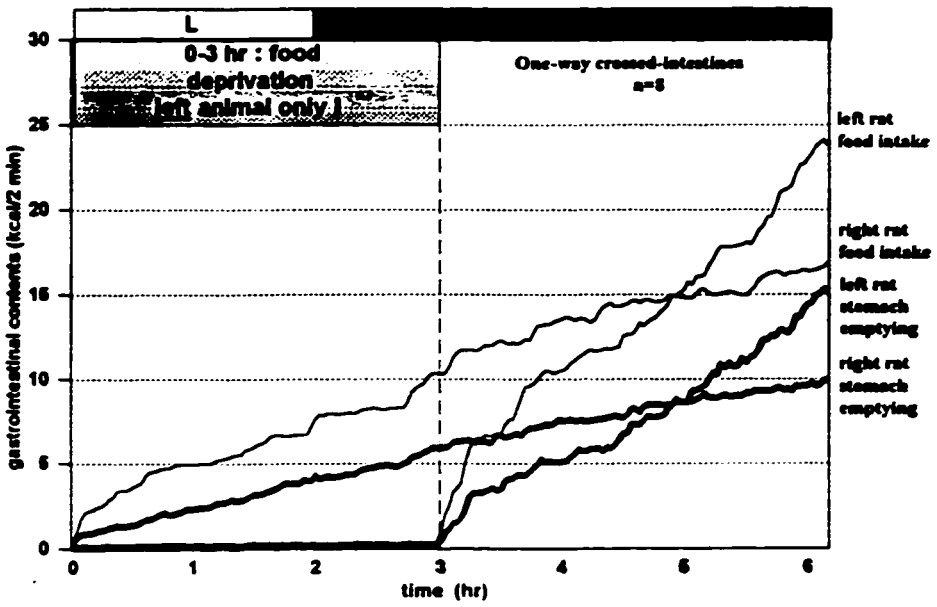

Fig. 8.2 Average curves for food intake and gastric emptying for the one-way crossed intestines rats. The left rat had been food deprived for the first 3 hours; the right rat was free-feeding for the full duration of the experiment.

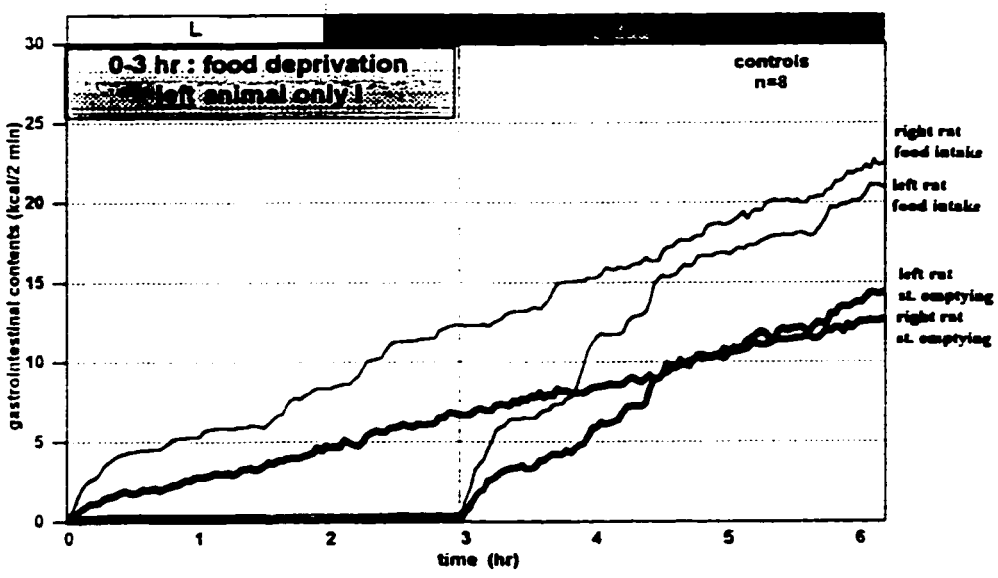

Fig. 8.3 Average curves for control rats (n=8). The left rat had been food deprived for the first three hours of the experiment.

No behavioral interaction between the rats of each pair was found for the food deprivation: the food intake of the control parabiotic right animal was not affected by the feeding state of its partner: 4.11 ± 0.61 (SEM) vs. 3.22 ± 0.41 kcal/hr ($p>0.24$ for 3-hr comparison, and $p>0.55$ on a 1-hr basis). However, in the one-way cross preparation the food intake of the right animal significantly decreased in the second half of the experiment (after its partner was allowed access to the food) from 3.45 ± 0.31 to 2.05 ± 0.50 kcal/hr ($p<0.05$, $F=5.73$ for 3-hr comparison, or $p<0.025$, $F=2.99$ for 1-hr comparison).

The average rate of gastric emptying for the first three hours (i.e. during the food deprivation of the left animal of each type of pair), compared with the average rate during the next three hours (i.e. while the left rat was allowed access to the food) was not significantly different for the control rats (2.23 ± 0.27, SEM, resp. 1.90 ± 0.24 kcal/hr, $p>0.36$), but showed a significant attenuation in the one-way cross animals (1.98 ± 0.15, resp. 1.23 ± 0.13 kcal/hr, $p<0.025$, $F=13.52$).

Crossed-intestines rats that were both free-feeding for the full period showed no significant difference in emptying rate between the first and second 3-hr period ($p>0.17$); a comparison between these 2 groups of one-way crossed-intestines (Left rat free-feeding vs. left rat deprived) showed that during the first 3 hours the difference in average emptying rate was highly significant (0.95 ± 0.13, resp. 1.98 ± 0.15 kcal/hr, $p<0.00025$, $F=25.94$). However, when the comparison was made for the first three hours that the left rat had been allowed to feed (i.e. hours 1-3 for the free-feeding group, vs. hours 4-6 for the group where the left animal had been deprived), the differences did not reach significant levels (0.95 ± 0.13 vs. 1.23 ± 0.13, $p>0.15$). The direct comparison between the second 3-hr period was still significantly different (0.67 ± 0.14, resp. 1.23 ± 0.13 kcal/hr, $p<0.025$, $F=8.46$).

The pattern of gastric emptying over the 1-hour periods of controls (see Table 8.1) did

not show any significant changes in control rats (P.0.74), but did change significantly in the one-way cross ($p<0.01$, $F=3.63$). An ANOVA factorial analysis showed that the differences were significant for both the first and the third hour, compared with the fifth resp. sixth hour.

Similarly, a direct comparison for the two successive 3-hr periods between one-way cross and control rats also showed no difference in the first three hours ($p>0.21$), but a significantly lower emptying rate for the next three hours in the one-way cross group ($p<0.025$). The more detailed comparison (via one-tailed t-test) between the emptying pattern of one-way cross and control rats over successive 1-hour periods gave the following results (Table 8.1):

	1	2	3	4	5	6
crossed-intestines	2.34	1.61	1.99	1.70	1.03	0.97
(SEM)	(0.26)	(0.16)	(0.43)	(0.32)	(0.20)	(0.23)
controls	2.76	2.05	1.89	1.72	1.81	2.17
(SEM)	(0.46)	(0.36)	(0.29)	(0.68)	(0.66)	(0.48)
p value	p>0.22	P>0.14	p>0.42	p>0.49	p>0.14	p<0.025

Table 8.1. Average gastric emptying rate (kcal/hr) for the right rats of one-way crossed intestines pairs compared with control parabiotic rats for the 6 successive 1-hr periods of the experiment. The left rat was food deprived for the first 3 hours. Values are averages ± SEM (n=8).

A regression analysis evaluating the effects of food intake of the left rat on food intake of its partner showed no significant interaction between control pairs ($p>0.45$), but a significantly negative effect for one-way cross animals ($p<0.05$). Similarly, the effects of the rate of gastric emptying of the left rat did not affect that of the right animal in control

pairs ($p>0.49$), but had a highly significant negative effect on that of the right rat in the one-way crossed animals ($p<0.005$).

8.4 Discussion

The results suggest that the attenuation of gastric emptying in the right animal of the one-way crossed intestines preparation (when both partners are allowed freely access to food) is not caused by adaptations in the right rat to the surgery that led to changes in the expression of signals involved in the regulation of gastric emptying. This is in full accordance with earlier data that found no significant changes in intestinal weight 3 to 4 months after the crossed-intestines surgery (194), suggesting that also no major morphological adaptation had occurred. The present data show that when the left animal is denied access to food, so that the transplanted jejunal segment (still connected to the right rat with blood vessels and nerves) does not receive any nutrient stimulation, the right animal will empty food from its stomach at a rate that is not different from control rats. The stomach and most of the intestinal tract of the left animal must have been empty after a deprivation period of 10 hours (seven as a standard deprivation, plus an extension of three more hours); the results of the 24-hr measurements in this model show that even before the end of the seven hours food deprivation its stomach is already empty. This suggests that all physiological control systems that regulate gastric emptying are fully functional in these animals, and that no long-term changes in receptor sensitivity have occurred that would alter their response to nutrients in the GI tract or in the bloodstream. After the left rat starts eating a significant reduction takes place slowly in both gastric emptying rate and food intake in its partner that is directly correlated with the levels of food intake and emptying rate in the left animal. Whether changes in intestinal transit time in the right rat are also the result of the generation of inhibitory feed-back signals via

stimulation of and absorption from the transplanted 30 cm-segment, or do show the effects of a specific adaptation after all, remains to be determined.

Oddly, the present data show that the reduction in gastric emptying took a considerable amount of time (one to two hours) to reach significant levels, although an immediate stimulation of intestinal receptors in the transplanted segment must have occurred as soon as the left rat started eating and started emptying food from its stomach; the (short-term) data appear to be inconsistent with those collected with the one-way crossed-intestines preparation over a 26-hour period. An immediate effect of the increased level of inhibitory feed-back would have been expected if neural signals originating from this segment were responsible for the regulation of gastric emptying in the right animal. Since the right animal had been emptying more nutrients from its stomach in the first three hours of the experiment than normal (in the control situation when the left rat was also allowed to eat), a higher level of stimulation of its own (shortened) intestinal tract should have added inhibitory feed-back to the existing signals created in the crossed segment after its partner started to feed. An after-effect of the extended food deprivation of the left rat is not likely; the left rat's high gastric emptying rate would rapidly make up for a possible further diminished level of nutrients in the crossed segment or in the right rat's bloodstream after the end of the food deprivation period. The only explanation for this result would be that some other factor is involved, most likely further inhibitory signals arising from post-absorptive sources (424;426;35;239). Still, the difference with the normal emptying pattern of the right rat (Chapter 7), measured after a food deprivation of seven hours for both rats, seems hard to explain.

Since a change in the rate of emptying was noticeable in the graphs (Fig. 8.2) from the beginning of the fourth hour onwards but without reaching significant levels within two hours, the fact that the emptying rate of the right animal was not significantly different

between the first three hours of feeding in the ad lib group (where the left rat had also access to food in the first 3 hours), compared with the second three-hour period in the deprivation group (where the left rat only started feeding after three hours) may also suggest that the noise level in the data was relatively high in the present experiment, so that the differences with the control group did not reach significant levels in an early stage of this short-term experiment.

Although more time than expected was needed to establish clear differences in emptying rate between the two conditions (left rat deprived vs. feeding), the results give support for the hypothesis that the characteristically slow emptying pattern of the right, under-feeding rat in the one-way cross (in a free-feeding situation for both animals) is partly mediated via a high level of nutrient stimulation of the transplanted segment, probably combined with elevated levels of nutrients in the wall of the small intestine and in the bloodstream after nutrient absorption from the crossed intestinal segment takes place.

Chapter 9: Effects of intravenous nutrient infusion on gastric emptying rate.

9.1 Introduction

Gastric emptying is regulated via a variety of inhibitory feed-back signals. Much attention has been focused on the effect of intestinal nutrient stimulation. Since the rate of gastric emptying should not exceed the capacity of the intestines for nutrient and fluid absorption, it is important that coordination between these events takes place so that ingested food can be efficiently digested by the gastrointestinal system. Until recently, the post-absorptive effects of nutrients on gastric emptying, however, have received considerably less attention in the literature. This is somewhat surprising, since the rate of gastric emptying has a direct effect upon the metabolic cycles within the various body tissues via nutrient uptake from the gut into the general circulation. The levels of circulating nutrients in the body are generally regulated via the maintenance of blood glucose levels by the liver and via release of hormones such as insulin, but nonetheless a marked increase in nutrient plasma levels can occur after a meal. Therefore, a regulation of the rate of gastric emptying via post-absorptive inhibitory feed-back would be highly beneficial in a homeostatic regulatory system that is aimed at the maintenance of blood nutrient levels around relatively constant values.

Elevated blood glucose levels have been shown to inhibit gastric emptying. Especially in diabetic patients this effect has been well documented; more recently it has been shown that the elevated blood glucose values can be held responsible for this effect (24;47;70;144;234;257;357;367). A number of studies suggest that this effect could be at least partially mediated via stimulation of glucose-sensitive cells in the portal vein and activation of hepatic vagal afferents (350;352-354).

The effects of amino acids and lipids on gastric emptying are not well described.

Intravenous infusion of Intralipid has been shown to delay gastric emptying (44); intravenous amino acids can prolong intestinal transit time (239). One study found after 6 hours of intravenous infusion of a standard parenteral diet or of an amino-acid mixture a delay in the rate of emptying of a test meal; the effect of an amino-acid mix enriched with branched-chain amino acids was less pronounced (35).

The influence of parenteral nutrients on food intake have received considerably more attention (128;235;285;286;424-426;454). Intravenously administered nutrients have a clear inhibitory effect on food intake; generally the compensation for the infused nutrients (via a reduction of a similar number of calories as was infused), however, is incomplete (285;424;425). A mixture of nutrients is more effective in reducing food intake than single macronutrients alone (285;425); especially fat is relatively ineffective in inhibiting food intake (128), resulting in only about 40 % compensation for an infusion over 6 days, whereas rats compensate fully for amino acid infusions. Glucose causes an intermediate reduction of ca. 55 % (425). Compensation is better for infusion during the feeding period than during the light phase (426). Since food intake was usually measured over long intervals, however, the time required for the expression of these inhibitory effects are not known, so that a physiological mechanism underlying the reduction in food intake has not been fully identified.

Since food intake and gastric emptying are functionally linked, some similarities between the effects of intravenous nutrient infusion on food intake and on gastric emptying could be hypothesized. To further investigate this issue, and to investigate how quickly eventual inhibitory effects would occur, gastric emptying was measured continuously via gamma camera scintigraphy while the rats received an intravenous caloric infusion or a control saline infusion.

9.2 Methods

9.2.1 Subjects

Eight pairs of male Lewis rats (Sprague Dawley, Indianapolis, IN) weighing 846 ± 17 g (SEM) were equipped with a permanent heart catheter (390). Briefly, a catheter, made of silastic medical-grade tubing (i.d. 0.57 mm, o.d. 0.9 mm) was inserted in the external jugular vein, just before the junction between the anterior jugular, the acromiodeltoid and the cephalic vein. A small ring of silicon glue 41 mm from the tip of the catheter was used to stabilize the cannula at the point of insertion The tip of the catheter was located at the entrance of the right atrium. The other end of the catheter was pulled subcutaneously to the head of the animal, and attached to a bent piece of stainless steel tubing manufactured from a 21G hypodermic needle. This was attached to the skull via four small stainless steel screws that were implanted on the four sides of the bregma and imbedded in a thin layer of acrylic dental cement. Between experiments the catheter was filled with ca 0.1 ml of a mixture made of 6 gram polyvinylpyrrolidone (PVP) in 10 ml of a heparin solution (500 E/ml). The open end of the stainless steel tubing was closed by a small sealed polyethylene cap (i.d. 0.75 mm, o.d. 1.45 mm), that was protected from biting by the parabiotic partner by a small piece of Tygon tubing (i.d. 3/32 in, o.d. 5/32 in). The rats were given at least two weeks to fully recover from any effects of the surgery.

On non-experimental as well as experimental days the rats were fed the liquid diet Ensure Plus. They were kept on a restricted feeding schedule with food access between 16.00 and 09.00, with lights off between 18:00 and 06:00.

9.2.2 Experimental Design

The animals were prepared for the study between 14.00 and15.00. The PVP solution was replaced by saline, and the rats were equipped with a polyethylene infusion line (i.d. 0.75 mm, o.d. 1.45 mm), filled with saline. The part of the infusion line that could be reached by the animals was protected against biting by a 20 cm piece of Tygon tubing (R-3603, i.d. 3/32 in, o.d. 5/32 in) that was attached to the infusion line by a concentric piece of silastic tubing (Dow-Corning 602-205, i.d. 0.040 in, o.d. 0.085 in). The infusion lines were connected at the free end to a piece of stainless steel tubing similar to the connector piece on the skull of the rats, and sealed with a small polyethylene cap. The infusion pumps were prepared with the filled syringes, with a 21 G Luer-Lock needle with a short piece of polyethylene infusion line attached. The pumps were started before 15.45 to avoid irregular functioning during start-up. At the beginning of the experiment, the cap from the end of the infusion line was removed and the connection with the syringe was made via the piece of stainless steel tubing.

The experiments began at 16.00 and ran for 7 hours, with lights on between 16.00 and 18.00. The rats were allowed free access to the radiolabeled food at 16.00. The nutrient infusion was started at 16.00 and ran continuously until the end of the experiment. The animals received the infusions pair-wise, to prevent any possible interference via the small amount of blood that is exchanged between the partners via the parabiotic connection. The infusions itself were randomly divided over the different pairs on the different experimental days.

9.2.3 Infusion

The pairs received one of the following four treatments:

1. A control infusion of Ringers solution, at a speed of 0.944 ml/hr

2. A 50 % Dextrose solution (Baxter, 2 kcal/ml) This was diluted 4:1 with Ringers solution and infused with a speed of 0.944 ml/hr, effectively infusing 1.61 kcal/hr or a total of 11.23 kcal over the full experiment.
3. A mixed essential and non-essential amino acids solution, 10 % Travasol-C (Travenol, Canada, 0.4 kcal/ml), infused at a speed of 1.97 ml/hr. Total calories infused from the amino acid infusion were 0.8 kcal/hr, or 5.6 kcal over the full seven hour.
4. A lipid infusion (Intralipid, Pharmacia Canada, 2 kcal/ml). Diluted 4:1 with Ringers solution and infused with a speed of 0.944 ml/hr, or 1.61 kcal/hr (or 11.23 kcal over the full 7-hr study).

The infusions were administered from 30 ml-syringes by a Razel A-99H syringe pump (settings 23 for the amino acids, 11 for the other treatments). A small delay of 2 min (for the faster amino acid infusion) to 4 minutes occurred for the infusion to replace the saline solution in the infusion lines by the nutrient solution and finally to reach the animal's bloodstream.

9.2.4 Data Acquisition

Continuous data collection took place in 2-min. intervals, using a dynamic planar protocol, with a resolution of the gamma camera (Odyssey, Picker) of 128 x 128 x 16 pixels.

Since nutrient infusion has an inhibitory effect on food intake, the fact that the study was performed with free-feeding animals carried a substantial risk that some of the rats would not have a sufficiently high level of food intake and of gastric labeled nutrient contents to be able to determine the gastric emptying rate successfully. Also large differences in food intake in the early stages of the experiment (when generally food intake is high) could

have caused differences in emptying rate based on the acceleration of emptying during feeding (177). Therefore the criterion was added to the study so that data were excluded from the analysis from rats that had eaten less than 14 kcal over the 7 hr duration of the study. Since the average emptying rate in control rats was found in earlier studies to be close to 2.5 kcal/hr, even this would carry the risk that animals with a normal emptying rate would not carry enough food in their stomach for extended time periods. An additional evaluation of the gastric filling over the time course of the experiment (measured using ROI 2, see Chapter 3) confirmed the somewhat arbitrary choice of 14 kcal of food intake during the 7 hr experiment, showing that the rats had a sufficient amount of food in their stomachs to allow determination of the rate of gastric emptying.

9.3 Results

The different infusions appeared to have a differential effect on the rate of gastric emptying (Fig. 9.1).

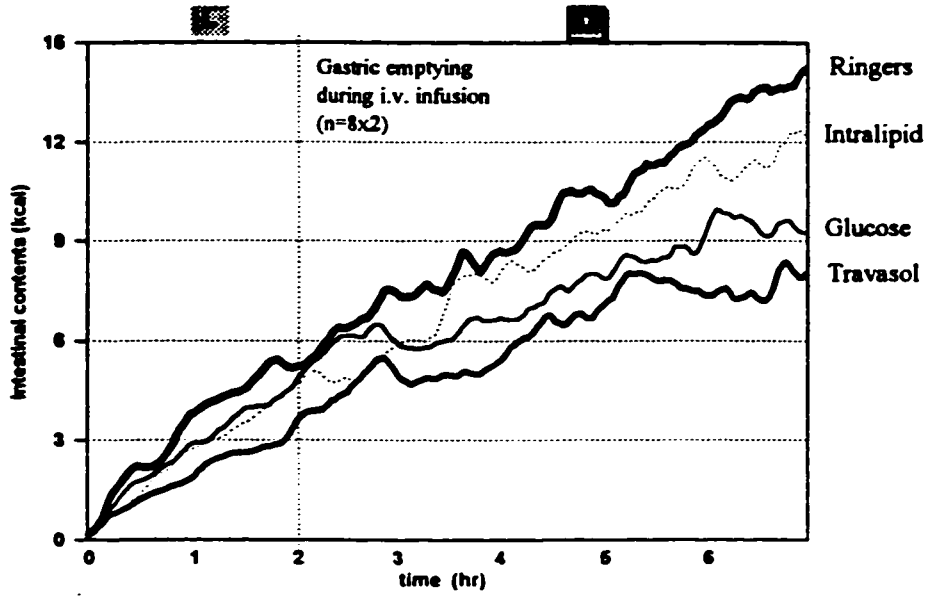

Fig. 9.1 **The effects of different macronutrient infusion on gastric emptying.**

At the end of the 7-hr infusion period the control group (Ringers-infusion) had emptied 14.0 ± 0.3 kcal, the rats infused with glucose emptied significantly less (9.1 ± 0.4 kcal, $p<0.025$, $F=8.81$), as did the Travasol group (7.6 ± 0.2 kcal, $p<0.0025$, $F=15.79$). No significant differences were found with the Intralipid-infused rats (12.0 ± 0.2 kcal, $p>0.30$). A direct comparison between the effective nutrient infusions showed that there was no significant difference in inhibition of gastric emptying between a 11.2 kcal glucose infusion and a 5.6 kcal travasol infusion ($p>0.29$). The effect took more than 5 hours to reach significance: at that time point the control animals has emptied 9.80 ± 1.22 kcal, the glucose group 7.55 ± 1.13 ($p>0.20$), the Travasol 6.72 ± 0.84 ($p>0.05$), and the Intralipid group 8.94 ± 0.80 ($p>0.56$).

Although the food intake appeared to be negatively affected by nutrient infusion, which could have affected the outcome of the different treatments, the effects remained limited:

compared to the saline infusion (23.4 kcal ± 0.6), food intake was not significantly different for glucose (18.7 ± 0.6, p>0.10), Travasol (18.8 ± 0.5, p>0.10) or Intralipid (19.5 ± 0.4, p>0.20).

The animals appeared to be influenced to some extent by the experimental treatment: the total number of emptied calories over the seven hours of the study in control rats that were not infused, compared to the rats that were infused with Ringers dropped from 17.3 ± 1.9 to 14.0 ± 1.5, however, this effect did not reach significance (p>0.07).

9.4 Discussion

The present data appear to form a direct link to previous data measuring food intake during intravenous (i.v.) infusion (424){Walls & Koopmans 1992 ID: 11328}. The relative effectiveness of the three macronutrients in inhibiting food intake is reflected in exactly the same order in the inhibition of gastric emptying: travasol is highly effective in both respects, whereas intralipid did not affect gastric emptying within seven hours of i.v. infusion and had only a limited effect on food intake. Glucose had in both cases an intermediate effect. These results are intriguing, since they suggest that the effects of i.v. infused nutrients on food intake may be related to a similar inhibition of gastrointestinal motility. If one follows gastric emptying over a full day period, a continuous suppression of the rate of gastric emptying may have a pronounced effect on the inhibition of daily food intake. Interestingly, the 100 % caloric compensation that was found in food intake measurements during amino acid infusion (425) has a similar effect on the emptying rate: a total of 5.6 kcal was infused over seven hours, and the reduction in gastric emptying compared to the controls is in a comparable range. The effects of the other nutrient infusions are too close to the control infusion in the early stages of the experiment (Fig. 9.1) to warrant strong statements about their correlation with the food intake data.

In the previous food intake studies (424;425), infusion of glucose led to a decrease in food intake that was only about 50% of the calories infused. Since twice as many glucose calories were infused in the present study as Travasol calories, the decrease in gastric emptying for these two nutrients should be approximately the same and, in fact, there was no significant difference in gastric emptying rate between the rats infused with glucose and those infused with the amino acid solution. In contrast, infusions of Intralipid had only a minor, non-significant effect on food intake during the first day of infusion and it is clear from the present data on gastric emptying that the Intralipid curve is not significantly different from the Ringers control infusion curve during the first 7 hours of infusion.

Interestingly, the infusions did not have any significant effect over the first 5 hours of the study. This is somewhat surprising, since a more direct interaction between metabolic events and gastrointestinal motility was hypothesized as being physiologically important. However, examination of Fig 9.1 shows that differences in the rate of gastric emptying that become significant at 6-7 hours are already present at 30 min, albeit non-significantly. Since the sample mean is the best estimator of the population mean at any one time, these data suggest that the effect of iv nutrients is present but relatively small soon after the infusion begins. They suggest that plasma nutrient levels can have a large, long-term effect on gastric emptying. Apparently, absorbed nutrients take several hours to assert a significant effect. This suggests th t during short-term studies of less tha a few hours the inhibitory effects of i.v. nutrients on gastrointestinal motility may not be recognized to full extent. The fact that these effects appear to develop only slowly over time implies that they have to be factored in when an assessment is made of an experimental treatment that has effects on plasma nutrient levels that last longer than a few hours.

A few factors could not be fully controlled: first of all the level of food intake is high in the first two hours after the food deprivation. This could have induced relatively fast

gastric emptying during the meals in all the animals, regardless of any post-absorptive effects. Also an effect of the light-dark cycle can not be excluded. Recently evidence has been found for the involvement of the circadian system in the expression of vagal and sympathetic activity (298;299). Suppression of vagal outflow in the light phase could have altered the response to the infusions. A repeat of the experiments, but starting the infusions in the dark phase, could clarify this issue. It is also possible that a certain nutrient requires a specifically elevated level with respect to other nutrients to exert its effect. In the present situation the animals were free-feeding, (425)causing a temporarily elevated influx of nutrients into the bloodstream during each meal, especially in the early part of the experiment. This would "dilute" the effect of any infused caloric substance by partly integrating it in the normal rise of plasma levels. During the inter-meal intervals, after some of these circulating nutrients have been absorbed in the tissues, a relatively higher effect of the infused nutrient could be expected.

Chapter 10: General Discussion.

The main results of the present studies were the finding that, when measured over longer time periods, gastric emptying takes place at a fairly stable rate throughout the 24-hr day (Chapters 5, 6), in spite of large fluctuations in food intake and gastric distension. The emptying rate appears to be regulated between relatively narrow boundaries. Intestinal feed-back signals may be important, but the nutrient infusion experiment (Chapter 9) suggested that also post-absorptive feed-back signals have a major influence on gastrointestinal motility. The hypothesis that food intake is regulated via inhibitory signals caused by gastric distension, and that in turn gastric distension would be regulated via the rate of stomach emptying, could not be fully confirmed based on the present data set. However, the most critical experiments in this respect, the 24-hr study with crossed-intestines rats (Chapter 7) also failed to refute the hypothesis; the data also demonstrated that gastric emptying is not simply being driven by food intake.

The 24-hr measurements (Chapter 6) showed that gastric distension increases over the first part of the night. This is partly caused by the fact that in that phase food intake is also at its highest level, but illustrates at the same time that the absolute level of distension alone can not act as "the" satiety signal. The 24-hr experiments with the crossed-intestines preparation also illustrated that there is no direct relationship between the level of gastric distension and the absence or occurrence of feeding behavior. Modulation of the effect of a distension signal by circadian rhythms or via synergistic interaction with hormonal or paracrine factors, however, remains a possibility; in that case the low level of food intake by the right rat of the crossed intestines pair, despite a low level of gastric distension, could be caused by such an effect, created via a high level of stimulation of its upper gut..

The modification of the standard scintigraphic techniques that was used in the

experiments described in this dissertation made it possible to explore some uncharted territory. The main accomplishments of this adaptation were the fact that measurements could be made accurately of cumulative food intake, gastric filling and gastric emptying simultaneously. Contrary to most gastric emptying studies, these measurements could be made in a natural, free-feeding situation, where the animals were allowed to take several self-initiated meals in succession, and the measurements could be made during as well as between these meals. Contrary to standard techniques, where gastric retention of a single labeled meal can only be measured for the time that there is enough label in the stomach to allow a reliable estimation of the emptying rate, the technique that was applied in the present experiments made it possible to continue these measurements over considerably longer time periods (up to more than a full day) within the same animal. Again, contrary to many single-meal studies that have to make estimations of 24-hour emptying based on a few data points, the actual measured rate of gastric emptying over a full day was similar to the observed food intake of the rats over such a period, greatly enhancing the physiological relevance of the results. With the present state of technique, the practical use of scintigraphy in a free-feeding model remains limited by the fact that extensive manual motion corrections have to be applied to the data set to diminish the effects of movements of the animals on the quality of the data. A single best-fitting ROI (instead of the labor-intensive manual recombination of several curves generated by multiple ROI's) could generate acceptable, but noisy quantitative data when a large number of animals is tested; statistically the effects of random movements on the measured gastric emptying rate will become smaller for larger numbers of animals The development of efficient motion-correction programs, implemented on the gamma camera workstations, would make the method more readily acceptable as a research tool.

Measurement of gastric emptying over a full 24-hr day should generate values for the

total number of calories emptied that are not much different from the food intake over that period. The capacity of the stomach for storage of food is limited, and animals appear to regulate their food intake during the night when they do most of their feeding to achieve coverage of a major part of their energy needs during the light phase by storing food in the stomach. The 24-hour experiments with control rats (Chapter 5) shows that they may be following this strategy: gastric emptying continued with the same rate during both the night and day, and even at the end of the light phase most of the animals still had some food left in their stomach. The fact that no significant difference was found between gastric contents or emptying rate in the first two hours of the 26-hr experiment (when still some unlabeled food may have been present in the stomach) and the last two hours (when all food in the GI tract could be assumed to be labeled) further illustrates this point.

Since different food sources have different energy contents, a certain involvement of learning behavior is likely. Rats eating a standard lab chow tend to fill their stomach to maximum capacity at the end of the dark phase (398;403). An intact animal could theoretically regulate its food intake to a great extent via a combination of signals generated by circadian rhythms, learning and the generation of satiety signals via stomach distention. When a rat is fed daily with the same standard diet, it is conceivable that it could learn to fill its stomach up to certain levels at specific times. A strategy for its food intake that is successful in meeting its daily energy needs could then be repeated on the following days. Such a hypothesis would require signals from mechanoreceptors in the stomach to be coupled with a sophisticated internal time keeping system; experimental support for such a metering system could be found in the fact that the meal patterns of individual rats over several consecutive days tend to be quite similar, and also by the fact that rats can not immediately adapt fully to changes in caloric density by adding cellulose fibers to solid food.. Standardization of feed as normally happens in a laboratory setting

makes the effects of different treatments quantifiable, but could also give the animal unnatural clues for its food intake regulation. Under normal circumstances an omnivorous animal like the rat could be expected to take several meals from a variety of food sources, which would make its more difficult to estimate total caloric value and regulate total daily intake based on volume alone.

It can be hypothesized that the lipolysis-lipogenesis cycle that has been observed over the day/night period (207-209) may reflect to some extent the nutrient contents of the stored food in the stomach, so that the stomach contents of solid lab chow may not have sufficient caloric value to cover the energy needs in the light phase and the animal will have to rely on its fat stores for its energy needs during that period. Although measurements of lipogenesis or lipolysis were not made during the present experiments, the continuous gastric emptying of the liquid diet Ensure Plus that was used for all experiments did not appear to support the occurrence of such a cycle as a fully endogenously regulated phenomenon. In general, the energy requirements will be higher during the night when the animal is active, so that an elevated lipogenesis in that period (during an assumed constant level of nutrients taken up from the gut and available in the circulation as suggested by the present experiments) would be the opposite of the physiological needs of the rat in that period, whereas lipolysis in the light period, while the nutrient influx from the gut into the bloodstream is still unaltered, does not seem to make physiologically sense either. Alternatively, a change in the direction of the metabolic cycles could be endogenously regulated via changes in the balance between sympathetic and parasympathetic activation or changing hormone levels; in that case the hypothesized direct correlation with periods of high vs. low food intake (207) would not occur. Experiments with R.Q. determination during long-term enteral or parenteral nutrient infusion could help to elucidate this problem.

Somewhat puzzling in that respect is the feeding behavior of the left rat of the one-way crossed intestines preparation (Chapter 7). Although its stomach contents do not appear to be at maximum values at the end of the night and the stomach contents drop to a low level well before the end of the light period, the animal does not appear to be able to compensate by eating more at the end of the night. It is possible that this reflects the typical (strain-specific) activity and eating pattern of the Lewis rats (447;448), that appear to be regulated via the generation of circadian rhythms by the nucleus suprachiasmaticus (SCN) (446;449). Contrary to the feeding pattern of the Wistar rat (398), both the control rats and the left rat of the one-way cross preparation reached their maximum stomach contents around midnight, well before the end of the dark phase. A cyclic fluctuation over the day of SCN neuronal activity (which is well documented in direct electrophysiological recordings of single SCN neurons (132;274)), could hypothetically cause a minimum in the satiating effects of gastric distension to be reached halfway through the night, so that maximum gastric contents would be reached around that period. An increase in satiety signals from gastric distension would than lead to a gradual decrease in food intake, so that in the case of the left rat of the one-way cross gastric contents at the end of the night would not be matched with its ongoing high rate of gastric emptying in day time. If gastric distension is indeed a major source of satiety signals, its expression can not be on a constant level throughout the day-night period, as is demonstrated by the fact that in the beginning of the feeding period (at the end of the light phase and the beginning of the night) meals are terminated at levels of gastric contents that are well below the levels later in the night, when new meals are initiated. A major effect of unlabeled liquid contents in the stomach on gastric volume and distension is not likely, as has been demonstrated by the strong correlation between gastric surface area and gastric contents over the 24 hour period (Chapter 5). Again, this would suggest a modulatory effect of the SCN on the

expression of gastric distension-related satiety signals, although another possibility would be that mechanoreceptors in the stomach slowly adapts to the higher level of distension. This, however, should cause a slow increase over the night in stomach contents for as long as food intake takes place (i.e. especially towards the end of the dark phase) and fails to explain why food intake slows down after midnight even as the stomach continues to empty its contents at a fixed rate throughout the rest of the night.

One of the most intriguing findings of the present series of experiments is the relatively constant rate of gastric emptying over the full 24-hr period when measured over hours. Especially in the control rats or in the right rats of the one-way cross this level is quite similar between the different rats; in the left rats of the one-way cross the fast rate of gastric emptying causes larger differences that are mainly related to the amount of food that is left in the stomach: these animals have more often than the controls a relatively empty stomach, which automatically leads to a low value for gastric emptying in that period. A constant level of gastric emptying would be physiologically highly functional for the animals, since it would tend to minimize the fluctuations in nutrient influx into the bloodstream over the day and night. Large fluctuations in especially plasma glucose levels would thus be prevented and the pressure on regulatory systems to maintain constant levels of circulating nutrients would be diminished. It was shown in Chapter 5 that the increased rate of gastric emptying during feeding (177) is partially compensated by an attenuation of the emptying rate directly after the meal. This was also indirectly demonstrated by the fact that over the 24-hr period the average emptying rate is not significantly different during the night, when a high feeding activity occurs, compared to the day when feeding is low (or even absent, during the 7-hr food deprivation). The similarity of the results of this experiment with the data presented by Kaplan et al (177) gave indirectly a validation of the very different techniques that were used in these two

experiments (gastric infusion/withdrawal via intubation techniques, versus gamma camera scintigraphy).

The working hypothesis for the present dissertation, that regulation of gastric emptying is the main mechanism for general regulation of food intake, and that gastric distension (regulated directly via gastric emptying) is one of the main factors in the regulation of meal patterns, could not be directly confirmed by the data. The level of stomach contents over the day and night was found to be actually higher in the control rats than in the overeating left animals of the one-way cross, and the underfeeding right rats of the one-way cross had actually the least amount of food in their stomach. This could suggest that post-gastric signals may be more directly involved in satiety than was assumed in the present hypothesis: stimulation by and absorption of nutrients by the transplanted 30 cm jejunal segment would then generate a higher level of satiety in the right rat of the one-way cross, whereas the relative decrease in nutrient availability in its left partner would lead to an increase its food intake. The results of the nutrient infusion experiment appear to agree with the first half of the hypothesis, in so far that the effects on food intake (424;425) and the effects on gastric emptying appeared to be strikingly similar. The data suggested that the rate of gastric emptying is set relatively independently from the rate of food intake and gastric filling; the alternative option that food intake is the driving force behind the process of gastric emptying could not explain the immediate differences in gastric emptying between the left and right rat of the cross-intestines pair. The gastric distension-hypothesis, however, fails to give a full explanation for the observed differences in food intake in this preparation. If gastric distension would be a major factor in satiety, at least some other factor (apart from circadian modulation) must be involved. Although not investigated in the present series of experiments, a tentative explanation may be found in the expression of the peptides CCK and amylin. Their release, caused by resp. the

presence of nutrients in the gut and an elevation of blood glucose levels, has been shown to decrease the rate of gastric emptying (55;192;232;457-459), and also to exert a potent inhibitory effect on food intake in physiological doses and in a strong synergistic manner (45;228;230;275-277;339;457). Amylin and CCK may exert their effect via modulation of the expression of vagal activity: CCK is believed to affect vagal afferent nerves directly (68;99;101;369-374;376;377), amylin (and possibly also CCK) appears to have a hormonal function (227;229). A number of recent studies indicate that amylin may have its effect via a direct action on the area postrema (231;337;339;457), which is located outside of the blood-brain barrier; a modulatory role for both CCK and amylin on efferent vagal outflow seems therefore likely. This putative regulatory system would explain the contradictions in a number of studies, including the present series. For instance, it could help to explain the fact that pancreatic insulin secretion (which is accompanied by amylin release) has been found to act as a satiety signal in normal rats (452-454), whereas in streptozotocin-induced diabetic rats (that have no functional β-cells and therefore no amylin secretion) insulin infusion under isoglycemic conditions leads to an increase in food intake (440-442). In the one-way crossed-intestines right rat, because of nutrient stimulation of and absorption from the transplanted segment, elevated levels of circulating amylin as well as release of CCK could be expected; this would lead to a strong inhibition of gastric emptying and an elevated expression of gastric distension as a satiety signal, which would be in keeping with the effects on food intake and gastric emptying.

A number of other current theories about meal initiation and meal termination could not be supported by the present data. Meals initiation in the rat is usually accompanied by a relatively small (ca 10 %) decrease in plasma glucose levels that appears to be functionally coupled to the start of feeding behavior; it has been suggested that this change in plasma glucose would be caused by a decrease in nutrient availability so that the start of the new

meal would reflect a reaction to a temporarily energy deficit (39-41;207;225)strongly suggest that the rate of gastric emptying does not change in the last minutes before the meal. Another possibility would be a change in nutrient absorption; since only the passive uptake from the gut of highly diffusible molecules is thought to be influenced by changes in blood flow to the intestines (124) to counteract the preprandial dip in glucose levels leads to a postponement of the meal (41), however, suggests that this effect is somehow integrated in a sequence of events that must be successfully completed for a new meal to be initiated. Other theories that suggest that meal initiation is part of a homeostatic regulatory mechanism as a reaction to a decrease in plasma levels of glucose ("glucostatic theory", (241-243)), amino acids (aminostatic theory, (256)) or availability of intracellular ATP (106-109). Although all these theories are supported by experimental evidence, the present data suggest that such fluctuations in nutrient availability generally do not occur as a result of decreased gastrointestinal motility and a decreased influx of nutrients into the intestine and a subsequent absorption into the bloodstream. This holds true especially during the early night phase, when nutrient contents of the GI tract are increasing and food intake is at its maximum.

Meal termination must be caused by a signal that is directly associated with meal intake. The data from Chapter 5 (measurements in 1-min intervals) support the theory that an important signal is created by gastric distension: although an increase in gastric emptying rate occurs during feeding activity, the major part of the incoming food is still stored in the stomach, thus causing a rise in intragastric volume and stomach distension. For small meals the observed increases in stomach contents are relatively small, whereas at other times considerably more food is stored in the stomach before cessation of feeding occurs. The satiety signal is therefore more likely created by an integration of signals from different sources; the increase in emptying rate during the meal would also contribute to

the signal via increased stimulation of intestinal receptors and increased uptake of nutrients from the gut. The effects of a small decrease in blood glucose on meal initiation (41) show that the rat is able to detect relatively small changes in its blood glucose level; an increase in blood glucose levels of similar magnitude is therefore not likely to remain undetected by the animal. Stimulation of glucose-sensitive hepatic vagal afferents (350;352-354)could be part of this integrated response to the incoming food.

The effects of the nutrient infusion appears to have similar effects as could be hypothesized for the increased rate of gastric emptying during feeding: a marked reduction of gastric emptying took place after several hours of amino acid infusion, mimicking the strong inhibitory effects of amino acid infusion on food intake (424){Walls & Koopmans 1992 ID: 11328}. The data also show a similar effectiveness for lipids and for glucose in reducing food intake and inhibiting gastric emptying, strongly suggesting that these functions share some common pathway, and would support the hypothesis that the rate of gastric emptying is an important factor in the regulation of food intake. The results also suggest that there may be a post-absorptive component in the effects of intestinal proteins: although the absolute level of protein in a normal diet is not more than ca 15 %, the effects of the amino acid infusion were more pronounced than that of glucose. Also glucose could have part of its effect via post-absorptive mechanisms; the hormonal responses to an elevated glucose level, such as insulin and amylin release, are likely to play a major role in the inhibition of food intake. Based on the present data, the inhibitory effects of lipids are for the major part restricted to their effects on intestinal receptors. The effects on gastric emptying were still not significant even after seven hours of intravenous infusion still not significant, which is in accordance with the relatively small effect on food intake (424;425).

A similar dual effect of intestinal and post-absorptive stimulation could be involved in

the inhibitory effects on gastric emptying of the right rat of the one-way crossed intestines preparation when its partner is allowed access to food. The short-term deprivation of the left rat led to a marked increase in gastric emptying rate of the right animal to values similar to normal control rats. As soon as the left rat was allowed access to food, the increased level of post-gastric stimulation (via nutrients entering the crossed intestinal segment) led to a reduction of the right rat's emptying rate over a period of 1-2 hrs to its characteristic values. This again supports the theory that gastric emptying takes place mainly independently from food intake and is regulated via intestinal and possibly post-absorptive inhibitory feed-back signals.

A number of follow-up experiments are suggested by the present findings. The present experiments measured the regulation of gastric emptying of a single concentration of a high-caloric liquid diet. Repetition of the experiments with a labeled solid food source would determine whether the present conclusions are more generally applicable to natural feeding situations. For instance, the increased rate of gastric emptying during feeding has only been investigated with liquid or semi-solid food. Whether during intake of solid food also an increased amount of gastric contents are being emptied remains to be determined; similar findings with solid food would considerably strengthen the case for a possible role of this mechanism in the expression of normal satiety. Similarly, a repeat of the 24-hour gastric filling in control rats with high-caloric or low-caloric solid food would clarify the issue whether during the night rats will fill their stomach to a larger extent if the caloric value of the food is decreased. In addition, the hypothesized modulatory influence of circadian rhythms on satiety signals generated by gastric distension, could be efficiently researched using the present gastric filling analysis in a model of SCN-lesioned rats. The prediction could be made that these animals will terminate their meals at a relatively constant level of distension, while their meals are being spread out more evenly over the

day. Another hypothesis that could easily be tested via the direct measurement of gastric filling would be the relationship between meal size or post-meal gastric contents and post-meal intervals. For this experiment 24-hour data from free-feeding animals under ad lib conditions would be required.

In summary, the present experiments appear to support the hypothesis that the regulation of gastric emptying is one of the main underlying mechanisms in the regulation of food intake. The direct effects of gastric distension as a major source of satiety signals and its hypothetically important role in meal termination could not be confirmed based on the present data alone; additional factors will have to be involved if the role of this mechanism as an important source of satiety signals is to be upheld. In its present form the analysis technique that was developed for this dissertation has been shown to generate detailed, accurate information about the regulation over time of gastric filling and emptying in parabiotic rat models.

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