CONTROL MECHANISMS OF MAMMALIAN PEPSINOGEN SECRETION

Irvin M. Modlin, M.B.ChB, FCS(SA), FRCS (Ed), FACS

July 1989

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

This thesis, "Control Mechanisms of Mammalian Pepsinogen Secretion," has been submitted in fulfillment of the Ph.D. requirements for the University of Capetown (UCT).

July 1989

Irvin M. Modlin, M.D.

ACKNOWLEDGEMENTS

ABSTRACT

Section I

HISTORY AND REVIEW

1. History of Digestion 2
2. Theodor Schwann 17
3. John Langley 24
4. An Overview of Pepsinogen 35

Section II

METHODOLOGY

5. History and Review of Pepsinogen Measurement 66
6. Ussing Chamber 79
7. Pepsinogen Assay 82
8. A Micro Method for the Assay of Cellular Secretory Physiology in Parietal Cells 95
9. Isolated Gastric Glands in the Study of Gastric Secretory Relationships 112

Section III

EXPERIMENTAL

10. Evidence for Dual Modulation of Pepsinogen Secretion using Isoproterenol, Carbachol, CCK-8, Forskolin, 8 BROMO cAMP and A23187 in Isolated Gastric Glands 136
11. Vasoactive Intestinal Peptide Augmentation of Cholecystokinin Stimulated Pepsinogen Secretion in Isolated Gastric Glands 152
12. Effect of Trifluperazine, A Calmodulin Antagonist, on Pepsinogen Secretion in Isolated Gastric Glands 168
13. Pepsinogen Release in Isolated Gastric Glands and Parietal Cells by a Prostaglandin El Analogue 185
14. Dissociation of Pepsinogen and Acid Secretion in the Guinea Pig Gastric Mucosal Sheet 196
15. Effects of Cholecystokinin and Cholinergic Receptor Blockade on Guinea Pig Gastric Mucosal Sheet Pepsinogen Secretion 212
16. Pepsinogen Release and Acid Secretion from Human and Guinea Pig Gastric Mucosa Compromised by Hypoxia, Endotoxin or Critical Illness 231

CONCLUSION 258

ACKNOWLEDGMENTS

This study was undertaken on the advice and with the supervision of Prof. S. Marks and has been pursued under his direction. It has been sustained by his constant counsel, incisive comments and witty encouragement, without which it would surely have foundered. Apart from his primary role in this particular body of work I wish to gratefully acknowledge his original powerful and initiating influence in activating my curiosity in the scientific investigation of the gastro-intestinal tract. I find it hard to express adequately my deep gratitude to him for his role as my cybernin over a period of many years.

Considerable thanks are due to Prof. Steven Bloom of the Royal Post Graduate Medical School, London, who spent much of his valuable time inducting me into the mysteries of peptide hormones and their role in the gut. His patience and forbearance in the education of a surgeon was nothing less than formidable. His optimism in the belief that a gut hormone might well influence pepsin secretion has been borne out.

To Prof. John Walsh and the late Mort Grossman of CURE I owe an enormous debt of gratitude for their courage in attempting to inculcate into a surgical neophyte the arcane fields of peptide chemistry and gut physiology. Both imbued me with the appreciation of how a clear and focussed question diligently pursued could provide both balance and perspective to the understanding of a subject. Mort allowed me to recognise that chemical regulators were the "message" of the future and John allowed me to glimpse the nature of their translation.

Throughout my pursuit of this topic, Howard Spiro has been a source of gastroenterological joy and philosophic delight. He chided me at my assumptions, humbled me with the purity of his recall and never ceased to be helpful as he encouraged me forward into theory whilst enjoining me to look backward into the interpretation of gastric history.

In a similar yet different fashion I owe an extraordinary debt of gratitude to Howard Rasmussen. A magus in the departments of physiology and endocrinology he exhibited Sisyphusian tolerance in shepherding me through the membrane barrier into the cabalistic metascience of secondary and tertiary messenger systems. He allowed me to appreciate the true meaning of synarchic relationships whether they be applied to calcium messenger systems or the teacher-pupil inter-reaction. Rarely has one so ill prepared as I been so carefully nurtured, so appropriately stimulated.

In my peripatetic course towards some general understanding of the subject of pepsinogen, I have been fortunate to have

had the support and guidance of many wise and generous scientists and clinicians. The lucidity of presentation and the perspicuity of thought that Basil Hirschowitz introduced me to, allowed me to comprehend the nature of the term "seer" as applied to gut science. His kind support and generous advice have greatly amplified my understanding not only of the subject of pepsinogen secretion but also that of gastric physiology. In no less a fashion am I indebted to Hugh Baron of the Royal Post Graduate Medical School in London, who in his own inimitable style gave advice, support and criticism worthy of one far more knowledgeable and deserving than myself. His lessons were my first formative experience in the understanding of the pleasures that the pursuit of knowledge might generate.

Apart from my "gastric" mentors I wish to gratefully acknowledge many who have provided the infrastructure which has allowed this project to come to fruition. William Collins, Cushing Prof. of Neurosurgery and Chairman of the Dept. of Surgery at Yale who has been so remarkably tolerant and supportive of the idea of a general surgeon spending time wandering thorough the realms of cells and secretion. Without his material and temporal support none of this work would have been possible. I am also beholden to John Terblanche, Professor and Chairman of Surgery at The University of Cape Town who established the unique inter-departmental discipline of an integrated gastro-intestinal and surgical division and thus enabled me to pursue much of this gastro-intestinal work from within the framework of his surgical department. For hisconfidence and substantative support, both in appointing me to the unit during critical phases of this study and providing much needed resources, I am indeed most grateful. Similarly I owe thanks to the deans of the medical school of both Yale and the University of Cape Town - Lee Rosenberg and J.P.Van Niekerk respectively. Their concern and interest in allowing me to pursue this venture has been amply demonstrated by the sabbatical time allotted to me by Prof. Rosenberg and the substantial advice and support afforded to me by Prof. Van Niekerk. This has enabled the complex scientific and geographical logistics of the project to be dealt with in an atmosphere of academic warmth and intellectual growth.

As a coda, I recognize that ultimate thanks are due to Schwann who conceived the idea of pepsin and Langley who so critically defined the logic of its site and role as a zymogen. Without their pioneering work this dissertation would not have been possible. In conclusion it must therefore be acknowledged that pepsin was and still is The Primary Intellectual Ferment.

ABSTRACT

Introduction

The objective of this thesis was to delineate aspects of the control mechanisms of mammalian pepsinogen secretion. In order to accomplish this goal, a comprehensive study was undertaken which would establish an historical perspective of the subject, validate appropriate methodology and then seek to answer specific questions regarding the physiology and pathophysiology of pepsinogen secretion. More specifically, the objectives of this thesis were:

1. To review the historical background of the subject of pepsinogen in the context of the physiology of digestion with specific emphasis on the work and lives of the two major initial proponents of pepsinogen research (Schwann and Langley).

2. To provide a contemporary overview and evaluation of the current status of pepsinogen pathophysiology.

3. To modify and adapt experimental models necessary for the study of pepsinogen and acid secretion in mammalian gastric mucosa and cells.

4. To establish and validate a pepsinogen assay sensitive and reproducible enough for use in mammalian mucosal and cellular secretory systems.

5. To delineate the fundamental (second messenger) control mechanisms (cyclic AMP and calcium calmodulin) of pepsinogen secretion in the isolated gastric gland model.

6. To define whether the process of pepsinogen secretion is independent of acid secretion in intact mucosal preparations.

7. To identify different classes of pharmacological agents which would inhibit pepsinogen secretion and/or release.

8. To identify whether conditions present in critically ill patients liable to mucosal "stress ulceration" might influence the release of pepsinogen.

Historical

The introductory chapters provide an historical background of digestion. Particular attention has been paid to the evolution of the understanding of digestion as a physiological process, the recognition of acid secretion as an active process, the

subsequent identification of hydrochloric acid and the eventual realization that "pepsin" is involved in the digestive process. The history of pepsinogen is considered with respect to both its discovery and the development of the different methodologies utilized for its measurement. Langley & Schwann's contribution and contrasting backgrounds are considered in detail.

Overview

The overview section considers the current state of knowledge of pepsinogen and covers a wide spectrum of biochemical, pathophysiological and clinical aspects. It provides a basic framework against which the specific areas addressed in this thesis should be viewed.

Methodology

The comprehensive methodology section includes a number of innovative procedures utilized in the thesis. "The microtitre plate" was developed to facilitate the study of parietal and chief cells, and the isolated gastric gland technology adapted for usage in the microtitre plate system. The Ussing chamber preparation was modified for a guinea pig model, and the system adapted to enable generation of a measurable pepsinogen signal. The original hemoglobin substrate-pepsinogen assay of Anson was modified substantially by use of isotope-labelled albumin as substrate. The resultant increase in sensitivity allowed for more accurate measurement of pepsinogen secretion or release by mammalian mucosa under experimental conditions.

Experimental

The first two chapters of the experimental section describe the validation of the gland and cell systems in terms of both secretory capacity and the ability to respond to different agonists. The pepsinogen assay was also validated. In addition, evidence in support of the interaction of the cyclic AMP and calcium calmodulin systems in the chief cell in the regulation of pepsinogen secretion is presented. No unique data was obtained and the results confirmed that the work was consistent with that reported by other investigators.

The effect of a putative specific calmodulin antagonist on pepsinogen secretion was examined. This experiment was based on the rationale that one of the most potent agonists of pepsinogen secretion was cholecystokinin and that this peptide functioned via the calcium calmodulin system. Trifluperazine, however, resulted in a paradoxical increase in measured pepsinogen. It is possible that the results observed reflect the nonspecific effects of trifluperazine and are more suggestive of release via cell damage rather than a physiological secretory response.

Since the prostaglandin E1 analogue (misoprostol) had been widely reported to be efficacious in peptic ulcer disease, an investigation of its effect on acid and pepsin secretion was undertaken. The inhibitory effect on acid secretion was confirmed. Prostaglandin E1, however, was demonstrated to be a significant agonist of pepsinogen secretion.

A different line of investigation was pursued in an attempt to define an agent capable of adequately inhibiting pepsinogen secretion. Firstly, the question of whether pepsinogen secretion by the chief cell could be completely dissociated from parietal cell acid secretion was addressed. In Ussing chamber-mounted mucosal sheets, the proton pump inhibitor omeprazole was used both alone and in conjunction with the pepsinogen agonist, cholecystokinin. These studies demonstrated that histamine-stimulated acid secretion and cholecystokinin-stimulated pepsinogen secretion were separate and independent events. With this information as a baseline, a second series of studies was undertaken using the specific cholecystokinin receptor antagonist L364718. The results obtained demonstrated that the agonist effect of cholecystokinin on pepsinogen secretion could be selectively and effectively blocked by a specific cholecystokinin receptor antagonist used at dosages which were physiologically acceptable. In addition, when contrasted with pepsinogen secretion stimulated by a cholinomimetic, it was evident that this inhibition was cholecystokin receptor specific.

The final experiment examined the possibility of a significant increase in pepsinogen release when gastric mucosa is compromised by either hypoxia or endotoxemia. The guinea pig Ussing chamber mucosal model provided both morphological and biochemical evidence of mucosal damage and increased pepsinogen release under these circumstances. A correlation was attemped by contrasting pepsinogen levels in the gastric juice of critically ill ICU patients with samples obtained from postoperative patients in relatively healthy and stable condition. This last study, while failing to provide irrefutable evidence of a role for pepsinogen in a damaged or "stressed" mucosa provides some information consistent with the hypothesis that increased levels of pepsinogen occur and may well be related to the pathophysiological evolution of mucosal damage in critically ill patients.

Section I

HISTORY AND REVIEW

CHAPTER 1

History of Digestion

As one wanders down the corridors and shelf stacks of the library one is left in awe at the compendium of man's knowledge. It is here that the heritage of civilization and science is studied, interpreted and handed on to succeeding generations. In the environment of the laboratories and libraries of great institutions of learning throughout the world, scholars work to extend the frontiers of thought and to enlarge man's perception of his world and the processes involved in its function. The labors are often mighty yet the information gleaned in many instances only minuscule. Indeed, as Thomas Gray so aptly framed it,

"But knowledge to their eyes her ample page.
Rich with the spoils of time did ne'er unroll ...."

History is however replete with wonderful examples of the mysteries of the pursuit of information, and the extraordinary and often turbulent lives of those who sought to frame the questions of life.

The particular area that has interested me for some considerable time has been the science and history of digestion. The exact origins of human knowledge regarding the intake of food and the speculations on the digestive process are lost in the mists of antiquity. Hippocrates (460-377 B.C.) in De Prisca Medicina states: "It was the necessity which men in the first stages of society must have felt of ascertaining the properties of vegetable productions as articles of food that gave rise to the science of dietetics; and the discovery having been made that the same system of regimen does not apply in a disordered as in a healthy condition of the body, men felt compelled to study what changes of the aliment are proper in disease and it was the accumulation of facts bearing on this subject which gave rise to the Art of Medicine." (translated by Francis Adams - 1849).

THE NATURE OF DIGESTION

In the earliest of times physicians had believed that the different organs were the seat of separate spiritual agencies which in a divine fashion controlled bodily function. The Ancient Greeks had proposed that digestion was in fact a process of concoction or heating, and in this evolution food was converted initially to chyle and then to the four humors: blood, phlegm, yellow and black bile prior to utilization by the mortal body. Hippocrates had in fact called the process of digestion pepsis and proposed that it was not dissimilar to the preparation of food by cooking (1). He felt that this conversion was accomplished by the heat of the stomach. Galenic physiology proposed a similar theory but in a more complex form. It held that successive cooking processes occurred sequentially in the stomach, intestine, and liver until food was finally converted into blood. Alternative doctrines as suggested by Plistonicus, a pupil of Praxagorus indicated that digestion might be due to putrefaction. This proposal was still entertained as late as

1763 by the English physician William Cheselden.

The concept of the stomach as an organ actively secreting acid and pepsin which was responsible for digestion was not appreciated until the early 19th century. The early Greeks were not aware of acids in the modern chemical sense but identified them only as bitter-sour liquids. Thus, Diocles of Carystos (circa 350 B.C.) specified sour eructations, watery spitting, gas, heartburn, and epigastric hunger pains radiating to the back, with occasional splashing noises and vomiting as symptoms of a melancholic gassy illness originating in the stomach (2). Some 300 years later Celsus (30 B.C. - 25 A.D) recognized that certain foods were acidic and recommended that "if the stomach is infested with an ulcer...light and gelatinous food must be used...and everything acrid and acid is to be avoided" (3).

There appears to have been little further development in understanding the function of the stomach and the nature of digestive processes until the 16th century. Aureolus Theophrastus Bombastus von Hohenheim, (Paracelsus), was born in Ensiedeln near Zurich in 1493 (4). He was an alchemist physician and a proponent of chemical pharmacology and therapeutics who proposed some major reforms in medical thinking. After training in Ferrara (1515) he taught at Freiburg and Strassbourg (1525) and was appointed Professor of Medicine in Basel in 1527. He publically burnt the books of Galen and Avicenna and rejected the old notion of four elements (earth, air, fire, and water). Instead he proposed that the forces of energy that governed the universe were archaei and maintained that physiological processes, diseases and drugs were chemical changes governed by the chief archaeus. Paracelsus believed there to be acid in the stomach and that it was necessary for digestion. He considered that the acid in the stomach of humans came from the drinking of acidic spa waters (acetosum ensurinum, hungry acid) and that its action was part of a catalytic process necessary to prevent the formation of precipitations and concretions within the body. As a response to his novel assertions and his forthright style, the authorities of Basel forced him to leave. He subsequently died from a wound sustained in a tavern brawl at Salzburg in 1541. Paracelsus' observations were, however, far in advance of his time and although many of his assertions appear as successful guesses or evidence of remarkable intuition, he clearly recognized the importance of chemistry and its relation to disease; rejecting Galenism and the mysticism of humors and health. The question of acid in the stomach and its exact nature was, however, still moot (5). Alchemists and chemiatrists had made progress in the identification of many substances. Johann Tholle who wrote under the pseudonym of the mythical 15th century monk Basil Valentine had published extensively regarding ammonia, sulfuric acid, and sugar of lead. He is reported to have identified hydrochloric acid and prepared it by mixing oil of vitriol (sulfuric acid) with common salt (6). Thus, by the middle of the 17th century Jean Baptiste

von Helmont was able to write of an acid ferment in the stomach responsible for digestion. Von Helmont was born in Brussels in 1577 and became a Doctor of Medicine in 1609 after spending some time as a Capuchin friar (7). Von Helmont was a mystic and like his master Paracelsus, believed that each material process of the body was presided over by a special archaeus or spirit which he named Blas. His doctrine held that physiological processes are in themselves purely chemical, being due in each case to the agency of a specific ferment (or gas). Von Helmont founded the IatroChemical School of Physicians which maintained that the principal archaeus (Blas) was controlled by the sensitive and motive soul, anima sensitiva motivaque, in the pit of the stomach (solar plexus) and that from this site all chemical physiological processes were regulated (8). He believed that digestion began in the stomach by the intermittent fermentation of acid and a ferment and that thereafter a number of other fermentation processes including that of bile in the duodenum took place. As a child, von Helmont had observed "a great sharpness in the throat of sparrows" which he fed. He presumed this to be part of the acid digestive process of the bird. In addition, he had noted that distilled liquid of sulfur could digest his leather glove. In further observations he recognized that acid alone did not digest food in vitro and he postulated the existence of another agent typified as a ferment. Von Helmont proposed the existence of an acid ensurinum as a normal component of the human stomach although he felt that it was derived from the spleen. He proposed that gastric acid was a requirement for digestion and that it was neutralized by the alkaline gall in the duodenum (9). In subsequent studies he proposed that this acid might be a mineral acid such as nitric or hydrochloric acid. He produced spiritus salis marini (spirits of sea salt) (hydrochloric acid) by distillation of salt and clay and noted that it could dissolve human kidney stones (Duelech) in much the same manner as juice from a bird's stomach. The son of von Helmont published his work - Ortus Medicinae - four years after his death in 1648 (10).

The physiological chemistry of von Helmont was divested of much of its mystic and spiritual overtones by the Leyden Professor Francois de la Boe-DuBois better known as Sylvius (1614-1672). Nevertheless, as late as 1757, Stahl considered the role of the supernatural to be of some importance in the physiology of digestion. Sylvius however, explained digestion on a chemical basis but excluded the mysticism which had permeated von Helmont's work (11). As Professor of Medicine at Leyden from 1658-1672 he was in charge of the first chemical laboratory and had learned from Glauber about the interaction of acids and alkalis. He ignored von Helmont's suggestions of a specific gastric ferment and maintained that digestion took place in the stomach by saliva which was also the major source of intestinal juice. At this time, considerable attention had been focused on the salivary glands by the studies of Niels Stensen of Copenhagen who was a pupil of Sylvius. This physician-priest described the

excretory duct of the parotid gland in 1661 prior to proceding on to numerous other important physiological observations. He achieved immortality as one of the fathers of modern geology and was ultimately appointed Bishop of Titiopolis in 1667. Sylvius supported pancreatic juice as the main factor in the digestive process and his pupil Regner de Graaf in 1664 produced experimental pancreatic fistulae to study this proposal. Unfortunately, de Graaf concluded that pancreatic juice was acid in character and he and Sylvius hypothesized that bile and pancreatic acid in combination fermented and digested food.

These chemical views as propounded by Paracelsus, von Helmont and Sylvius represented the doctrine of the Iatrochemists. They were, however, strongly opposed by the Iatromathematical school who maintained that all physiological happenings should be treated as rigid consequences of the laws of physics. This group included men such as Descartes, Borelli, Sanctorius, Pitcairn, and Boerhaave. Giovanni Alphonso Borelli (1608-1679) was a Neapolitan mathematician who published De Motu Animalium in 1680. He regarded all physiologic processes as mechanical in nature and he proposed the erroneous idea that a contracting muscle increased in bulk due to the fermentation within its substance which was initiated by a liquid discharged into it by nerves (12). The existence of the Iatrophysical doctrine was further propounded by a pupil of Malpighi-Georgio Baglivi (1668-1706) - whom Clement XI had appointed to the chair of Medical Theory in the Collegio Della Sapienza. Baglivi experimented on muscular physiology and was the first to distinguish between smooth and striated muscle. "He pushed the mechanical allegory to the extent of dividing the human machine into innumerable smaller machines: likened the teeth to scissors, the chest to a bellows, the stomach to a flask, the vicera and glands to sieves, the heart and vessels to a water work" (12). Baglivi, however, dispensed with all these esoteric theories when he looked after patients. He was an extremely effective physician and a true follower of Hippocrates at the bedside. "To frequent societies" he said, "to visit libraries, to own valuable, unread books, or shine in all the journals does not in the least contribute to the comfort of the sick" (12).

Thus, Borelli and his disciples favored the view that the stomach was but a mechanical mill grinding up its contents into chyme. Mobius denied the existence of gastric acid and Archibald Pitcairn interpreted all function in terms of mechanical activity. The members of the Iatromathematical school cared little for the new science of chemistry and their postulates faded into such sterile eccentricities as Archibald Pitcairn's proposal to base the whole of medical practice upon mechanical principles. Thus, in 1727, Pitcairn questioned the Iatrochemical group "...why upon the digestion of food upon the stomach, which is easily (as) digestible as the food, yet the stomach itself should not be dissolved" (13). Despite the fact that he was a successor of Sylvius,

held the Professorship of Medicine at Leyden, and wrote Elementa Chemia in 1732, Boerhaave, a pupil of Pitcairn also denied the idea of either a gastric acid (von Helmont) or a pancreatic acid (Sylvius).

Albert von Haller (1708-1777) was a pupil of Boerhaave and Professor of Anatomy, Medicine and Botany in Gottingen from 1736-1753. He was an infant prodigy who wrote Latin verses and a Chaldee grammar at ten years of age. In his seventeen years in Gottingen he wrote some 13,000 scientific papers and ultimately produced his Elementa Physiologicae Corporis Humanae (1759-1766) which confirmed his reputation as the master physiologist of his time. Von Haller recognized the use of bile in the digestion of fat but declared that gastric juice was not acid, alkaline, or a ferment and believed that any acid found in the gastric juice was from the degeneration of food.

THE NATURE OF DIGESTIVE AGENTS

Thus, by the end of the 17th century, there were two main schools of thought regarding the nature of digestion. The one maintaining it as an act of trituration while the other perceived it as a chemical action occurring either via fermentation or, alternatively, by substances either in saliva or secreted by the lining of the stomach.

Attempts to define the nature of digestive agents were aided by the ingenuity of investigators in developing methods of obtaining gastric juice and secondly, by the development and recognition of indicator dyes. These vegetable derived substances changed color when exposed to appropriate acids or alkali and allowed for identification of the nature of the material being tested. In particular, controversy centered around the chemical nature of the acid in the stomach and the debate as to whether it was primarily secreted by the stomach or in fact derived in some way from ingested food.

METHODS OF OBTAINING GASTRIC SECRETION

A. Animal Necropsy

In 1692, Viridet experimented upon dogs, cats, squirrels, hares, pigs, and eagles (14). He killed the animals either after a meal or fasting, opened the abdomen and collected the gastric contents. Viridet noted that "solutio heliotropii" could, by turning red, indicate the presence of acid. In a classic study, he killed a specially fattened pig and poured solutio heliotropii down its throat. The blue color was noted preserved even to the entrance of the stomach, but that in the stomach an intense red solution was evident. Viridet noted that the stomach smelled of acid and that this resembled the odor of fermentation. He also noted that in humans one might recognize acid in the esophagus but this was in fact due to regurgitation of stomach contents. "We experience it by an acid in the mouth. The condition was not

a natural one."

B. The Use of Sponges

In 1752, Reaumur, the French naturalist noted that birds of prey vomited indigestible objects such as feathers and bones (15). He therefore experimented on a tame buzzard by feeding it small hollow metal tubes containing different varieties of food. When the tubes were recovered, it was apparent that the food had dissolved without putrefaction leaving a bitter yellow fluid. In order to study the nature of this fluid, Reaumur placed sponges in the small metal tubes and on recovery squeezed these to obtain the gastric juice. The latter was a sour fluid which turned blue paper red. Reaumur attempted to study in vitro digestion by incubating the gastric juice with meat. The meat, however, failed to completely digest although he noted that the gastric juice had prevented the "onset of corruption". He interpreted the failure of this study to indicate that either digestion required a high temperature or that the gastric juice required constant renewal. Alternatively, it was possible that in the in vitro situation the evaporation of a volatile acid had taken place. At this stage, Reaumur's pet buzzard died and he thereafter ceased his studies in birds.

C. Self Emesis

In 1760, Reuss found that even with preliminary alkalization of the stomach, the ingestion of a meal of meat and vegetables resulted in acid secretion: "the vomit, having an acid taste and turning an infusion of 'campanules a feuilles rondes' red" (16). Gosse in 1783 repeated these studies more elegantly. He had as a child developed the faculty of aerophagy and self-induced emesis, whereas Reuss required to take an emetic. By inducing emesis at specific times after eating, Gosse was able to demonstrate that digestion began within thirty minutes of eating and was concluded by approximately two hours. Gosse, however, was not able to find acid or alkaline gastric juice, and suggested that secretion occurred by a mechanical process whereby food stretched the internal lining of the stomach. He was, however, able to report that there were different varieties of food, some of which were partially digestible and others completely digestible (17).

In 1777 Edward Stevens presented his inaugural thesis "De Alimentorum Concoctione" to the University of Edinburgh (18). This young man, who may have been the half-brother of Hamilton, had been born in the Leeward Islands (British possession) graduated from Kings College in New York (now Columbia University) and pursued his studies in Scotland. He was the first to apply Reaumur's studies to humans and was the first person to perform an in vitro digestion experiment successfully. He thus proved that the gastric juice itself contained the active principle necessary for the assimilation of food. Stevens obtained the services of a Hungarian,

Hussar, who was visiting Edinburgh and whose means of livelihood was to entertain the populace by swallowing stones and then rejecting them. Using perforated silver spheres so constructed as to hold meats, vegetables, worms and leeches, Stevens observed that all were digested when passed through the rectum. He did not use a sponge and thus, did not attempt to extract gastric juice and evaluate the presence of acid (19).

In 1780, Lazzaro Spallanzani who was the Professor of Natural History in Pavia, published his extensive observations in this area (20). He had used Reaumur's methods in fish, frogs, snakes, cattle, horses, cats, dogs, and himself. In 1783 he finally proved that digestion in vitro as well as in vivo was a chemical process but he asserted that gastric juice was neutral (21). Spallanzani's studies bear comment since they were performed in great detail and with considerable care. He himself initially swallowed linen bags containing food and bread and collected them for examination after they had been passed per anum. Later, he substituted small metal tubes to avoid any possibility of trituration. In both instances, he could find no trace of food remaining. At this time it was felt that there existed three types of fermentation - vinous, acid, and putrid. Since he could find no evidence of fermentation, he postulated that digestion was by an acid or a putrefactive principle. The latter he disregarded since gastric juice prevented putrefaction and in his experiments, there had been no evidence of putrefaction (22). In his own experience, he noted that ingestion of large quantities of strawberries and wine interfered with his sleep by producing severe eructation from the stomach to mouth accompanied by a sharp acid taste. He interpreted this acidity, however, to be an abnormal phenomenon (23). In 1784, he collaborated with his colleague Scopoli who was Professor of Chemistry in an attempt to elucidate the nature of gastric juice. Scopoli reported that the juice from the crows which Spallanzani was studying contained pure water, some soapy and gelatinous animal substances, sal ammoniam, and earthy matter similar to that found in all animal liquids (24).

Spallanzani, however, obviously was uncertain as to his findings regarding the acidity of gastric juice. He corresponded and worked with a number of colleagues in an attempt to resolve this question. In 1785, Spallanzani collaborated with Carminati who was the Professor of Medicine at Pavia. Carminati was the first to detect the acidity of the contents of a meal (25). He said "it is clear that the human gastric juice is neutral as the physiologists such as von Haller and Spallanzani have taught; that this is true in crows, in dogs, and in cats which eats and digests with equal facility both animal and vegetable substances; and that in fact this humor consists of a water, a small amount of marine salt, and an animal substance...I had already recognized in twenty crows that the gastric juice was neutral when I obtained in July two crows whose gastric juice distinctly

turned tincture of heliotrope red, produced immediate and complete curdling of milk, and in every way proved similar to that of carnivorous animals. The novelty of this led me to enquire into its cause and I found that the birds for many days past had been fed exclusively on meat...The same happened in dogs and cats fed entirely on meat for ten or more days as in the crows. Their gastric juice had all the properties of that of crows on a meat diet". Spallanzani was advised to test birds on à meat-free diet and he too found marine acid in the juice squeezed from sponges fed to five ravens fed on vegetables for fifteen days. Later, Brugnatelli in 1786 and some years afterwards Werner in 1800 found the contents of the stomachs of sheep, cats, fish and birds to be acid. At one stage, Brugnatelli who had used the metal tube technique of Reaumur, noted the presence of iron in the stomach of one owl. After correspondence with Carminati who suggested the use of wooden tubes, all trace of iron disappeared.

Despite the relatively clear evidence produced by Scopolli and Spallanzani that there was acid in the stomach and that it was, in fact, hydrochloric acid, considerable controversy existed. Indeed, many of the investigators of this area reversed themselves a number of times during the study of the subject. Of particular interest are the comments of the eminent British investigator, John Hunter (26). In 1772, he stated, "In all animals, whether carnivorous or not upon which I made observations or experiments to discover whether or not there was an acid in the stomach (and I tried these in a great variety), I consistently found that there was an acid but not a strong one, in the juices contained in that viscus in a natural state". After more than a decade of further experimentation, John Hunter, in 1786, assessed the situation somewhat differently in that he regarded gastric acid as originating from a sugar which is secreted by the stomach (27). "I should not be inclined to suppose that there is any acid in the gastric juice as a component or an essential part of it." Thus, even amongst the minds of the most eminent physicians of the day confusion reigned, not only as to the presence of acid but the exact nature of the substance.

DIGESTION AND PEPSIN

One of the first persons to formally recognize the existence of a basic digestive process was Johan Van Helmont (1577-1644) (10). It is reported that his initial interest was aroused by the fact that when as a young child he had fed sparrows and let them get hold of his tongue he had noticed an intense acidity in the bird's throat. Subsequently he noted that the sulphurous substance present in the digestive juice of these birds was able to partially dissolve the leather of his glove to a fluid. Based on the current chemical knowledge of his time he therefore believed that the digestive agent present in the stomach might be an acid. He also postulated that this acid would be specific for each

species of animal studied. Of particular relevance however was the fact that Van Helmont was strongly opposed to Paracelsus' theory of heat as a digestive agent. He stated that "Hunger and strong and molesting digestion should be accompanied by excessive heat. This is not the case..... when food is brought into contact with a warm surface there is no trace of digestion and it putrifies" (28). Van Helmont went on to state that "Warmth acts in a uniform way, the specific vital ferments act according to the exigency of the species and living being in question". Thus Van Helmont, although he had no formal biochemical evidence recognized that it was probable that there was a specific digestive agent(s) in the digestive juice. The vital faculty of transforming food ("Vera atque formaliter") Van Helmont called "Ferment" ("Eamque fermentorum nomine designavi"). In further discussion of the issue he stated that "a digestive ferment, therefore, has the essential power by reason of its vital acidity of causing transmutations". As one can easily recognize Van Helmont at this stage had confused fermentation with digestion and was unable to separate the two different processes simply because of the lack of biochemical information available at this time. His observations however were substantive for the 16th century and a gigantic leap forward in the understanding of digestion as compared to the previous demonic interpretations of the process. He thus broke through the mythological fabric of medical science which had been erected by the Greeks and Romans over the previous 1000 years. Subsequently Descartes, Sylvius and Willis further interpreted his views and correlated the theory of digestion with that of fermentation. At this stage most investigators were still concerned with the digestive or fermentative process in the stomach being related to an acid compound. The idea of alternative agents being capable of participating in the digestion of food had not been formally appreciated.

Nehemiah Grew (1626-1711) had observed the glands in the stomach and in a published lecture stated "By the joynt assistance of the glandulous and nervous membranes, the business of chylification seems to be performed. The mucous excrement provided by the former, as an animal corrosive, preparing, and the excrement of the nerve by the latter as an animal ferment, perfecting the work" (29). There was thus some preliminary contemplation of the possibility of animal ferments in the subject of digestion. Subsequent contributions by the Iatro-Mathematical school of physiologists attempted to explain digestion on a mechanical basis. Thus Pitcairn suggested that digestion in the stomach took place by a grinding force and that this explanation was sufficient to allow the discard of the previous theory which required "the assistance of a Daemon or a Stygian Liquor" (13). Theories of digestion ebbed and flowed over the next 100 years. Herman Boerhaave (1668-1738) of the Eclectic School supported the mechanical theories of digestion and spoke with some considerable authority for Dutch medicine of the early 18 Century. The French as represented by Borden of

Montpelier (1722-1776) supported vitalism and believed that a "vital principle" was the cause of all the phenomena of life including digestion. In England Edward Rigby (1747-1821) stated that the stomach was the seat of "phlogistication" where free heat was produced. Some few years after the death of Rigby, Prout in his epic presentation of 1823 before the Royal Society would demonstrate the gastric secretion of hydrochloric acid and relate its presence to the clinical symptomatology of dyspepsia (30).

It is clear from the preceeding information that up until the early 19th Century there had been little formal understanding of the process of digestion. Although demons and spirits were now relegated to a background position, the science of digestion was as yet poorly defined (31). Prout, Tiedemann and Gmelin had independently identified free hydrochloric acid in gastric secretion. In a separate but subsequent publication William Beaumont had confirmed these observations in man (32). The postulate of a separate animal ferment or digestive agent was substantiated in 1831 by the identification of the diastatic action of saliva by Leuchs. Subsequently in 1834 Eberle had prepared an artificial gastric juice. The quintessential observation however was made in 1836 by Schwann who postulated the existence of pepsin (discussed in detail Chapter 2). Subsequently in 1839 Wasmann isolated pepsin and established the premise for protein digestion. Claude Bernard in 1846 commented extensively on the digestive ferments of the pancreas and in 1854 Epstein and Grutzner postulated the existence of pepsinogen. In 1859 Meisner investigated the products of the gastric digestion of proteins. The nature of the secretory mechanisms of the proteolytic zymogens were first clearly evaluated by Heindenhein when he studied proteolytic zymogens in the pancreas.

It remained however for Langley of Trinity College, Cambridge to formalize the study of pepsinogen and the mechanisms of its secretion from the gastric mucosa (discussed in detail Chapter 3). Although to Schwann must go the credit for first postulating the existence of the digestive enzyme, Langley deserves full credit for his meticulous analysis of the morphology of the chief cells and their mechanisms of secretion. His observations though almost 100 years old have stood the test of time and proved to be valid far beyond what he could have believed possible at the time that he studied the question with the primitive means at his disposal. In 1880 Langley published and presented a series of precise and detailed observations which concluded "The fresh gastric glands contain no pepsin; they do however, contain a large quantity of pepsinogen; consequently the granules of the chief cells consist wholly or in part of pepsinogen". His drawings and sketches quite clearly attest to his clear understanding of the nature of zymogen secretion and some of the mechanisms of its stimulation. In 1898 Gillespie published the first photograph of pepsinogen granules in the gastric mucosa of Salmo Salar (29). Little was written on

the subject of pepsinogen for the subsequent 30 years until Herriot in 1938 was able to isolate it in a crystalline form (33). He studied the chemical properties of the enzyme and confirmed many of Langley's observations on the activation of pepsinogen.

Although Brucke (1861) had first proposed the theory that pepsin was reabsorbed from the gut lumen into the blood and subsequently excreted in the urine, little further understanding of this process has been acquired (34). There still remains some controversy as whether pepsinogen is directly secreted into the blood or whether it represents an ill understood backdiffusion process. Initial work had suggested that it might be transported on white blood cells or alternatively absorbed on to fibrin. Some investigators (Van Slyke 1893) believed that pepsin circulated combined with an inhibitor complex and Fuld and Hirayma (1912) had demonstrated to their satisfaction the existence of anti-pepsin - pepsin complexes (31). It was theorized that these complexes were set free in the kidney and that this accounted for the ability to measure pepsinogen in the urine. Indeed the physiological explanation for the existence of a proteolytic enzyme of this type in the blood has not yet been clearly elucidated nor will it be further considered in this dissertation.

REFERENCES

1. Hippocrates. The Genuine Works of Hippocrates. Translated by F. Adams, Vol I.:155-161. London: Syderham Soc., 1899.

2. Basel. Diocles of Carystos: Disease, Cause and Cure. Cited by Galen. In: On the Affected Parts. Translated by R.E. Siegel, Book III, Chapt. 10:91. S. Karges, 1976.

3. Celsus, A.C. Of the disorders of the stomach and their cure. In: De Medicina Book IV, Chapt. V. Translated by J. Griene, 196-201. London: Wilson and Durham, 1756.

4. Foster, M. von Helmont and the rise of the chemical physiology. In: Lectures on the History of Physiology. 120-143. Boston: Cambridge University Press, 1924.

5. Baron, J.H. The discovery of gastric acid. Gastroenterology 76:1056-1064, 1979.

6. Reti, L. How old is hydrochloric acid? Chymia 10:11-23, 1965.

7. Partington, J.R. Jean Baptiste van Helmont. Ann Sci, 1:359-384, 1936.

8. Pagel, W. J.B. van Helmont's reformation of the Galenic Doctrine of digestion and Paracelsus. Bull. Hist. Med. 29:563-568, 1955.

9. van Helmont, J.B. Opscula Medica Inaudita I. de Lithiasis. Edited by F.M. van Helmont. Amsterdam: Elsevier, 1648.

10. van Helmont, J.B. Ortus medicinae, id est initia physicae inaudita. Progressus medicinae novus. In: Morborum Ultionem Ad Vitam Longam. Edited by F.M. van Helmont. Amsterdam: Elsevier, 1648.

11. Foster, M. Sylvius and his pupils. In: Lectures on the History of Physiology. p 144-171. Cambridge University Press, 1924.

12. Garrison, F.H. The seventeenth century. In: The History of Medicine. 4th Ed., p 257-258. W.R. Saunders Co., 1963.

13. Pitcairn, A. A dissertation upon the motion which reduces the aliment in the stomach to a form proper for the supply of blood. In: The Whole Works. Translated by G. Sewell, J.T. Desaguliers. 2nd Ed., p 106-138. London: J. Pemberton, 1727.

14. Viridet, J. Tractatus novus medico. Physicus de Prima

Coctione Praecipueque de Ventriculi Fermento. Leonard Chouet, Chapt. XI, p 90-100, Chapt. XX, p 216-228. Geneva, 1692.

15. Reaumur, R.A.F. Sur la digestion des oiseaux. In: Memoires de l'Academie Royale des Sciences, 1752: p 266-307, p 461-495. Paris, 1756.

16. Loeper, M. Histoire de la Secretion Gastrique. Edited by Masson et cie. p 73-81. Paris, 1924.

17. Gosse, E. Experiments on digestion. In: Spallanzani, L.: Dissertations Relative to the Natural History of Animals and Vegetables. Translated by T. Beddoes. p 317-328, London: John Murray, 1784.

18. Stevens, E. De Alimentorum Concoctione. M.D. Thesis, Edinburgh, 1777. Transcribed in Thesaurus Medicus. Edited by W. Smellie. Vol 3, p 471-495. Edinburgh: C. Elloitt, G. Robinson, 1785.

19. Day, S.B., Swanson, R.A. The important contribution of Dr. Edward Stevens to the understanding of gastric digestion in man and animals. Surgery, 1962, 52:819-836.

20. Spallanzani, L. Dissertazioni di Fisica Animale e Vegetabile. Modena, Societa tipografica, 1780.

21. Baron, J.H. Spallanzani, Scopoli e l'acido cloridrico. Il giornale della 1° feltimana italianadella malatie digesive. N.7.2. 1979.

22. Prescott, F. Spallanzani on spontaneous generation and digestion. Proc R Soc Med, 23:495-510, 1929.

23. Grondona, F. Des experiments de Lazzaro Spallanzani sur la digestion aux applications therapeutiques du suc gastrique. Clio Medica (Amsterdam), Vol 8. 4:285-294, 1973.

24. Scopoli, G.A. Chemical analysis of the gastric fluid of the crow. In: Spallanzani, L: Dissertations Relative to the Natural History of Animals and Vegetables. Translated by T. Beddoes. p 266-268. London: John Murray, 1784.

25. Walder, A.I. A historical review of the nature of the gastric fluid. Surgery, 52:546-553, 1962.

26. Hunter, J. On the digestion of the stomach after death. Philos Trans R Soc London, 62:447-454, 1772.

27. Hunter, J. Observations on certain parts of the animal oeconomy. In: Some Observations on Digestion. p 147-189. London, 1786.

28. Pagel, W. Religious and philosophical aspects of van Helmont's science and medicine. Bull Hist Med (suppl 2), p 1-44, 1944.

29. Gillespie, A.L. The natural history of digestion. London: Walter Scott, 1898.

30. Modlin, I.M. From Prout to the proton pump. Surg Gyn Obs. In press, 1989.

31. Hirschowitz, B.I. A study of the physiology of pepsinogen in the human. M.D. Thesis. University of Witwatersrand, 1953.

32. Baron, J.H. One hundred and fifty years of measurements of hydrochloric acid in gastric juice. Br Med J, 4:600-601, 1973.

33. Herriott, R.M. Ioslation, crystallization and properties of swine pepsinogen. J Gen Physiol, 21, 501, 1938.

34. Brucke, E. Beitrage zur Lehre von der Verdaaung. V. Die verdauende Substanz im Urin. S.B.Akad. Wiss Wien, 43, 618, 1861.

CHAPTER 2

Theodor Schwann

Theodor Ambrose Hubert Schwann was born in Neuss, Germany on the seventh of December 1810. At primary school and at the progymnasium, Schwann was a delightful and brilliant child who was both diligent and modest. He had, however, little interest in the attractions of society and lacked self confidence. Indeed, his shyness and withdrawn manner, coupled with his close family life and religious background, led to a somewhat sheltered and separate upbringing. He was, however, brilliant in all branches of learning and showed a major aptitude for mathematics and physics. As a result of his quiet personality and his intellectual brilliance it was not surprising that he moved towards the church and in 1826 entered the Jesuit College of Three Crowns in Cologne (1).

His early life is almost saintly in its configuration and proceeded predictably until he came under the influence of an exceptional religious teacher, Wilhelm Smets. Until then Schwann had only been acquainted with the strict aspects of piety although he was endowed with a brilliant intelligence and a lively sensibility. Smets, however, taught him a completely different kind of religion (2). He revealed an entirely new aspect of God and especially the singular fact of the liberty of man with regard to the whole of nature. During his exposure to Smets, Schwann learned the concept of the elevation of man by personal perfection. As a result of this interaction Schwann became further enamoured with the study of reason as opposed to pure theology and decided to pursue a medical career. At this time his personal philosophical position focused towards that of Christian rationality and he based his philosophy more on the tradition of Descartes and Leibniz. To further his medical studies he moved to Bonn where in October 1829 he entered the University and enrolled in the premedical curriculum. In 1831 he obtained his bachelors degree and in the autumn of 1831 he moved to Wurzburg where he studied for three semesters and attended numerous clinical lectures (3).

While at the University of Bonn, Schwann had spent time in the laboratory of Johannes Muller and had also extensively attended his lectures on physiology. When Schwann left Wurzberg in 1833 for Berlin it was to work with Muller, who had since been appointed to the chairs of anatomy and physiology. In Berlin, Schwann attended clinical demonstrations and under Muller's guidance prepared a dissertation on the necessity of air for the development of chicken eggs. On 31 May, 1834 he obtained his M.D. degree and subsequently on 26 July of that year he passed the state examination. Immediately thereafter Schwann became one of Muller's assistants and subsequently devoted all his time and efforts to research (4). Schwann worked for Muller in Berlin from 1834 until 1839. During this time he was helpful in writing sections of the Handbuch Der Physiologie of which Muller was the author. The Handbuch was not merely a compilation by a senior editor. Muller's style was to critically examine all the information that he printed. He therefore repeated the experiments of others and suggested

new types of investigations, thus opening avenues not previously studied. Schwann played a major role in this textbook which demonstrates evidence of numerous contributions both of previously resolved ideas and a number of exciting new postulates (5). The book also contains details of studies which clearly show Schwann's innovative skills. His first experiments can be dated on the basis of his laboratory notebook, which begins 16 April 1835. In these, Schwann envisioned experiments in which it would be possible to subject the physiological properties of an organ or of a tissue to physical measurement.

One such method involved the measurement of the secretion of a gland; another a design to study muscle function. In the latter he measured the length of muscle contracted by the action of the same stimulus for different loads to further compare the intensity of the contraction with that of the stimulus. In order to accomplish this he developed a muscular balance and essentially established the basics of the first tension-length diagram.

His ability to undertake these complex kinds of experimental work was probably influenced to a large extent by his religious background. Throughout his life, Schwann's serious involvement with religion and mysticism proved to be a major factor in his work (6). Although he remained a practicing Catholic, Schwann developed an extreme mechanistic tendency. It is thought that this change in focus was influenced by the death of his mother in 1835. This mechanistic tendency was particularly helpful to him in the impressive work which he accomplished in Muller's laboratory in Berlin between 1834 and 1839.

Muller remained a convinced vitalist and recourse to experimentation for him was simply a means of studying the effects of a vital force peculiar to a specific organ. He had limited chemical or physical background and subsequently detached himself from physiology and devoted his time almost entirely to comparative morphology. Schwann, however, with his extreme rationalistic and mechanistic tendencies, was able to almost single-handedly inaugurate the quantitative period of physiology in Muller's laboratory. His subsequent work bears clear evidence of this skill. In a milieu where idealistic philosophy was still dominant, the examination of vital forces as physical phenomena and the expression of their action as a quantitative entity came almost as a revelation to the scientific community. A group which included Emil Dubois-Reymond and Hermann Von Helmholtz dissociated themselves from the notion of vital force and moved towards the study of molecular mechanisms.

Almost at the same time as he undertook his superb studies of muscle physiology, Schwann's research led him to the discovery of pepsin (7). This relatively minor observation however was almost completely lost in the spate of subsequent major investigative work that he embarked upon. He

demonstrated the need of oxygen for development of putrefaction and subsequently proved that alcoholic fermentation required oxygen and was dependent upon the work of a live organism - yeast (8). His laboratory notebook of 16 February, 1836 notes the multiplication of yeast cells and documents the relationship between alcoholic fermentation and the life cycle of yeast. However, the contribution to science for which Schwann achieved most fame relates to his insistence on the common cellular origin of every living thing (9). Prior to Schwann's publication in 1839 of his Mikroskopische Untersuchungen there had been a prolonged debate regarding the nature of the human organism and the search for a common structural principle of live entities. A number of famous scientists had variously suggested "fibres, molecules, or globules" as the basis of living organisms. Schwann however clearly described cells which had a layer around a nucleus (10). He furthermore described membranes and vacuoles which were interrelated with these cells. A major error in his theory, however, was the proposal that cells form around a nucleus within a blastema - an amorphous substance that can be intracellular or extracellular (11). It is well accepted that Schwann was the first to recognize the basic cellular composition of organisms with the cell as the vital element and the bearer of all the characteristics of life (12). His theory regarding the blastema however was erroneous.

Schwann subsequently spent a number of years studying cellular formation and development in considerable detail. His contributions to this area were highly regarded and widely accepted (13). In 1841 he received the Sommering Medal and in 1847 the Sydenham Society published an English version of Mikroskopische Untersuchungen which was translated by Henry Smith. In the introduction Smith presented the following judgement: "The Treatise has now been seven years before the public, has been most acutely investigated by those best competent to test its value, and the first physiologists of our day judge the discovery which it unfolds as worthy to be ranked amongst the most important steps by which the science of physiology has ever been advanced." Schwann himself however suffered a major disappointment in 1839 when he failed to be awarded a chair at the University of Bonn. His short and brilliant scientific career virtually halted at this time. He moved to Louvain and subsequently Liege where he, to a large extent, abandoned rationalism and became a mystic (14). The scientist gave way to the professor, inventor, and subsequently to the theologian. It appears that part of the transformation was initiated by the disappointments attending the circumstances surrounding his failure to be awarded a Professorial Position at Bonn. In addition, however, he was badly hurt by a number of papers published by Turpin, Wohler and Liebig who fiercely criticized his work on the role of yeast in alcohol fermentation. Indeed, this cruel treatment of Schwann by the scientific leaders of his time made it virtually impossible for him to pursue a further scientific career in Germany.

Years later, Pasteur clearly demonstrated that Schwann had been correct and that criticism of his work had been both inappropriate and utterly unjust.

These setbacks resulted in the cooling of Schwann's ardent rationalism and he became preoccupied with religious meditation. Much of this state of mind was presumably influenced by his brother Peter who was a Theologian. In 1839 Schwann accepted the Chair of Anatomy at Louvain, but his scientific impetus and enthusiam for investigation had been broken. Like Pascal before him, he abandoned rationalism to return to the God of his childhood, the "God of the Heart, not of reason." He remained a well regarded Professor at Louvain and subsequently in 1848 moved to Liege. The rest of his life was spent in a solitary existence darkened by episodes of major depression and anxiety.

During his stay in Louvain (1839-1848) Schwann developed a method to utilize a biliary fistula model for the study of the role of bile in digestion. He concluded that a lack of bile in the digestive tract was incompatible with survival. His papers of 1844 and 1845 on the biliary fistula were his last physiological works. When he moved to Liege in 1848 he became more of an inventor and developed a number of instruments used in mining technology. These included pumps for the aspiration of water in coal mines and respiratory apparatus for rescue operations (15). These instruments became the forerunners of the apparatus utilized for measurement of metabolism in man and of the devices used by underwater divers. At Liege, Schwann was mostly preoccupied with religious meditation. Although he worked extensively on Theoria, a complement to his Mikroskopische Untersuchungen, he was to fail in the completion of this last opus.

During a Christmas visit to a brother and sister living in Cologne he suffered a stroke and died on 11 January 1882 after two weeks of agony, during which he several times expressed the regret that he had not been able to publish the whole of his Theoria.

Schwann was a brilliant and complex man. Every subject that he addressed led to the identification of entirely new concepts and directions of research. His mind however was one which questioned constantly the nature of the involvement of man with the mysteries of God. It is probable that his personal disappointments in dealing with the intellectual dwarves and bureaucratic bigots who governed his day to day existence and research studies seriously embittered him. Certainly his early spiritual retirement into a world of religious mysticism can be interpreted as a manifestation of his serious disillusionment. His discovery of pepsin, observations on the vital necessity of oxygen and his recognition of putrefaction and fermentation as properties of organic function were masterpieces of intellectual endeavour. Yet all of these pale when viewed against the

initial observations which delineated the basic concepts of the cell. These precepts have withstood the test of time and form the basis of modern cell theory. When one contemplates the unique intellectual productivity of this man over a brief five-year period in Muller's laboratory, one is left to ponder to what heights Schwann might have aspired if the scientific hierarchy of his age had allowed him to flourish.

REFERENCES

1. Oppenheimer, Jane. Lives and letters of Theodor Schwann. Bull. Hist. Med. 37(1):78-83, 1963.

2. Florkin, Marcel. Relgion et christologie de Schwann. Rev. Med. Liege 14:272-281, 1959.

3. Florkin, Marcel. Le cas Schwann. Arch. Int Hist. Sci. 13:197-209, 1960.

4. Florkin, Marcel. Schwann Theodor Ambrose Hubert. In: Dictionary of Scientific Biography. Edited by C.C. Gillispie. Vol XII:240-245. New York: Charles Scribner's Sons, 1975.

5. Watermann, Rembert. Theodor Schwann, Entdecker des Pepsins und Begrunder der Zelltheorie. Orion (Munchen), Heft 12, 969-973, 1960.

6. Florkin, Marcel. La nevrose de Schwann. Rev. Med Liege 14:742-750, 1959.

7. Florkin, Marcel. La decouverte de la pepsine par Theodore Schwann. Rev. Med. Liege 12:139-144, 1957.

8. Florkin, Marcel. Les contributions de Theodore Schwann a l'etude de la fermentation alcoholique et de la putrefaction. Rev. Med. Liege 12:240-247, 1957.

9. The progenitor of cellular theory. (Editorial) J. Amer. Med. Ass. 179:650-651, 1962.

10. Mendelsohn, Everett. Cell theory and the development of general physiology. Arch. Int. Hist. Schi. 16:419-429, 1963.

11. Mendelsohn, E. Schwann's mistake. Bull. Signal. Hist. Sci. Techn. 17(1), No.7855, 1963.

12. Florkin, Marcel. La "Theoria" de Theodor Schwann. Rev. Med. Liege, 14:205-214, 1959.

13. Watermann, Rembert. Theodor Schwanns Beitrag zur Histochemie. Acta histochem. (Jena), 10:326-332, 1960.

14. Florkin, Marcel. Schwann et Liege. Rev. Med. Liege 14:709-719, 1959.

15. Foregger, R. Respiratory apparatus of Theodor Schwann. Anesthesiology 27:187-194, 1966.

CHAPTER 3

John Langley

John Newport Langley was born in Newbury in 1852, the second son of John Langley, a private school master. He was educated at Exeter Grammar School and entered Saint Johns College Cambridge in October 1871. Initially he studied mathematics, constitutional history and other literary subjects with the intention of competing for a place in the civil service. During the second year of his studies he changed his plans and gave up other subjects to read natural science. To a large part this decision was influenced by his exposure to Dr. Michael Foster, fellow and praelector of Physiology, of Trinity College who was at this time conducting classes in elementary biology, embryology and physiology. In May of 1873, Langley first attended Foster's lectures on the subject of physiology. Subsequently he spent all his working life at Cambridge studying physiology. In 1883, Foster became the first Professor of Physiology at the University of Cambridge and later on was knighted as Sir Michael Foster, became Secretary of the Royal Society and first editor of the Journal of Physiology. When Langley first met Foster the latter had already demonstrated his ability to attract and select the best recruits for biological science. He was widely remembered as a man of great personal charm, humor and shrewdness. More than twenty years later, Foster wrote of his initial meeting with Langley: "From the very first time I marked him as a man of whom something was to be made" (1). Langley himself, forty years after those early days, wrote that: "Foster lead a considerable number of his earlier pupils to a scientific career. He first aroused an interest in scientific problems and then sometimes gradually, sometimes suddenly, suggested that there was no better course in life than that of trying to solve them."

In 1873, Langley was elected to a foundation scholarship at Saint Johns and in December 1874 was placed in the first class of the Natural Science Tripos. At this stage, Langley was assisting Dr. Newell Martin, who was Foster's assistant. In 1875, having obtained his BA degree, he succeeded Martin as demonstrator since the latter had left Cambridge for Baltimore to be a Professor at Johns Hopkins University. In 1877, Langley was elected to a fellowship at Trinity College.

From 1875 until 1884, Langley was responsible for the organization and conduct of Foster's laboratory classwork. Initially, Langley had studied Jaborandi (pilocarpine) and its effect on the heart. In 1884, Langley was appointed to the permanent college staff at Trinity College as a lecturer in Natural Sciences and in the same year became a University lecturer in Histology. He held these positions until 1903 when he was elected Professor of Physiology in the University, upon Foster's resignation of the chair. The obituary of John Newport Langley, as written by W. M. Fletcher, in the 1926 Journal of Physiology, attests to the magnificent contribution of this man to the study of Physiology. "The main achievements of Langley's research work are familiar to the readers of the Journal of Physiology

and need little here in the way of description or comment. They stand permanently in their place, not merely as additions here and there to knowledge, but as indispensable stepping stones along which at this point, or that, the progress of knowledge has actually made its way. Each gain he made was a step placed securely and finally and few indeed of them as the road has become more firmly and widely trodden by others following have been found wrongly placed. All his chief works keep, and must always keep, their place in the significant history of Animal Physiology. The bare titles of his papers and books deployed there along the years give the plainest testimony to me to his unwearing, unhalting service to science. No single year in all that series, extending well nigh for half a century, from youth to age, appears without its contribution of effective work."

Langley's two main contributions to physiology can be considered under the headings of those dealing with the mechanisms of secretion and those establishing the main anatomical and functional lines of the autonomic nervous system. It is the former area of study to which this thesis is primarily addressed. It essentially covers the first fifteen years of his work, whereas the latter study of the nervous system encompassed the succeeding thirty years or more (Table 1). As in most studies, chance is often the starting point. Foster in 1874, had suggested that he examine the newly introduced drug, Jaborandi and its effect on the heart. He did this in frogs and mammals and his results lead him to further study its effect upon secretion first in the submaxillary gland of the dog and then subsequently into the pure research of the physiology of secretion which he followed avidly until almost 1890. As Fletcher comments: "A merely competent worker might have described the actions of pilocarpine and then have passed even usefully to the actions of similar and different drugs. Langley, however, opened up at every point the physiology of secretion, adapting his technical work not only to the convenience of his laboratory but to his intellectual needs." He made histological studies of gland structure, in activity and rest, and checked the interpretation of the appearance of killed and stained cells by simple but most effective direct observations of the living gland cells. He correlated these findings with experimental unravelling of the nervous influence upon the glands and the share in these taken by vasomotor phenomena. He further linked both sets of results with chemical estimations of the changing qualities of secretion under different circumstances. The well known steps he took and the variety of the work done are to be traced in the titles of his successive papers.

Before Langley's work was published, the accepted view had been that of Heidenhein that gland cells became more granular as secretion took place (2). Langley showed that the reverse was true: that granules were stored up during rest to be passed out in secretion not only in the pancreas, as previously been reported by Kuhne and Lea, but over a wide

range of glands (3). Langley's further studies indicated the beliefs that the secretory nerves might have special or trophic functions in the glands which could be explained in terms of purely vaso-motor changes in the gland circulation. Apart from these latter observations the results of his work in the secretory glands have stood the test of time. Much of this work was incorporated into a major article contributed to salivary glands in Schafer's Text Book of Physiology (4).

In a series of publications between 1879 and 1882, Langley established the basic morphology and secretory characteristics of the pepsin-forming glands of the oesophagus and the stomach (Table 1). The articles are masterful in their original thinking and lucid writing and the powerful interpretation which he was able to make with limited means at his disposal. Indeed, little further meaningful progress in the study of gastric pepsin secretion was made in the subsequent half century after these observations. Of considerable interest is his last publication on the subject of pepsinogen with Edkins in November of 1886 (5). The latter individual subsequently went on to make one of the most fundamental contributions to the understanding of human gastrointestinal physiology by providing evidence of the existence of gastrin. Indeed, for other reasons related to the confusion of his peers regarding the difference between gastrin and histamine, Edkins, like Langley, remained for almost the next half century a virtually unknown contributor to the science of the gut.

On March 24, 1881, Foster communicated to the Royal Society Langley's work entitled "On The Histology and Physiology of pepsin-forming glands" (6). He reported studies in Rana temporaria, Bufo vulgaris, Triton taeniatus, Triton cristatus and Coluber natrix. In these different animals he examined the structure of the resting stomach, noted the alterations which occurred during secretion and also developed a methodology for studying the relative amounts of pepsin contained by the different portions of the stomach. He furthermore was able to characterize the granules of pepsin within the chief cells and to delineate the nature of their secretion during secretory activity of the stomach. Of particular interest in this paper, Langley became the first individual to propose the use of the term oxyntic glands. Previously, glands of the stomach had variously been called fundus glands, peptic glands or rennet glands. In his own words: "It is only after great hesitation that I venture to employ a new term, but without a new term I find myself reduced to circumlocution or inaccuracy".

Langley and Sewall reported that the most striking event of secretory activity was the using up of cell granules. They noted that after about six hours the granules again began to increase and re-accumulated steadily until the cells were granular throughout (7).

It was also observed that during the winter months the

granules decreased in the oesophageal and gastric mucosa of the frogs that were being studied. This problem still causes difficulties to investigators of this particular area of physiology. In the course of his experiments, Langley concluded that the oxyntic glands formed pepsin and that the granules formed by the cells in the oxyntic glands were the same kind of granules as those secreted by the oesophageal glands. Using a simple but ingenious colorimetric test which embodied the principle of the digestion of fibrin, stained with carmine, Langley was able to differentiate between the pepsin content of different areas of mucosa. Almost fifty years later, Anson and Mirsky using a similar colorimetric method for the digestion of hemoglobin were able to measure pepsinogen activity. Similarly, Langley reported on the use of osmic acid as a morphological method for determining the amount and the nature of the granular content of these secretory cells. By use of the now well established technique of placing a sponge in the stomach and recovering it as a test of digestive function, Langley was able to observe that in newts stimulation of secretion resulted in emptying of the granules from the chief cells. He subsequently compared the nature of the gastric glands and their secretory function in frogs, newts and snakes, commenting that "the osmic acid specimens of the gastric mucus membrane of a hungry animal showed no great difference in the characters of the cells throughout the oxytic gland region."

His further studies delineated the different amounts of the pepsin content of the successive regions of the stomach of the various animal species that he studied. The conclusions which he derived from the studies were salient, specific and of considerable importance. He came to a number of important conclusions. Firstly, that pepsin is formed from the granules seen in the gland cells in the living states. In defence of this statement he cited a number of points:

(a) In each part of the animals studied the amount of pepsin contained in any part of the stomach was directly proportional to the amount of granules contained by cells of that part.
(b) In each of the animals studied he noted that the cell granules diminished in number and size during digestion.
(c) Thirdly that when a definite weight of gastric mucous membrane was taken at a stage of digestion when the granules were markedly diminished it contained a marked diminished amount of pepsin.

The second main conclusion was that the gland cells did not store pepsin as such but stored zymogen "out of which pepsin arises when the cell secretes". This latter observation was not dissimilar from the studies of Heidenhein. The latter had reported that "Cells of the pancreas do not store up trypsin but store up a substance which under certain conditions can give rise to trypsin" (8). Heidenhein, in

fact, introduced the word zymogen to include the antecedent of the ferment recognized in cases. Similarly, it had been reported by Ebstein and Grutzner that the gastric glands of mammals contained a certain quantity of a substance capable of giving rise to pepsin when acted on by sodium chloride or by dilute hydrochloric acid. Langley commented that in the gastric glands and the oesophageal glands of the frog and the newt, that the granules showed a general similarity (9,10). It seemed to him, therefore, that the microscopic appearance rendered it far more probable that the stored granules "should consist of a zymogen rather than that they should consist of a ferment or other ready formed secretory product". In a subsequent paper he discussed the entire question of the formation of pre-products by gland cells. He also stated "that since the pepsin of different animals probably differs somewhat in its properties, it is probable that both pepsin and its antecedents differ in different animals somewhat in chemical constitution". This remarkable observation has, in recent years, been considerably expanded with the analysis and chemical configurations being determined for a number of pepsin moieties from different species.

The third main conclusion was that during secretion, three chief phenomena could be recognized in gland cells, viz:

(a) a using up of granules
(b) a fresh formation of granules
(c) a growth of protoplasm - occur simultaneously.

Langley explained that the different morphological aspects of the gland cells depended upon the relative activity of these three processes. In support of this view he quoted exerpts from Heidenhein in which the latter reported that to account for the changes which he observed in the pancreas: "In the cells a continuous change takes place; a using up of a substance in the inner portion, an addition of substance to the outer portion. In the inner portion change to the granules into secretory constituents, in the outer portion employment of the nutritative material for the formation of a homogeneous substance which, on its part, is changed into granule material." As the reader will recognize these changes preceded by almost 75 years the clear understanding of the subcellular compartmentalization of cell function. They are indeed remarkable intellectual guesses and observations, of the site and function of the endoplasmic reticulum and Golgi apparatus of the cell. Both Langley and Heidenhein had understood in 19th century terms the formation of zymogens and their intracellular transfer prior to exocytosis. In fact, a careful reading of Heidenhein's interpretation of the morphological changes occurring during pancreatic secretion provides a startling understanding of the perspicuity of these individuals using the relatively crude investigative tools available at that time.

Heidenhein had reported that in the mammal the presence of digestible food caused a more rapid flow of gastric juice

than the presence of non-digestible food. He interpreted this to be a chemical stimulation caused by the absorbed products of digestion. Langley felt that the same principle could be used to explain the stimulatory mechanisms by which gastric glands are activated when the animal is fed with digestible or non- digestible food. His numerous experiments with newts and frogs brought him to the conclusion that peptone formed by the zymogens might be the particular product which caused increased gastric secretion. However, in experiments performed by the injection of peptone into either the lymph sac or stomach of frogs he was unable to arrive at a clear view as to whether peptone itself caused gastric secretion. He finally concluded that the absorbed products might cause "an increase in use-up of the granules" so that during the later parts of digestion there might be some additional factor responsible for the formation of granules. He postulated that this process involved an additional factor present in some of the digestive products which was "converted into a fit state to be assimilated by the gland cells". In a series of experiments which he performed to evaluate the nature of this digestive product, he arrived at the conclusion that when the stimulus is removed, the gland cells form granules although no digestive products have been absorbed. The observation "that the more rapid formation of granules which takes place when digestible substances are given", he attributed to an increased supply of those substances which serve as a food to the cells. Langley was obviously addressing the question of synthesis and his observations, though carefully directed, did not allow him to quantify the process. Subsequent studies of the synthesis of pepsinogen have required the development of complex radio-isotopic labelling procedures for amino acids and have only been available in the last 25 years. Indeed the subject is still poorly understood.

In his last conclusion, Langley addressed the question of exocytosis, a process as yet unknown to scientists of his day. "The difference in the changes produced by secretion in different gland cells is due partly to variations in the relative rates with which the using up of granules, the growth of protoplasm, and the formation of granules go on, both in each cell as a whole and in various parts of it; partly also to variations in the power of the gland cells to move the granules towards the lumen." In studies in the newt and Triton cristasis, using a gastric sponge as the stimulatory agent, Langley was able to determine that in both cases acid secretion of a good digestive power was obtained but the observable diminution in the number of granules in the cells was very small compared to the amount of acid secreted. These observations allude to two issues. Firstly, the recognition of a process by which granules are moved outside of the cell and secondly, an early indication of the recognition of the fact that acid and pepsin secretion, though related, might occur at somewhat different rates.

TABLE 1 LANGLEY & (PEPSIN) SECRETION

1873- LANGLEY. On the Physiological Action of Jaborandi. Proc. Camb. Philos. Soc., April, 1975, p. 402.

1876- The Action of Pilocarpin on the Sub-maxillary Gland of the Dog. Journ. of Anat. and Physiol., vol. XI.pp.173-180, Oct, 1876.

1878- Some Remarks on the Formation of Ferment in the Sub-maxillary Gland of the Rabbit. One Figure. Journ. of Physiol., vol.I. pp 68-72, March,1878 (and Untersuch. a. d. physiol. d. Univ. Heidelberg. Bd. 1. p. 471, 1878).

- On the Physiology of the Salivary Secretion. Part 1. The Influence of the Chorda Tympani and Sympathectomy Nerves upon the Secretion of the Sub-maxillary Gland of the Cat. Jour. of Physiol., vol.I. pp. 96-104, March, 1878 (and Untersuch. a. d. physiol. Instut d. Univ. Heidelberg. Bd. 1. p. 476, 1878).

- On the Physiology of the Salivary Secretion. Part II. On the Mutual Antagonism of the Atropin and Pilocarpin, having especial reference to their relations in the Sub-maxillary Gland of the Cat. Journ. of Physiol., vol. I. pp. 339-369, Sept, 1878.

1879- On the Structure of Serous Glands in Rest and Activity. Proc. Royal Soc., vol. XXIX. pp. 377-382. Rec. Oct. 13, 1979. (Fuller account in Journ. of Physiol., vol. II. pp. 261-280. Two Plates. Dec. 1879)

- & SEWALL. On the Changes in Pepsin-forming Glands during Secretion. Proc. Royal Soc., vol. XXIX. pp. 383-388. Rec. Oct. 13, 1879. (Fuller account in Journ. of Physiol., vol. II. pp. 281-301. One Plate. Dec. 1879.)

1881- On the Estimation of Ferment in Gland-cells by means of Osmic Acid. Proc. Camb. Philos. Soc., vol. IV. pp. 74-75, Feb. 21, 1881.

- On the Histology and Physiology of Pepsin-forming Glands. Two Plates. Phil. Trans. Royal Soc., Part III. 1881, pp. 663-711. Rec. March 11. (Abstract. Proc. Roy. Soc., vol. XXXII. p. 20, 1881.)

1882- On the Destruction of Ferments in the Alimentary Canal. Journ. of Physiol.,vol.III.pp. 246-268, Jan.1882

- On the Histology of the Mammalian Gastric Glands, and the Relation of Pepsin to the Granules of the

Chief Cell. Journ. of Physiol., vol. III. pp. 269-291, Jan. 1882.

1883- & EVES. On certain Conditions which influence the Amylolytic Action of Saliva. Journ. of Physiol., vol. IV. pp. 18-23, Jan. 1883.

- On the Structure of Secretory Cells and on the Changes which take place in them during Secretion. Proc. Camb. Philos. Soc., vol. V. Nov. 12, 1883 (and Internat. Journ. Anat. and Histol., vol. I. p. 69, 1884).

1885- On the Physiology of the Salivary Secretion. Part III. The Paralytic Secretion of Saliva. Journ. of Physiol., vol. VI. pp. 71-92, April, 1885. (Prelim. account in Proc. Royal Soc., vol. XXXVIII. pp. 212-215. Rec. March 16, 1885.)

- On Variations in the Amount and Distribution of Fat in the Liver Cells of the Frog. Proc. Royal Soc., vol. XXXIX. pp. 234-238. Rec. Sept. 23, 1885.

1886- On the Structure of Mucous Salivary Glands. Proc. Roy. Soc., vol. XL. pp. 362-367. Rec. April 14, 1886.

- & EDKINS. Pepsinogen and Pepsin. Journ. of Physiol., vol. VII. pp. 371-415, Nov. 1886. (Prelim. account. Proc. Physiol. Soc., May, 1886, p. XV.)

1888- Note on "Secretory" Fibres in the Sympathertic Nerve supplying the Parotid Gland of the Dog. Proc. Physiol. Soc., Feb. 1888, pp. iii-iv. (Journ. of Physiol., vol. IX.)

- On the Physiology of the Salivary Secretion. Part IV. The Effect of Atropin upon the supposed Varieties of Secretory Nerve Fibres. Journ. of Physiol., vol. IX. pp. 55-64, August,1888.

1889- & FLETCHER. On the Secretion of Saliva, chiefly on the Secretion of Salts in it. Phil. Trans. Roy. Soc., vol. CLXXX, B. pp. 109-154, 1889. Rec. Aug. 17, 1888. (Abstract. Proc. Roy. Soc., vol. XLV. p. 16, 1888.)

- Note on the Preservation of Mucous Granules in Secretory Cells. Proc. Physiol. Soc., pp. v, vi, March, 1889. (Journ. Physiol., vol. X.)

- On the Physiology of the Salivary Secretion. Part V. The Effect of stimulating of the cerebral Secretory Nerves upon the Amount of saliva obtained by stimulating the Sympathetic Nerve. Journ. of Physiol., vol. X. pp. 291-328, May, 1889.

- On the Histology of the Mucous Salivary Glands, and on the behaviour of their Mucous Constituents. One Plate. Journ. of Physiol., vol. X. pp. 433-457, Oct. 1889.

1890- On the Physiology of the Salivary Secretion. Part VI. Chiefly upon the Connection of Peripheral Nerve cells with the Nerve Fibres which run to the Sub-lingual and Sub-maxillary Glands. Journ. of Physiol., vol. XI. pp. 123-158, Jan. 1890.

REFERENCES

1. Fletcher,W.M. John Newport Langley. In Memoriam. J. of Physiology. Vol LXI. No.1. March 18, 1926.

2. Heidenhein,R. Ueber die Pepsinbildung in dem Pylorusdrusen. Pfluger's, Archiv, Bd.X. 169-171, 1879.

3. Sheridon Lea of Trinity College, later fellow of Caius and an immediate contemporary of Langley's.

4. Langley,J.N. The Sympathetic and other related systems of Nerves. Text Book of Physiology. Ed. Schafer,E.A. Vol.II pp. 616-696, 1900.

5. Langley,J.N., and Edkins. Pepsinogen and Pepsin. J. of Physiol. Vol.VII. pp. 371-415. Nov. 1886.

6. Foster,M. (Langley,J.N.). On the Histology and Physiology of Pepsin-forming glands. Abstract. Proc. Roy. Soc. Vol XXXII pp.20. 1881.

7. Langley,J.N. and Sewall. On the Changes in Pepsin-forming Glands during Secretion. Proc. Royal Soc. vol. XXIX. pp. 383-388. Rec. Oct. 13, 1879. (Fuller account in Jour. of Physiol., vol. II. pp. 281-301. One Plate. Dec. 1879.)

8. Heidenhein,R. In the Handbuch d. Physiol. Ed. Hermann. Bd.v. s.202, 1880.

9. Grutzner. Neue Unters, u d. Bildung u. Ausscheidung d. Pepsins. 26, 1875.

10. Ebstein and Grutzner. Pfluger's Archiv. Bd VIII, s, 122, 1874.

CHAPTER 4

An Overview of Pepsinogen

INTRODUCTION

Pepsinogen is a potent digestive enzyme secreted by the chief cell into the gastroduodenal lumen. Such a powerful and abundant agent must affect the mucosa it bathes, and in fact pepsin is an active barrier breaker likely to play a significant role in the pathogenesis of peptic ulcer disease. Abnormalities in pepsinogen have also been linked epidemiologically to gastric carcinoma and its precursors. Thus, in addition to elucidating mammalian secretory physiology, an understanding of the biology of pepsin and the control of its secretion may prove crucial for the treatment of these diseases. Furthermore, pepsinogen secretion is closely analogous to other exocrine functions, so that data on pepsinogen-related disorders may illuminate the role of exocrine dysfunction in diseases such as pancreatitis.

HISTORICAL NOTES

In 1836, the German physiologist Theodor Schwann described a water-soluble factor in gastric juice which digested egg white. He called it "pepsin," after the Greek word for digestion [110]. Crude gastric pepsin extracts were used by 1869 to treat dyspepsia, vomiting, and diarrhea on the supposition that undigested food caused disease [43]. (Such use continues among health faddists [51].) In 1878, Heidenhein demonstrated that canine pyloric pouches secreted an acid-dependent digestive factor which was probably pepsin [44]. Langley [71] described the histology of pepsin secretion in 1881, demonstrating the depletion of secretory granules simultaneous with the loss of peptic activity from frog gastric and esophageal homogenates.

Northrop crystallized pepsin in 1930, the first demonstration that enzymes were proteins [83]. The newly purified pepsin was soon implicated in ulcerogenesis. Howes [55] showed in 1936 that although acid alone did not prevent the healing of feline gastric ulcers, pepsin and acid together did. In 1942, Schiffrin found that cat intestinal segments filled with acid did not ulcerate until luminal pepsin was added [109]. Subsequent research would analyse the biochemistry of pepsin, the control of its secretion, and its role in disease.

BASIC BIOLOGY

The Chemistry of Pepsinogen and Pepsin

Pepsin is stored as inactive pepsinogen, a forty kilodalton, 375 amino acid polypeptide. After exocytosis, gastric acid hydrolyzes forty to fifty amino acids from pepsinogen, exposing its active site to create bioactive pepsin [34,128]. Once formed, pepsin catalyzes the generation of more pepsin [47].

Active pepsin is a single polypeptide chain of approximately

320 amino acids folded by three disulfide bonds [47]. A paucity of basic amino acids [47] renders pepsin most active and stable in acid. Pepsin digests protein, preferentially cleaving peptide bonds in which the carboxyl group comes from tyrosine, tryptophan or phenylalanine [143].

Much can be deduced about human pepsin by analogy to related acid proteases in other species [2,59,121]. All are of similar molecular weight and structure. In each, two essential aspartyl residues are juxtaposed by a dominant central fold and hydrogen bonded together [143]. Human pepsin is most active from pH 1.8 to pH 3 [138], suggesting that one aspartate is acidic (pKa 4.5) and one in basic form (pKa 1.1) (Fig. 1).

Isozymes of Pepsinogen and Pepsin

The pepsinogen molecule differs significantly between species [36,127] and each species exhibits several isozymogens [41,149]. Human pepsinogen is separable by gel electrophoresis into seven isozymogens with different pH optima and isoelectric points [86,140,141] (Fig. 2). Homologous pepsinogens with similar electrophoretic mobilities in different species are often closer in structure than the isozymogens of one species [34].

Each pepsinogen yields one or more pepsins with different properties [68,147]. Conversely, some pepsins are derived from more than one pepsinogen [34,68,147]. At least twelve classifications of human isozymogens exist [107]. The nomenclature used here is based on the work of Samloff [107].

Samloff numbered pepsinogens according to their migration toward the anode on an electrophoretic gel. Pepsinogen 1 is the fastest (most electronegative) and pepsinogen 7 the slowest. Pepsin 1, originating from pepsinogen 1, is unlike other human pepsins in being stable and active above pH 5 [86]. Pepsinogens 1 through 5 are collectively named pepsinogen I. The less electronegative pepsinogens 6 and 7 are called pepsinogen II. A "slow moving protease" dominates gels of fetal mucosa and occurs frequently in gastric cancers [51], but is structurally unrelated and not unique to gastrointestinal mucosa [107].

Diversity in isozyme activity may facilitate digestion of different proteins [138]. The hyperactive pepsin 1 may be significant in ulcerogenesis. In addition, isozymogen ratios may prove useful clinical markers for ulcers and gastric cancer.

Histology of Pepsinogen-Producing Cells

Fortuitously, the classification of pepsinogens by electronegativity is biochemically and histologically significant. Antibodies to pepsinogen I and pepsinogen II do not crossreact, suggesting significant structural

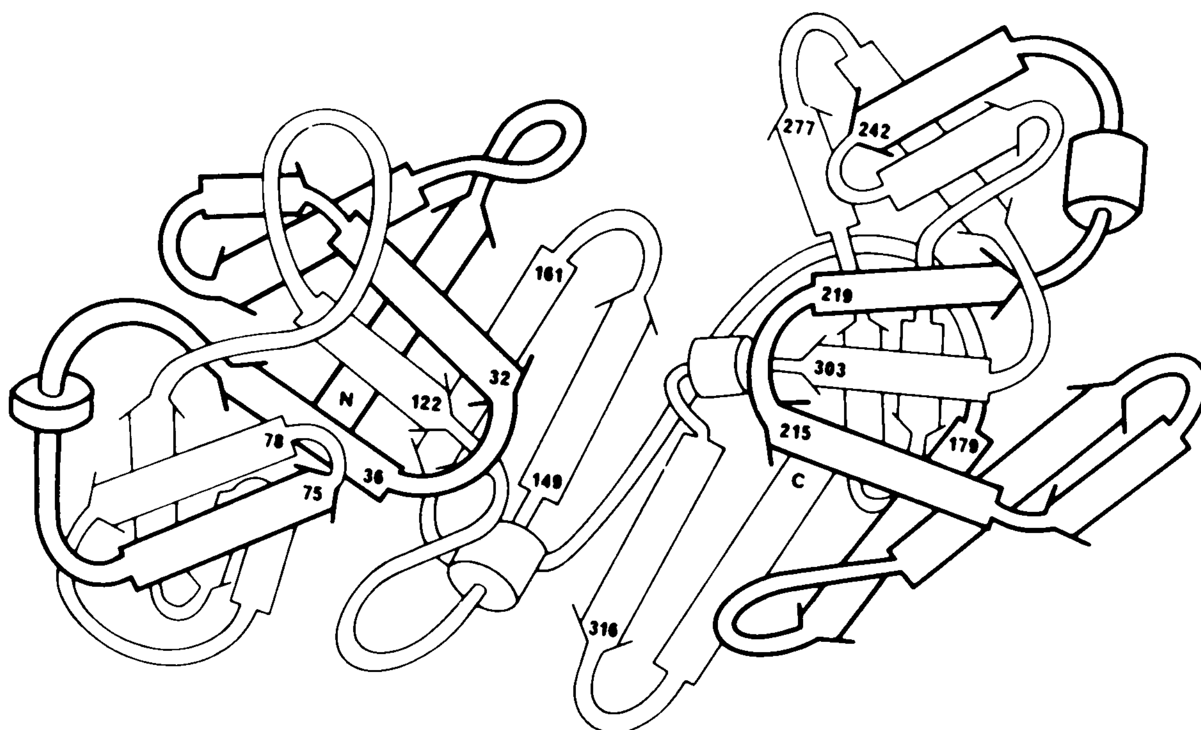

Figure 1. Schematic sequence construct of the tertiary structure of human pepsin, based on homologies with porcine pepsin [2] and other acid proteases [59,121]. The pepsin molecule is a prolate ellipsoid divided by a central bioactive cleft into two lobes.

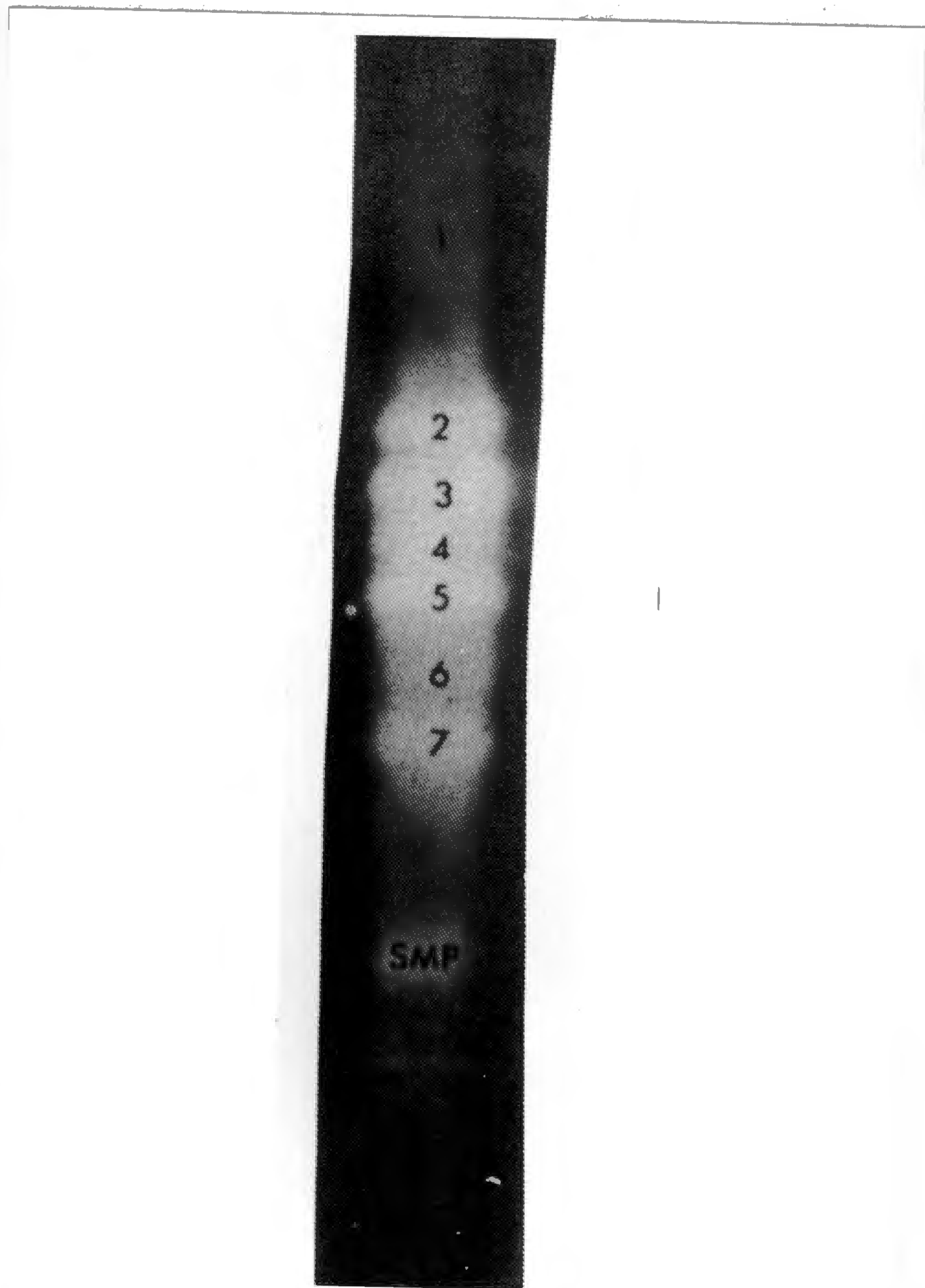

Figure 2. Agar gel electrophoresis of human plasma. Human serum acid proteases are separable into seven pepsinogen isozymogens and a "slow moving protease (SMP)."

differences. Immunofluorescence demonstrates pepsinogen I only in the body and fundus of the human stomach [99] while antibodies to pepsinogen II also stain antrum, duodenum, distal esophagus, and gastric cardia [101]. Although pigs [74] are similar to humans, cats and dogs [73], amphibians, reptiles and fish [58] secrete pepsinogen I and II cosynchronously from esophagus through duodenum.

The human gastric gland is a microscopic invagination of fundic mucosa (Fig. 3). Chief cells predominate at the base of the gland but are sparse toward its luminal opening [94]. It also contains parietal cells, mucus cells, undifferentiated cells and endocrine cells. Difficulties in the isolation of pure chief cells from these mixed glands have impeded pepsinogen research.

Five cell types produce pepsinogen in humans. These are the chief cell and mucus neck cell in the gastric body and fundus, the pyloric gland cell in the antrum, the cardiac gland cell in the cardia, and the Brunner's gland cell in the duodenum [131].

The chief cell is a representative exocrine cell (Fig. 4). Apical zymogen granules store pepsinogen [38] while a profuse basal endoplasmic reticulum synthesizes new pepsinogen. The luminal surface has microvilli and joins adjacent cells by tight junctions. A basement membrane lies behind the cell [58]. Mucus neck cells are less differentiated than surface mucus cells and include stem cells from which the gastric mucosa regenerates. Like chief cells, mucus neck cells contain zymogen granules and a prominent endoplasmic reticulum [58,99].

The glands of the pylorus, cardia and duodenum lack chief cells [58] but produce pepsinogen. Canine pyloric [39] and duodenal [24] pouches secrete pepsinogen and antibodies to pepsinogen II stain these areas as well as the gastric body and fundus [101]. All pepsinogen-secreting cells make pepsinogen II but only chief cells and mucus neck cells secrete pepsinogen I [99,101]. Unlike chief cells, the pepsinogen II-producing cells of pylorus, cardia and duodenum have few zymogen granules [131].

Ectopic gastric mucosa may contain pepsinogen I and II [72,75]. Pepsinogen II is also secreted by the prostate [20] into the semen [113] but its function there is unknown.

In fish, reptiles and amphibians, the oxynticopeptic cell produces both acid and pepsinogen from esophagus through duodenum [58]. Although easy to study, amphibian pepsinogen secretion may thus differ substantially from mammalian processes.

Measurement of Pepsinogen and Pepsin

Northrup assayed pepsin by the digestion of casein, gelatin

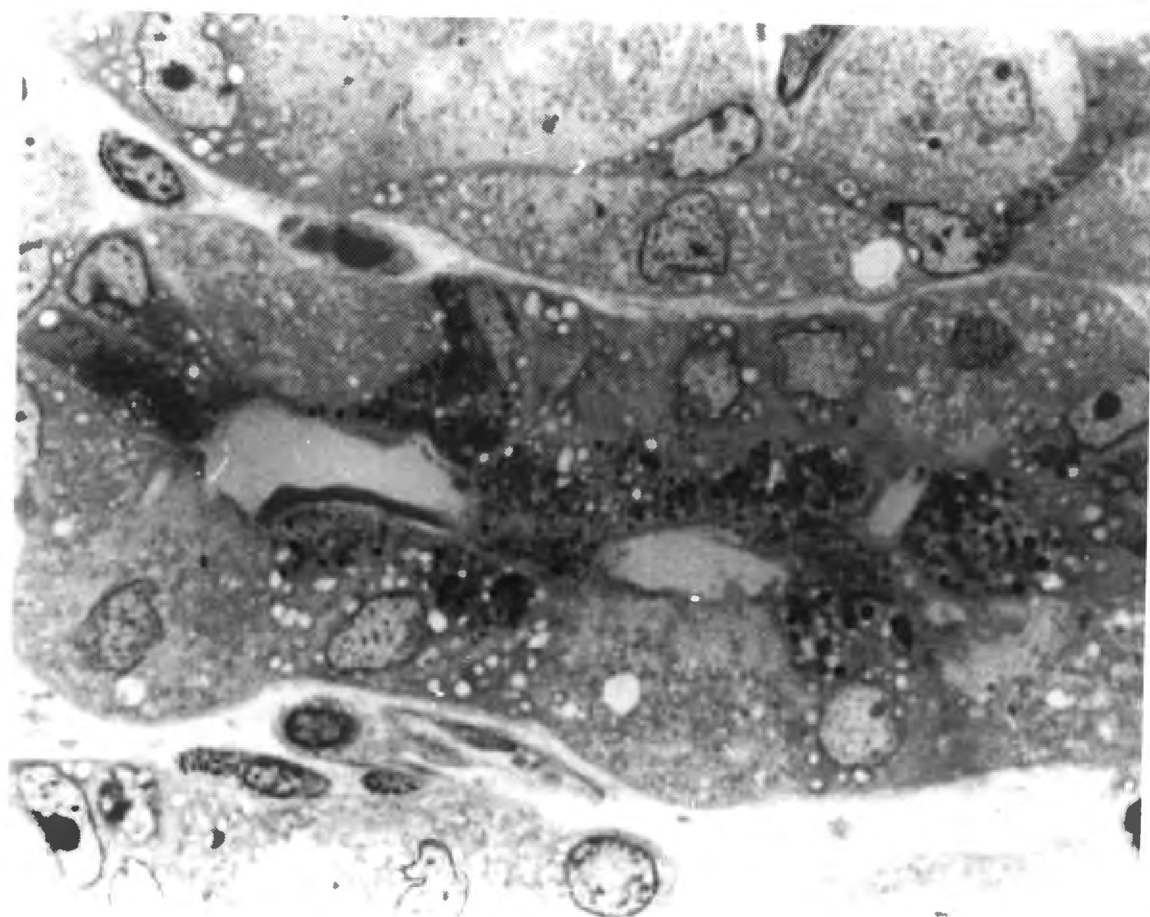

Figure 3. Light photomicrograph (original magnification 313x) of human gastric glands containing numerous different cell types. The chief cells are predominantly located in the deeper parts of the gland. At an average they constitute 20-25% of the cell population of the gastric glands. (Courtesy of Dr. Jeff Sussman)

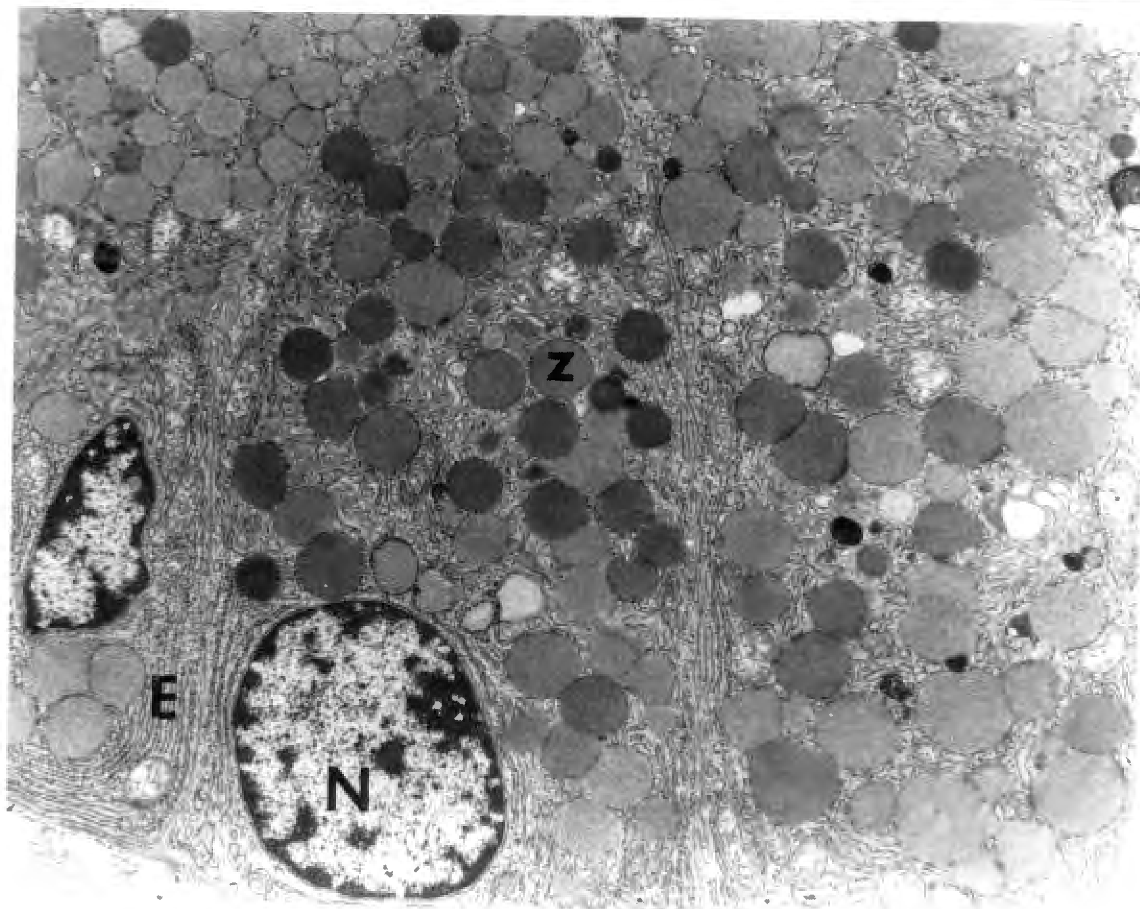

Figure 4. Transmission electron microscopic photograph (original magnification 4500x) of the human chief cell. A profuse endoplasmic reticulum (E) and numerous zymogen granules (Z) are evident as is the nucleus (N). (Courtesy of Dr. Jeff Sussman)

and egg white [83]. In 1932, Anson and Mirsky described the now standard measurement of digested hemoglobin with a colorimetric tyrosine assay [3]. Isotopic measurement of radiolabelled substrate may be faster and more accurate than colorimetry [8,62]. The use of digestion (bioactivity) to measure pepsinogen requires that other digestive enzymes be inactivated by acid, which does not denature pepsin [111]. However, the possiblity of another acid-stable enzyme must always be considered.

Pepsinogen isozymogens can be measured directly in humans by radioimmunoassay or electrophoresis. Quantitative RIA's cannot differentiate similar isozymogens. RIA's for human pepsinogen I [102,135] and II [106] have been applied to human diseases but poor cross-reactivity with non-human pepsinogens renders these assays useless in animals. Electrophoresis is specific but requires larger samples and cannot be well quantitated [70]. Although used in genetic urine studies, electrophoresis is thus also not generally applicable experimentally. In vitro research consequently does not distinguish the isozymogens.

Pepsinogen Genetics

Although all humans with intact stomachs possess pepsinogen I, its component isozymogens vary [68,81]. Single [105] and multigenic [35,37,68,84] models have attempted to explain urine electrophoresis results. However, in addition to its imprecision, urinary pepsinogen electrophoresis does not correlate with serum pepsinogen I levels or electrophoresis in gastric mucosa [81,87]. Variations in renal isozymogen handling may also invalidate urine studies [70,126].

Serum studies are more reliable. Twin studies attribute one third of pepsinogen I variation to heredity and two thirds to environment [130]. Despite confounding factors, siblings of patients with peptic ulcers and high serum pepsinogen I clearly tend to exhibit elevated pepsinogen I. Siblings with high pepsinogen I also have more ulcers [94]. Elevated pepsinogen I and duodenal ulcers may cosegregate as autosomal dominants [95].

As well as illuminating an important physiologic process, an understanding of the genetic control of pepsinogen synthesis should facilitate pharmacologic intervention and provide disease markers in affected families. A prepepsinogen I gene from a human DNA library [118] has not yet been demonstrated in vivo.

THE CONTROL OF PEPSINOGEN SECRETION

Contrasting in vitro and in vivo Effects

Early observations on pepsinogen stimulation in humans and intact animals were frequently inconsistent and contradicted subsequent in vitro work. Such discrepancies may stem from

neuroendocrine interference in vivo, from defects in in vitro models, or from differences in dosages or species response.

Pentagastrin stimulates pepsin secretion in man [53.132] and dogs [85] but has little effect on isolated rabbit glands [77]. The difference is probably due to neural action since vagotomy weakens pepsigogic stimulation by pentagastrin in dogs [40] and humans [33,42]. (Cholinergic replacement reverses this [53].)

Histamine stimulates pepsinogen release and H-2 blockers inhibit this response in vivo in humans [30,53,114], dogs [32,40,85,145] and cats [32]. However, histamine does not release pepsin from isolated rabbit gastric glands [5,66] or rabbit mucosal biopsies [61]. Guinea pig chief cell suspensions secrete slightly more pepsinogen at high histamine doses [122].

Denervation is unlikely to explain these differences since histamine releases pepsinogen from denervated canine gastric pouches [40] and isolated rat stomachs [15]. Damage to chief cell receptors during collagenase digestion is not likely when parietal cells remain intact. Also, organ cultured rabbit gastric mucosa not exposed to collagenase secretes pepsinogen in response to histamine only when phosphodiesterase inhibitors prevent cAMP degradation and thus potentiate histamine [57].

An otherwise weak pepsigogic response to histamine may be augmented in intact mammals by some other agent, secreted in response to histamine, which stimulates more pepsinogen secretion. The dilution of such factors in isolated tissues would minimize the pepsigogic potency of histamine in vitro.

Acid may be the missing link. Histamine strongly stimulates adjacent parietal cells to secrete acid [142] and acid causes pepsinogen secretion in humans [17], denervated canine gastric pouches [60] and in organ cultured rabbit gastric mucosa [61]. Potentiation of this effect in isolated rabbit gastric glands by cAMP-mediated agents suggests that acidity may stimulate a cAMP dependent protein kinase [82]. Gland and cell suspensions are buffered to a constant pH while in intact mucosae the unstirred layer of mucus slows the diffusion of acid, perhaps allowing it to exert a mediated secretagogue effect.

Pepsinogen secretion is inhibited by PGE2 in humans [67] and CCK in dogs [79] but these agents stimulate secretion in vitro [11,48,62]. These differences may represent species variations or pharmacologic rather than physiologic responses.

Serotonin augments pepsinogen secretion in intact dogs [145] and in cod [54], probably via sympathetic neurons releasing adrenergic agonists [9]. It has not been studied in vitro. Unlike other agents, secretin acts similarly in vivo and in

vitro. It stimulates pepsinogen secretion and potentiates the actions of histamine and gastrin in man [14,137] and in dogs [79]. Secretin has proven a potent pepsigogue in vitro only at pharmacologic doses far above physiologic levels [14,19].

Models for Study

Whole animal studies are methodologically simple and have obvious clinical relevance. However, unrecognized neuroendocrine feedback loops may contaminate such experiments. In particular, vagal stimulation and a "wash out phenomenon" related to acid hypersecretion may obfuscate human and intact animal studies [135]. Furthermore, acid in the duodenum releases pepsinogen in man by neurohumoral reflexes not yet elucidated [14].

Isolated gastric mucosa is a simpler model. Gastric biopsies can be maintained in organ baths [61]. Alternatively, a mucosal sheet may be mounted in an Ussing chamber for short term culture. This is technically difficult for mammalian tissue [8,98]. However, frog esophageal mucosa secretes pepsinogen copiously, is physically stronger than mammalian gastric mucosa, and survives in less controlled conditions [116]. Such techniques are useful but yield nonuniform preparations. Furthermore, amphibian secretion may not reflect mammalian physiology. Berglindh has isolated rabbit gastric glands by type I collagenase digestion of minced gastric mucosa [11]. Isolated glands may be further digested to cells, using chelators and additional collagenase to break intercellular linkages [119]. Cell suspensions may be enriched for chief cells by culture [108] or by elutriation according to volume and weight [119,148].

Gland and cell suspensions yield reproducible results uninfluenced by neuroendocrine factors. Enriched chief cell preparations may also avoid local paracrine effects from parietal or endocrine cells. Unfortunately, digestion eliminates tissue polarity [4] and may damage receptors [119]. Cell culture may select abnormal cells [46]. Finally, these models rarely distinguish pharmacologic from physiologic effects. Thus, in vitro observations require confirmation in intact mucosa or whole animals before physiologic mechanisms can be reliably deduced.

The Categorization of Pepsinogen Secretagogues

Many hormones, nucleotide derivatives and neurotransmitters stimulate pepsinogen secretion. Pairs of stimulants with less than additive effects are presumed to act through the same pathway while those with additive effects are assumed independent. Potentiating agents probably activate different receptors but have interacting second messengers within the cell. Blockers like propranolol and atropine confirm such groupings.

Two sets of pepsigogues have been defined which are additive if not synergistic with respect to one another [5,6,91,115]. The first includes beta adrenergic agonists like isoproterenol [66,116] as well as prostaglandins [10], secretin and VIP [6,91,108]. Cholinergic stimulants [66,108,116,150] and bombesin [116], CCK, cerulein and gastrin [48] form the second group. Figure 5 summarizes this schema for pepsinogen secretion.

Group I: The cAMP-mediated Secretagogues

Isoproterenol and epinephrine are classic cAMP-mediated beta adrenergic agonists which in other cell systems stimulate membrane-bound adenylate cyclase via membrane receptors to form cAMP. The cAMP activates an intracellular protein kinase which initiates a cascade of enzymatic phosphorylations [69].

Two lines of research suggest that adrenergic agonists stimulate pepsinogen secretion via cAMP. First, isoproterenol increases the cAMP content of rabbit gastric glands and stimulates adenylate cyclase activity in rabbit [66] and frog [116] gland homogenates and rabbit chief cell suspensions [66]. Second, cAMP and its analogs release pepsinogen from rat [91,150], guinea pig [10] and rabbit [66] gland suspensions as well as frog esophageal mucosa [116,117] and canine chief cell monolayers [108]. Forskolin, a potent adenylate cyclase activator derived from coleus roots [112], stimulates rabbit glands to secrete pepsinogen [50] while cholera toxin, another adenylate cyclase agonist, releases pepsin from guinea pig chief cells [90].

Secretin and VIP may also stimulate pepsinogen secretion through cAMP. Both pure secretin and VIP cause pepsinogen secretion in canine chief cell monolayers [108], rat glands [91], and guinea pig chief cells [135]. These agents also increase cAMP levels [122] and stimulate adenylate cyclase [91]. Secretin and VIP potentiate carbachol and CCK which do not act through cAMP [6,90]. Isobutylmethylxanthine, which inhibits cAMP degradation, increases the potency but not the efficacy of secretin [91]. These findings strongly suggest that cAMP mediates the pepsigogic effects of secretin and VIP.

Although PGE2 was reported ineffective in rabbit glands [66], Berger and Raufman [11] found several prostaglandins to stimulate pepsinogen secretion in guinea pig glands. PGE2 was not potentiated by the cAMP-related agents secretin or 8-bromo-cAMP but was potentiated by carbachol and by the calcium ionophore A23187. PGE2 also stimulates cAMP production in enriched canine chief cell suspensions [148]. Like isoproterenol, secretin and VIP, the prostaglandins probably act through cAMP in chief cells.

Group II: The Calcium-mediated Secretagogues

Cholinergic agonists and peptides of the CCK family stimulate pepsinogen release independently of cAMP [49]. The effect is probably mediated by changes in intracellular calcium. Carbachol stimulates pepsinogen secretion in vivo [132] and in vitro. Atropine blockade suggests a muscarinic receptor. Carbachol and isoproterenol are additive pepsigogic stimuli in rabbit glands [48]. Although bethanechol increases the cAMP content of frog esophageal glands in the presence of agents which block cAMP degradation [116], it is generally accepted that cholinergic agents do not increase cAMP or activate adenylate cyclase [49]. CCK, cerulein and gastrin similarly have no effect on adenylate cyclase or cytoplasmic cAMP [49] despite their pepsigogic potency [62]. It is, therefore, probable that cholinergic and some peptidergic influences are independent of cAMP. Since atropine does not antagonize CCK, at least two different receptors (muscarinic vs peptidergic) must influence this secretory control system. Bombesin may also stimulate pepsinogen secretion through this second control system via its own receptor which is blocked by a substance P analog {115,116].

Calcium is a potent intracellular signal for secretion in many other tissues [22,89]. Many calcium-mediated agents stimulate cleavage of membrane associated phosphatidylinositol into inositol trisphosphate and diacylglycerol. Inositol trisphosphate mobilizes calcium into the cytosol from endoplasmic reticular organelles. This increase in intracellular free calcium initiates secretory activity by binding to calmodulin which allosterically interacts with intracellular enzymes. The calcium ionophores A23187 and ionomycin mimic this response [116]. Diacylglycerol produces a less marked but sustained effect activating calcium/phospholipid-dependent protein kinase (C kinase) [63]. The phorbol esters have been used experimentally to activate C kinase and study this phase of calcium-mediated function [137]. Phenothiazines and related compounds block calmodulin-dependent processes [146].

The application of such probes and blocking agents to chief cells suggests that muscarinic and peptidergic stimulation of pepsinogen secretion involves both phases of calcium response. Incubation of frog esophageal mucosa [116] or isolated rate [91,150] or guinea pig [11] glands with the calcium ionophore A23187 stimulates secretion. Phorbol esters potentiate this effect [76]. Furthermore, the intracellular calcium antagonist nicorandil [78] and the calmodulin inhibitor chlorpromazine [108] inhibit calcium-mediated pepsigogic stimulation.

Whether the calcium for the first phase of the calcium-

mediated response originates outside or inside the chief cell is unknown. Verapamil (a calcium channel blocker) does not block carbachol, histamine or pentagastrin stimulated pepsinogen secretion in humans or rabbits [1,78]. Experiments in calcium-free media have been inconclusive [62,66,108], possibly because of calcium from dying cells or glassware [22].

Intracellular Control Systems

Agents related to cAMP have been reported to potentiate calcium-mediated agonists, even though these messenger systems were originally believed distinct [91]. The calcium and cAMP pathways interact extensively in other cells [88,89], and may intertwine in the chief cell as well. Forskolin increases rabbit gastric gland cAMP, but also increases cytoplasmic calcium, just as carbachol and CCK do [18]. Somatostatin blocks both cAMP and calcium-mediated agents in frog isolated peptic cells [45] and intact dogs [10], perhaps at a final common pathway [45] (Fig. 5).

Pepsinogen Synthesis

Little is known about the control of pepsinogen synthesis. Carbon-14 incorporation into suspensions of rabbit and human glands demonstrates the synthesis of protein, believed mostly pepsinogen [29]. Cimetidine seems to inhibit pepsinogen synthesis although no stimulation by histamine has been observed. Dibutyryl cAMP also stimulates pepsinogen synthesis, probably through the cAMP messenger system [29]. Pentagastrin, secretin and CCK cannot stimulate pepsinogen synthesis on their own, but each stimulates pepsinogen synthesis in rabbit gastric mucosa cultured with acetylcholine [123]. Pepsinogen synthesis in canine chief cell monolayers lags about thirty minutes behind secretion after stimulation by carbachol, VIP and CCK. Synthesis may be stimulated by the depletion of intracellular stores [28].

Pepsinogen synthesis in the frog esophagus exhibits marked seasonal variation [116]. The agents controlling this variation, when found, may prove pharmacologically useful. In addition, peptic ulcer disease in humans may also fluctuate with the seasons [144] and it is tempting to speculate on whether similar mechanisms might underlie both sets of variations.

PEPSINOGEN IN DISEASE

Although abnormalities in pepsinogen have not yet been shown to cause disease, it seems unlikely that such a potent digestive enzyme has no effect on the gastroduodenal mucosa. Clinical research has related pepsinogen isozymogen levels to preneoplastic and neoplastic diseases and peptic ulcers. Unfortunately, our understanding of pepsinogen secretion remains insufficient to explain the complexities of in vivo response and differences in isozymogen secretion.

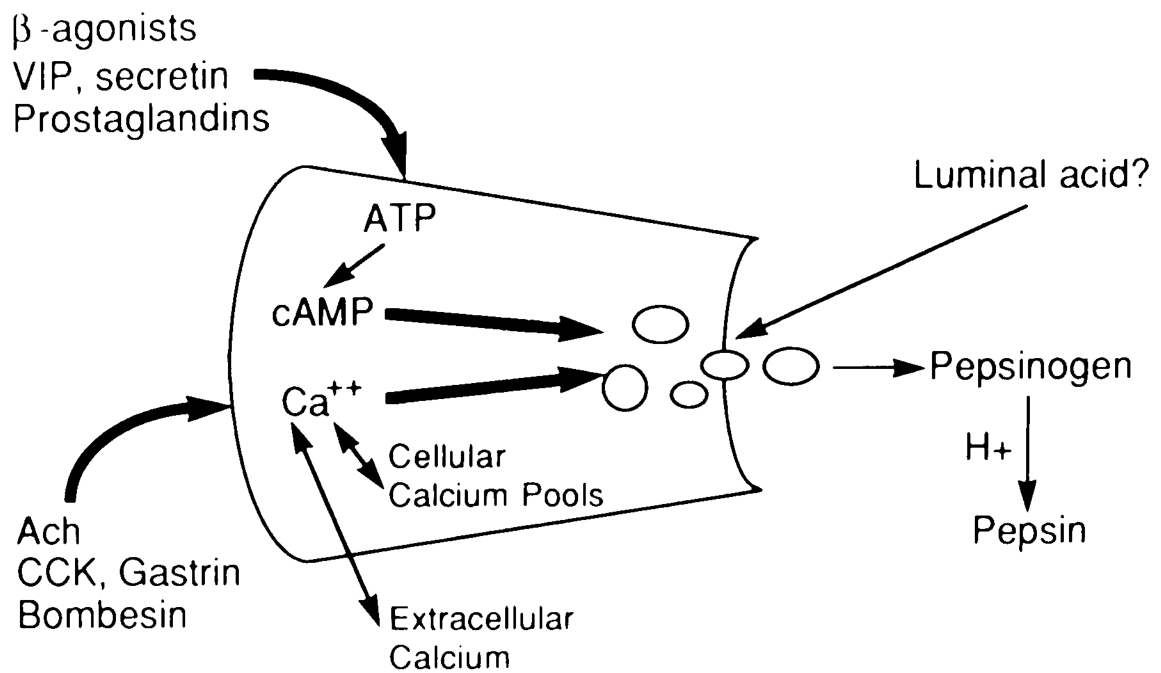

Figure 5. Pepsinogen secretion is stimulated in the chief cell by agents acting through cyclic AMP or through flux of calcium into the cytosol from intracellular organelles or extracellular fluid. Zymogen exocytosis may also be stimulated by luminal acid. Once secreted, pepsinogen is hydrolytically cleaved into pepsin and can autocatalyze this reaction.

The correlations between pepsinogen and disease states may represent causes, effects, epiphenomena or chance associations; lacking a theoretical framework, we can only collect observations and hypothesize.

The loss of peptic activity in frozen samples may obscure retrospective studies [16,27]. Serum studies are most likely to be valid since pepsinogen is stored more stably at a neutral pH. Glycerol or titration to neutrality may preserve samples [25].

Cancer and Preneoplastic States

The search for a relationship between pepsinogen and gastric cancer has been energetic. Six percent of gastric cancers stain histochemically for pepsinogen while no other neoplasm does [92]. Pepsinogen fractionation may yield more valuable clinical markers. Pepsin 5 dominates apparently normal gastric mucosa distant from the site of pathology in two thirds of gastrectomy specimens for cancer but in only 20% of specimens from ulcer patients [147]. Similar work demonstrated immunofluorescent staining of all gastric cancer specimens with "pepsinogen IV" [51], analogous to the isozymogen producing pepsin 5 [100].

More common are blood studies. Nomura [81] identified forty-eight patients who had blood frozen for another study before developing evidence of gastric cancer. Nearly a third, and 40% of those with "intestinal" carcinoma, had low serum pepsinogen I while only 6% of controls demonstrated low levels.

Patients with premalignant gastric lesions also may exhibit pepsinogen isozymogen abnormalities. One study found low serum pepsinogen I in 25% of subjects with intestinal metaplasia but in less than 3% of subjects without metaplasia [120]. Low serum pepsinogen I was four times as frequent in ethnic Japanese as in caucasians (who are less likely to develop gastric cancer).

Atrophic gastritis, which precedes intestinal metaplasia, also lowers serum pepsinogen, perhaps by reducing chief cell mass [13,26]. Pepsinogen fractionation is even more significant. Serum pepsinogen I is 97% specific and 91% sensitive in screening relatives of patients with pernicious anemia [129]. In fact, low serum pepsinogen I is a better test for atrophic gastritis than high gastrin because an atrophic antrum may secrete less gastrin. Atrophic gastritis and low pepsinogen I correlate similarly in other populations [124,136]. In fact, atrophic gastritis can be staged according to the pepsinogen I/II ratio [106].

The associations between low pepsinogen I and high pepsin 5 levels and preneoplastic and neoplastic conditions seem so impressive that patients with low serum pepsinogen I may require periodic gastroscopy [80,81]. However, how pepsin

abnormalities actually relate to gastric cancer remains unclear. Gastric tumors probably do not make large amounts of pepsin 5 [92]. If the low pepsinogen I seen with these diseases stems from a decrease in chief cell mass, then chief cells synthesizing pepsin 5 might survive better and produce a relative abundance of pepsin 5. Pepsin 5 could also be a cocarcinogen, either directly or through digestion of some dietary chemical. However, the correlations may reflect only a coincidental linkage between the genes for pepsinogen I or pepsinogen 5 and some sequence predisposing to gastric cancer.

Peptic Ulcer Disease and Pepsinogen

The second focus for clinical pepsinogen research has been on ulcerogenesis. Current ulcer therapy emphasizes acid control, but acid alone does not cause ulcers [23,109,134]. Pepsin permits the acid access to the mucosa [134,142]. Hypoxic and hyperacidic gastric mucosa requires pepsin to ulcerate [64,65].

High concentrations of hyperactive pepsins may breach the mucosal barrier in ulcer patients. Patients with duodenal ulcers have high plasma pepsinogen levels [77] and high intragastric peptic activity [97]. Conversely, low or normal pepsinogen levels may predict subsequent freedom from ulcers [21]. Furthermore, patients whose duodenal ulcers recur after vagotomy secrete more pepsinogen than those who are cured [52]. Carbenoxolone and bismuth do not block acid secretion but inhibit the secretion of pepsin and its activity and treat ulcers [7,23].

Serum studies are difficult to interpret since mucosal ulceration and necrosis may release pepsinogen. Furthermore, age, renal failure, smoking, gender, and ICU stress alter pepsinogen and pepsinogen I levels [81,93,139,141].

Nevertheless, pepsinogen I seems linked to peptic ulcer disease. Elevated serum pepsinogen I and duodenal ulcer disease are often inherited together [95]. Gastric ulcer patients have normal serum pepsinogen I while duodenal ulcer patients have high levels and patients with Zollinger-Ellison syndrome exhibit massive elevations of serum pepsinogen I [102,103]. Pepsinogen I levels may be bimodally distributed in duodenal ulcer patients, with one group having significantly higher levels [102]. This could represent disease more directly related to pepsinogen. The pepsinogen I/II ratio may also be important. Duodenal ulcers are associated with high serum pepsinogen I and gastric ulcers with high pepsinogen II and a low I/II ratio [104].

Isozyme fractionation of gastric juice refines these observations. Pepsin 1, derived from a pepsinogen I pepsinogen, is more stable and better able to digest mucus than other pepsins [86]. Patients with ulcers secrete increased amounts of pepsin 1 [133]. Pepsin 1 becomes more

prominent during the hypersecretory state characteristic of many ulcer patients. In healthy cats [149] and patients with peptic ulcers [141], insulin hypoglycemia and gastrin significantly increase the proportion of pepsin 1 secreted. It is unclear whether pepsin 1 secretion differs between duodenal and gastric ulcer disease [125,141].

CONCLUSIONS

Pepsin is an essential gastric enzyme, synthesized as pepsinogen and secreted into the lumen by chief cells and mucus neck cells in the gastric fundus as well as by the glands of the duodenum, pylorus and gastric cardia. Its secretion is modulated by two distinct yet interacting pathways. The first involves cAMP and is activated by adrenergic stimulation, prostaglandins, and such hormones as secretin and VIP. The second control mechanism responds to vagal and cholinergic stimuli, to bombesin and to CCK, cerulein and gastrin. It appears to be mediated by calcium entering the cytosol from interstitial fluid or intracellular organelles and acting through both calmodulin and calcium-dependent kinase pathways. The relative importance of the various calcium pools is unknown. Secretory modulation by interactions of the cAMP and calcium systems at the phosphatidyl inositol level requires further investigation.

Pepsinogen synthesis remains largely unexplored. Secretin, VIP, CCK and cholinergic agonists stimulate pepsinogen synthesis but the pathways through which they act are unknown.

Pepsin isozymes may be clinically significant. Pepsin 5 seems linked to the intestinal type of gastric carcinoma and pepsin 1 to ulcers. Whether these correlations are causal is unknown. Detailed epidemiologic studies may better define subgroups of neoplastic and peptic disease which are primarily pepsinogen-related. Refinement of currently speculative models of pepsinogen genetics [35,68] may determine whether these associations stem from genetic cosegregation or peptic actions.

Pepsin is a powerful digestive enzyme plentiful in the stomach. Its further study promises to illuminate not only the biology of secretion but also the genesis of "peptic" ulcer disease and gastric malignancy.

REFERENCES

1. Aadland, E., and Berstad, A. Effect of verapamil on gastric secretion in man. Scand. J. Gastroenterol. 18:969, 1983.

2. Andreeva, N.S., Fedorov, A.A., Gutshino, A.E., Riskulov, R.R., Schutzkever N.E., and Safro, M.G. X-ray crystallographic studies of pepsin. Conformation of the main chain of the enzyme. Molek. Biol. 12:922,1978.

3. Anson, M.L., and Mirsky, A.E. The estimation of pepsin with hemoglobin. J. Gen. Physiol. 16:59,1932.

4. Ayalon, A., Sanders, M.J., Thomas, L.P., Amirian, D.A., and Soll, A.H. Electrical effects of histamine on monolayers formed in culture from enriched canine gastric chief cells. Proc. Natl. Acad. Sci. USA 79:7009,1982.

5. Ballantyne, G.H., Zdon, M.J., Schafer, D.E., Fratesi, G.R., Roberts, J.R., Tyshkov, M., and Modlin, I.M.. Evidence for dual modulation of pepsinogen secretion using isoproterenol, carbachol, CCK-8, forskolin, 8-bromo-cAMP, and A23187 probes. Ann. Surg. 204:559,1986.

6. Ballantyne, G.H., Zdon, M.J., Zucker, K.A., Tyskoff, M., Fratesi, G.R., Schafer, D.E., and Modlin, I.M. Vasoactive intestinal peptide augmentation of cholecystokinin-8-stimulated pepsinogen secretion: Evidence for dual modulation of chief cell function. J. Surg. Res. 40:617,1986.

7. Baron, J.H., Barr, J., Batten, J., Sidebotham, R., and Spencer, J. Acid, pepsin and mucus secretion in patients with gastric and duodenal ulcer before and after colloidal bismuth subcitrate (De-Nol). Gut 27:486,1986.

8. Basson, M.D., Adrian, T.E., and Modlin, I.M. A new model for the study of mammalian pepsinogen secretion. Gastroenterol. 91,1987.

9. Bech, K. Effect of serotonin on bethanechol-stimulated gastric pepsin secretion in dogs. Scand. J. Gastroenterol. 21:537,1986.

10. Bech, K. Somatostatin increases the ratio between gastric mucosal blood flow and gastric acid and pepsin secretion in dogs. Scand. J. Gastroenterol. 21:215,1986.

11. Berger, S., and Raufman, J.P. Prostaglandin-induced pepsinogen secretion from dispersed gastric glands from guinea pig stomach. Am. J. Physiol. 249:G592, 1985.

12. Berglindh, T., and Obrink, K.J. A method for preparing isolated glands from the rabbit gastric mucosa. Acta Physiol. Scand. 96:150,1976.

13. Bock, O.A.A., Arapakis. G., Witt, L.J., and Richards, W.C.D. The serum pepsinogen level with special reference to the histology of the gastric mucosa. Gut 4:106,1963.

14. Brooks, A.M., Isenberg, J., and Grossman, M.I. The effect of secretin, glucagon, and duodenal acidification on pepsin secretion in man. Gastroenterology 57: 159,1969.

15. Bunce, K.T., Grewal, M., and Parsons, M.E. The effect of cimetidine on basal and stimulated pepsin secretion in the isolated whole stomach of the rat. Br. J. Pharmac. 73:41,1981.

16. Burget, D.W., Degara, C.J., and Hunt, R.H. Instability of pepsin in stored gastric juice - a cause for concern? Gut 25:A1153,1984.

17. Bynum, T.E., and Johnson, L.R. Stimulation of human pepsin output by topical hydrochloric acid. Digestive Diseases 20:607,1975.

18. Chew, C.S. Cholecystokinin, carbachol, gastrin, histamine and forskolin increase [Ca2+]i in gastric glands. Am. J. Physiol 250:G814,1986.

19. Chey, W.Y., Kim, M.S., Lee, K.Y., and Chang, T.M. Secretin is an enterogastrone in the dog. Am. J. Physiol. 240:G239,1981.

20. Chiang, L., Contreras, L., Chiang, J., and Ward, P.H. Human prostatic gastricsinogen: The precursor of seminal fluid acid proteinase. Arch. Biochem. Biophys. 210:14,1981.

21. Chuong, J.J.H., Fisher, R.L., Chuong, R.L.B., and Spiro, H.M. Duodenal ulcer: Incidence, risk factors, and predictive value of plasma pepsinogen. Digest. Dis. Sci. 31:1178,1986.

22. Churchill, P.C. Second messengers in renin secretion. Am. J. Phys. 249:F175,1985.

23. Colin-Jones, D.G. There is more to healing ulcers than suppressing acid. Gut 27:475,1985.

24. Cooke, A.R., and Grossman, M.I. Studies on the secretion and motility of Brunner's gland pouches. Gastroenterology 51:506,1968.

25. de Gara, C.J., Burget, D.W., Sivakumaran, T,. and Hunt, R.H. The effect of temperature and pH on the stability

of human pepsin in stored gastric juice. Scand. J. Gastroenterol. 21:650,1986.

26. De Los Santos, M., Thayer Jr.,W.R., and Spiro, H.M. Blood pepsin determinations in gastritis. Yale J. Biol. Med. 35:353,1963.

27. Deakin, M., Ramage, J.K., Paul, A., Gray, S., Billings, J., and Williams, J.G. Don't freeze pepsin! Gut 26:A546,1985.

28. Defize, J., and Hunt, R.H. Pepsinogen synthesis and secretion in chief cell monolayers. Gastroenterology 90:1391A,1986.

29. Defize, J., Pals, G., Frants, R.R., Westerveld, B.D., Meuwissen, S.G.M., and Eriksson, A.W. Pepsinogen synthesis and secretion in isolated gastric glands. J. Clin. Pathol. 37:531,1984.

30. Domschke, W., Lux, G., and Domschke, S. Furan H2-antagonist ranitidine inhibits pentagastrin-stimulated gastric secretion stronger than cimetidine. Gastroenterology 79:1267,1980.

31. Ellis, A., Hughes, S., and McConnell, R.B. Gastric neoplasms and pepsinogen phenotypes. Br. J. Cancer. 46:289,1982.

32. Emas, S., and Grossman M.I. Comparison of gastric secretion in conscious dogs and cats. Gastroenterology 52:29,1967.

33. Emas, S., and Aly, A.. Acid and pepsin responses to graded doses of pentagastrin in duodenal and corporeal gastric ulcer patients before and after selective proximal vagotomy. Am. J. Surg. 150:543,1985.

34. Foltmann, B. Purification, structure and activation of pepsinogens. In J. Kreuning, I.M . Samloff, J. Rotter, and A.W. Eriksson (Eds.) Pepsinogens in Man: Clinical and Genetic Advances. Prog. Clin. Biol. Res. New York: Alan R. Liss, Inc, 1985. Vol 173, pp. 1-13.

35. Frants, R.R., Pronk, J.C., Pals, G., Defize, J., Westerveld, B.D., Meuwissen, S.G.M., Kreuning, J., and Eriksson, A.W. Genetics of urinary pepsinogen: A new hypothesis. Hum. Genet. 65:385,1984.

36. Furihata, C., Saito, D., Fujiki, H., Kanai, Y., Matsushima, T., and Sugimura, T. Purification and characterization of pepsinogens and a unique pepsin from rat stomach. Eur. J. Biochem. 105:43,1980.

37. Gedde-Dahl Jr., T. On chromosome mapping of human pepsinogen gene loci. In Kreuning et al. (Eds.) op.

cit., pp. 101-106.

38. Gibert, A.J., and Hersey, S.J. Exocytosis in isolated gastric glands induced by secretagogues and hyperosmolarity. Cell Tissue Res. 227:535,1982.

39. Grossman, M.I., and Marks, I.N. Secretion of pepsinogen by the pyloric glands of the dog, with some observations on the histology of the gastric mucosa. Gastroenterology 38:343,1960.

40. Guldvog, I., Gedde-Dahl, D., and Berstad, A. "Non-active: pepsin secretion compared with stimulated secretion by bethanechol, histamine, pentagastrin, and 2-deoxy-D-glucose: The role of vagal innervation. Scand. J. Gastroenterol. 15:939,1980.

41. Hanley, W.B., and Boyer, S.H. Electrophoretic and functional heterogeneity of pepsinogen in several species. Nature 209:996,1966.

42. Haukland, H.H., Waldum, H.L., and Johnson, J.A. Effect of proximal gastric vagotomy on insulin-induced gastric H+ and pepsin secretion and serum group I pepsinogens. Scand. J. Gastroenterology 17:555,1982.

43. Hawley, J.S. Pepsin: Its physiological and therapeutical actions. New York: Monograph printed by George F. Nesbitt & Co.,1869.

44. Heidenhain, R. Ueber die pepsinbildung in den pylorusdrusen. Pflugers Archiv. 18:169,1878.

45. Heldman, A.W., and Matsumoto, H. Somatostatin-14 (SOM) inhibits pepsinogen secretion: Investigations into its mechanism of action. Gastroenterology 90:1791A,1986.

46. Heppner, G. Tumor heterogeneity. Can. Res. 44:2259.1984.

47. Herriott, R.M. Pepsinogen and pepsin. J. Gen. Phys. 45:57,1962.

48. Hersey, S.J., May, D., and Schyberg, D. Stimulation of pepsinogen release from isolated gastric glands by cholecystokininlike peptides. Am. J. Physiol. 244:G192,1983.

49. Hersey, S.J., Norris, S.H., and Gibert, A.J. Cellular control of pepsinogen secretion. Ann. Rev. Physiol. 46:393,1984.

50. Hersey, S.J., Owirodu, A., and Miller, M. Forskolin stimulation of acid and pepsinogen secretion by gastric glands. Biochim. Biophys. Acta 755:293,1983.

51. Hirsch-Marie, H., Loisillier, F., Touboul, J.P., and

Burtin, P.Immunochemical study and cellular localization of human pepsinogens during ontogenesis and in gastric cancers. Lab. Invest. 34:623,1976.

52. Hirschowitz, B.I. Factors influencing pentagastrin dose response of acid, chloride and pepsin in peptic ulcer. In A Miyoshi (Ed.) Gut peptides and ulcer Tokyo: Biomedical Research Foundation, 1983, pp. 357-365.

53. Hirschowitz, B.I. and Helman, C.A. Effects of fundic vagotomy and cholinergic replacement on pentagastrin dose responsive gastric acid and pepsin secretion in man. Gut 23:675,1982.

54. Holstein, B., and Cederberg, C. Effects of 5-HT on basal and stimulated secretions of acid and pepsin and on gastric volume outflow in the in vivo gastrically and intestinally perfused cod, Gadus morhua. Agents and Actions 15:290,1984.

55. Howes, E.L., Flood, C.A., and Mullins, C.R. The influence of pepsin and hydrochloric acid on the healing of gastric defects. Surg. Gynec. Obstet. 62:149,1936.

56. Huxtable, R.J., Luthy, J., and Zweifel, U. Toxicity of comfrey-pepsin preparations. N. Engl. J. Med. 315:1095,1986.

57. Ito, M., Noguchi, Y., Yakochi, K., Kishimoto, T., Tomomatsu, T., Katsumi, K., and Takeuchi, T. Effects of betazole hydrochloride and cyclic AMP in the pepsinogen secretion by rabbit gastric mucosa in organ culture. Digestion 23:169,1982.

58. Ito, S. Functional gastric morphology. In: L.R. Johnson (Ed.). Physiology of the gastrointestinal tract. New York: Raven Press. Chapter 17, pp. 517-550,1981.

59. James, M.N.G., and Sielecki, A.R. Structure and refinement of penicillopepsin at 1.8 A resolution. J. Mol. Biol. 163:299,1983.

60. Johnson, L.R. Regulation of pepsin secretion by topical acid in the stomach. Am. J. Phys. 223:847,1972.

61. Kapadia, C.R., and Donaldson, R.M. Macromolecular secretion by isolated gastric mucosa: Fundamental differences in pepsinogen and intrinsic factor secretion. Gastroenterology 74:535,1978.

62. Kasbekar, D.K., Jensen, R.T., and Gardner, J.D. Pepsinogen secretion from dispersed glands from rabbit stomach. Am. J. Phys. 244:G392,1983.

63. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and

Nishizuka, Y. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relationship to phosphatidyl inositol turnover. J. Biol. Chem. 254: 3692,1980.

64. Kivilaakso, E., Barzilai, A., Schiessel, R., Crass, R., and Silen, W. Ulceration of isolated amphibian gastric mucosa. Gastroenterology 77:31,1979.

65. Kivilaakso, E., and Silen, W. Pathogenesis of experimental gastric mucosal injury. N. Engl. J. Med. 301:364,1979.

66. Koelz, H.R., Hersey, S.J., Sachs, G., and Chew C.S. Pepsinogen release from isolated gastric glands. Am. J. Physiol. 243:G218,1982.

67. Konturek, S.J., Kwiecien, N., Obtulowicz, W., and Olesky, J. Prostaglandins and vagal stimulation in duodenal ulcer patients. Scand. J. Gastroenterol. 18:43,1983.

68. Korsnes, L., and Gedde-Dahl Jr., T. Genetics of pepsinogen I. Ann. Hum. Genet. Lond. 43:199,1980.

69. Krebs, E.G., and Beavo, J.A. Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. 48:923,1979.

70. Kreuning, J., Samloff, I.M., Rotter, J.I., and Eriksson, A.W. (Eds.) Discussion on genetic aspects of pepsinogen, phenotypes and serum levels. In Kreuning et. al. (Eds). op. cit. pp.107-113.

71. Langley, J.N. On the histology and physiology of pepsin-forming glands. Phil. Trans. Soc. Lond. 172:663,1881.

72. Liebman, W.M., and Bujanover, Y. Groups I and II pepsinogens in Meckel's diverticulum. J. Histochem. Cytochem. 26:867,1978.

73. Liebman, W.M., and Samloff, I.M. Immunochemical characterization and cellular localization of pepsinogens in cat and dog. J. Histochem. Cytochem. 26:1115,1978.

74. Liebman, W.M., and Samloff, I.M. Cellular localization of hog pepsinogens J. Histochem. Cytochem. 27:1112,1979.

75. Mangla, J.C., Kim, Y., Guarasci, G., and Schenk, E.A. Pepsinogens in epithelium of Barrett's esophagus. Gastroenterology 65:949,1973.

76. Matsumoto, H., Komiyama, K., Shirakawa, T., Heldman, A., Anderson, W., and Hirschowitz, B.I. Essential role of

Ca2+ in the action and interaction of cAMP and protein kinase C on pepsinogen (PG secretion). Gastroenterology 90:1538A,1986.

77. Mirsky, I.A., Futterman, P., and Kaplan, S. Blood plasma pepsinogen: II The activity of the plasma from "normal" subjects, patients with ulcer, and patients with pernicious anemia. J. Lab. Clin. Med. 40:188,1952.

78. Miyamoto, T., Itoh, M., Yokoyama, Y., Katsumi, K., and Takeuchi, T. Role of calcium ion for pepsinogen secretion stimulated by carbachol and CCK-8 in rabbit gastric mucosa. Gastroenterology 90:1554A,1986.

79. Nakajima, S., Nakamura, M., and Magee, D.F. Effect of secretin on gastric acid and pepsin secretion in response to various stimuli. Am. J. Phys. 216:87,1969.

80. Nomura, A.M.Y., Stemmerman, G.N., and Samloff, I.M. Gastric carcinoma and serum pepsinogen I. Ann. Int. Med. 94:542,1981.

81. Nomura, A.M.Y., Stemmerman, G.N., and Samloff, I.M. Serum pepsinogen I as a predictor of stomach cancer. Ann. Int. Med. 93:537,1980.

82. Norris, S.H., and Hersey, S.J. pH dependence of pepsinogen and acid secretion in isolated gastric glands. Am. J. Physiol. 245:G730,1983.

83. Northrop, J.H. Crystalline pepsin: I. Isolation and tests of purity. J. Gen. Physiol. 13:739,1930.

84. Pals, G., Biemond, I., Defize, J., Westerveld, B.D., Pronk, J.C., Mewissen, S.G.M., and Eriksson, A.W. Serum pepsinogen I levels in relation to pepsinogen phenotypes. In Kreuning et. al. (Eds.) op. cit. pp.91-100.

85. Passaro, E.P., and Grossman, M.J. Effect of vagal innervation on acid and pepsin response to histamine and gastrin. Am. J. Physiol. 206:1068,1964.

86. Pearson, J.P., Ward, R., Allen, A., Roberts, N.B., and Taylor, W.H. Mucus degradation by pepsin : Comparison of mucolytic activity of human pepsin 1 and pepsin 3: Implications in peptic ulceration. Gut 27:243,1986.

87. Petersen, G.M., Rotter, J.I., and Samloff, I.M. (1985). Quantitative serum pepsinogen: Genetics and relationship to urinary and gastric phenotypes. In Kreuning et al. (Eds.) op. cit. pp.79-90.

88. Rasmussen, H. The calcium messenger system (first of two parts). N. Engl. J. Med. 314:1094,1986.

89. Rasmussen, H., and Barrett, P.Q. Calcium messenger system: An integrated view. Physiol. Rev. 64:938,1984.

90. Raufman, J.P., and Cosowsky, L.N. Actions of cholera toxin on cellular cyclic AMP and pepsinogen secretion from dispersed chief cells. Gastroenterology 90:1598A,1986.

91. Raufman, J.P., Kasbekar, D.K., Jensen, R.T., and Gardner, J.D. Potentiation of pepsinogen secretion from dispersed glands from rat stomach. Am. J. Physiol. 245:G525,1983.

92. Reid, W.A., Thompson, W.D., and Kay, J. Pepsinogen in gastric carcinoma cells. J. Clin. Pathol. 36:137,1983.

93. Rotter, J.I., Elashoff, J.D., Samloff, I.M., Wong, F.L., and Reedy, T.J. Studies in the variation of quantitative serum pepsinogen I (PG I). Am. J. Hum. Genet. 32:150A, 1980.

94. Rotter, J.I., Rimoin, D.L., Samloff, I.M., McDonnell, R.B., Jensen, K.G., Gadeberg, O., and Hauge, M. The genetics of peptic ulcer disease - elevated serum group I pepsinogen concentrations in siblings and twins of ulcer probands. Gastroenterology 72:1165,1977.

95. Rotter, J.I., Sones, J.Q., Samloff, I.M., Richardson, C.T., Gursky, J.M., Walsh, J.H., and Rimoin, D.L. Duodenal ulcer disease associated with elevated serum pepsinogen I. N. Engl. J. Med. 300:63,1979.

96. Rubin, W., Ross, L.I., Sleisenger, M.H., and Jeffries, G.H. The normal human gastric epithelia: A fine structural study. Lab. Invest. 19:598,1964.

97. Rune, S.J. Proteolytic activity in the human stomach during digestion and its correlation with the augmented histamine test. Gut 7:69,1966.

98. Rutten, M.J., and Ito, S. Morphology and electrophysiology of guinea pig gastric mucosal repair in vitro. Am. J. Phys. 244:G171,1983.

99. Samloff, I.M. Cellular localization of group I pepsinogens in human gastric mucosa by immunofluorescence. Gastroenterology 61: 185,1971.

100. Samloff, I.M. Slow moving protease and the seven pepsinogens: Electrophoretic demonstration of the existence of eight proteolytic fractions in human gastric mucosa. Gastroenterology 57:659,1969.

101. Samloff, I.M., and Liebman, W.M. Cellular localization of the group II pepsinogens in human stomach and duodenum by immunofluorescence. Gastroenterology

65:36,1973.

102. Samloff, I.M., Liebman, W.M., and Panitah, N. Serum group I pepsinogens by radioimmunoassay in control subjects and patients with peptic ulcers. Gastroenterology 69:83,1975.

103. Samloff, I.M., Secrist, D.M., and Passaro, E. A study of the relationship between serum group I pepsinogen levels and gastric acid secretion. Gastroenterology 69:1196,1975.

104. Samloff, I.M., Stemmerman, G.N., Heilbrun, L.K., and Nomura, A. Elevated serum pepsinogen I and II levels differ as risk factors for duodenal ulcer and gastric ulcer. Gastroenterology 90:570,1986.

105. Samloff, I.M., and Townes, P.L. Pepsinogens: Genetic polymorphism in man. Science 168:144,1970.

106. Samloff, I.M., Varis, K., Ihamaki, T., Siurala, M., and Rotter, J.I. Relationships among serum pepsinogen I, serum pepsinogen II and gastric mucosal histology. Gastroenterology 83:204,1982.

107. Samloff, Foltmann et al. (1985). Discussion on pepsinogen classification, nomenclature and genetics of electrophoretic variants. In Kreuning et al. (Eds.) op. cit., pp. 31-40.

108. Sanders, M.J., Amirian, D.A., Ayalon, A., and Soll, A.H. Regulation of pepsinogen release from canine chief cells in primary monolayer culture. Am. J. Physiol. 245:G641,1983.

109. Schiffrin, M.J., and Warren, A.A. Some factors concerned in the production of experimental ulceration of the gastrointestinal tract in cats. Am. J. Dig. Dis. 9:205,1942.

110. Schwann, T. Ueber das Wesen des Verdauungsprocesses. Arch. Anast. Physiol. wiss Med. pp. 90,1836.

111. Schlamowitz, M., and Peterson, L.U. Studies on the optimum pH for the action of pepsin on "native" and denatured bovine serum albumin and bovine hemoglobin. J. Biol. Chem. 234:3137,1959.

112. Seamon, K.B., Padgett, W., and Daly, J.W. Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc. Nat. Acad. Sci. USA 78:3363,1981.

113. Seijffers, M.J., Miller, L.L., and Segal, H.L. Pepsinogen I in semen. Chromatographic separation of pepsinogen I from human semen. Proc. Soc. Exp. Biol.

Med. 118:405,1965.

114. Sewing, K.-Fr., Billian, A., and Malchow, H. Comparative study with ranitidine and cimetidine on gastric secretion. Scand. J. Gastroenterol. 16(Suppl. 69): 45,1981.

115. Shirakawa, T., and Hirschowitz, B.I. Interaction between stimuli and their antagonists on frog esophageal peptic glands. Am. J. Phys. 249:G668,1985.

116. Shirakawa, T., and Hirschowitz, B.I. Cellular messengers of stimulants of pepsinogen secretion from isolated frog esophageal mucosa. Am. J. Physiol. 250:G361,1986.

117. Simpson, L., and Hirschowitz, B.I. Differing responses to peptic-cell stimuli by summer and winter frogs. Fed. Proc. 38:885,1979.

118. Sogawa, K., Fujii-Kuriyama, Y., Mizukami, Y., Ichihara, Y., and Takahashi, K. Primary structure of human pepsinogen gene. J. Biol. Chem. 258:5306,1983.

119. Soll, A.H. The actions of secretagogues on oxygen uptake by isolated mammalian parietal cells. J. Clin. Invest. 61:370,1978.

120. Stemmerman, G.N., Ishidate, T., Samloff, I.M., Masuda,H., Walsh, J.H., Nomura, A., Yamakawa, H., and Glober, G. Intestinal metaplasia of the stomach in Hawaii and Japan: A study of its relation to serum pepsinogen I, gastrin and parietal cell antibodies. Dig. Dis. 23:815,1978.

121. Subramanian, E., Swan, I.D.A., Liu, M., Davies, D.R., Jenkins, J.A., Tickle, I.J., and Blundell, T.L. Homology among acid proteases: Comparison of crystal structures at 3 Angstroms resolution of acid proteases from Rhizopus chinensis and Endothia parasitica. Proc. Natl. Acad. Sci. USA 74:556,1977.

122. Sutliff, V.E., Raufman, J.P., Jensen, R.T., and Gardner, J.D. Actions of vasoactive intestinal peptide and secretin in chief cells prepared from guinea pig stomach. Am. J. Phys. 251:G96,1986.

123. Sutton, D.R., and Donaldson, R.M. Synthesis and secretion of protein and pepsinogen by rabbit gastric mucosa in organ culture. Gastroenterology 69:166,1975.

124. Tamm, A., Villako, K., Harkonen, M., and Karonen, S.-L. Serum pepsinogen I and the state of gastric mucosa in an Estonian population sample. Scand. J. Gastroenterol. 19:1091,1984.

125. Taylor, W.H. Pepsins of patients with peptic ulcer.

Nature 227:76,1970.

126. ten Kate, R.W., Pals, G., Donker, A.J.M., Eriksson, A.W., and Meuwissen, S.G.M. Renal clearance of pepsinogen A (PGA) in healthy volunteers. Gastroenterology 90:1660A,1986.

127. Twining, S.S., Huibregtse, K., and Glick, D.M. A pepsinogen from dog stomach. Comp. Biochem. Physiol. 75B:103,1983.

128. Van Vunakis, H., and Herriott, R.M. Structural changes associated with the conversion of pepsinogen to pepsin: I. The N-terminal amino acid residue and amino acid composition of the pepsin inhibitor. Biochim. Biophys. Acta 22:537,1956.

129. Varis,K., Samloff, I.M., Ihamaki, T., and Siurala, M. An appraisal of tests for severe atrophic gastritis in relatives of patients with pernicious anemia. Dig. Dis. Sci. 24:187,1979.

130. Varis, K., Samloff, I.M., Koskenvuo, M., Rotter, J., and Sarna, S. Serum pepsinogen I and II in mono- and dizygotic twins. Hepatogastroenterol. 29:95,1982.

131. Varis, K.S. Peptic cells. In Kreuning et al. (Eds.) op. cit. pp 177-184.

132. Vatn, M.H., Schrumpf, E., and Myren, J. The effect of carbachol and pentagastrin on the gastric secretion of acid, pepsin and intrinsic factor in man. Scand. J. Gastroenterol. 10:55,1975.

133. Venables, C.W. The secretion of pepsin 1 in peptic ulceration and its relationship to activity. Br. J. Surg. 58:864,1971.

134. Venables, C.W. Mucus, pepsin, and peptic ulcer. Gut 27:233,1986.

135. Waldum, H.L. The influence of nervous and humoral stimulation on serum group I pepsinogens. In Kreuning et al. (Eds.) op. cit., pp. 115-127.

136. Waldum, H.L., Jorde, R., and Burhol, P.G. The effect of a test meal on serum group I pepsinogens (PGI) and serum gastrin in persons with normal gastric H+ secretion and in persons with achlorhydria. Scand. J. Gastroenterol. 15:267,1980.

137. Waldum, H.L., Walde, N., and Burhol, P.G. The effects of secretin on gastric H+ and pepsin secretion and on urinary electrolyte excretion in man. Scand. J. Gastroenterol. 16:999,1981.

138. Walker, V., and Taylor, W.H. Ovalbumin digestion by human pepsins 1, 3 and 5. Biochem. J. 176:429,1978.

139. Walker, V., and Taylor, W.H. Cigarette smoking, chronic peptic ulceration, and pepsin 1 secretion. Gut 20:971,1979.

140. Walker, V., and Taylor, W.H. Pepsin 5 in gastric juice: Determination and relationship to the akali-stable peptic activity. Gut 20:977,1979.

141. Walker, V., and Taylor, W.H. Pepsin 1 secretion in chronic peptic ulceration. Gut 21:766,1980.

142. Wallace, J.L., and Whittle, B.J.R. Role of mucus in the repair of gastric epithelial damage in the rat: Inhibition of epithelial recovery by mucolytic agents. Gastroenterology 91:603,1986.

143. Walsh, C. Enzymatic Reaction Mechanisms. San Francisco: W.H. Freeman & Co., 1979, pp 98-104.

144. Walsh, JD, Wolf, S. Geographical and environmental aspect of peptic ulcer. Am J Med 1960;29:754-760.

145. Wazna, M.F., Stein, T., and Wise, L. Studies on the role of serotonin in gastric pepsin secretion. Surg. Gynec. Obstet. 146:929,1978.

146. Cheung, W.Y. (Ed.) Calcium and Cell Function Vol I. Mew York: Academic Press,1980. Vol. I, Chapter 16, pp. 329-379.

147. Whitecross, D.P., Armstrong, C.,Clarke, A.D., and Piper, D.W. The pepsinogens of human gastric mucosa. Gut 14:850,1973.

148. Wollin, A., Soll, A.H., and Samloff, I.M. Actions of histamine, secretin and PGE2 on cyclic AMP production by isolated canine fundic mucosal cells. Am. J. Physiol. 237:E437,1979.

149. Wright, C.L., Shaw, B.,Sanders, D.J., and Reed, J.D. Variations in the proportions of individual pepsins secreted by the cat in response to vagal stimulation and hypoglycemia. Clin. Sci. Mol. Med. 48:297,1975.

150. Yahav, J., Lee, P.C., and Lebenthal, E. Ontogeny of pepsin secretory response to secretagogues in isolated rat gastric glands. Am. J. Physiol. 250:G200,1986.

Section II

METHODOLOGY

CHAPTER 5

History and Review of Pepsinogen Measurement

A) HISTORICAL CONSIDERATIONS

Initially the issue of accurately measuring pepsin revolved around the rate at which pepsinogen was transformed into pepsin and whether this was a standard reproducible percentage. Gottlieb, in 1924, reported that pepsinogen was rapidly converted at pH's of 1-3 but slow at pH's 3-4 and very slow above that [1]. His methodology was somewhat inaccurate since he used fibrin as a substrate. Subsequently Ege and Menck-Thygesen reported that a conversion began at pH 5.1 and was 10 times more rapid at a pH of 4.1 [2]. This observation was confirmed by Sumner and Somers who reported that at pH 2 conversion of pepsinogen to pepsin was instantaneous [3]. Herriot in 1938 had stated that the conversion of crystalline pepsinogen commenced at pH 6 and became increasingly rapid at a lower pH [4]. Hirschowitz and others reported that pepsinogen was rapidly, autocatalytically and irreversibly transformed into pepsin at a pH of 2.0 and that assay should take place as shortly as possible thereafter [5].

In the measurement of pepsin using its acid protease activity, a number of techniques were utilized. The first technique involved the activity measurement in substrate concentration high enough to give maximal rate. The theory was that the amount of substrate split would be proportional to the amount of enzyme present. Indeed this was the principle of the technique employed in the original hemoglobin-based assay [6].

An alternative technique related to the activity measurement in substrate solutions so dilute that the monomolecular law was simulated. This technique was of no particular advantage in the measurement of pepsin since it was only of use where the substrate was relatively insoluble.

The third possibility was the use of the principle that for a given substrate the time required to decompose a given fraction would vary in inverse proportion to the amount of enzyme present. This theoretical consideration was never of particular use in the measurement of pepsin since the kinetics of the equation were not well characterized for the pepsin digestive reaction, and it was not initially possible to express the activity of the enzyme as a velocity constant. For practical use, therefore, the first principle of activity measurement in substrate concentrations high enough to give maximal rates has always been used as the primary methodology for the measurement of pepsin.

The various methods that have been historically utilized in peptic assay are listed below. (Reproduced with kind permission of Dr. B. Hirschowitz)

Date	Author	Method
1778	Stevens	Meat liquefied by gastric juice.
1839	Wasmann	Discovery of pepsin-digestion of meat and egg-white.
1861	Brucke	Fibrin flakes, egg-white.
1881	Grutzner	Solid fibrin.
1886	Gehrig	Carmine stained fibrin.
1894	Mett	Egg-white tubes.
1894	Hammerschlag	Egg-white precipitation with Esbach's reagent.
1907	Fuld & Levison	Edestin.
1907	Jacoby & Solms	Ricin.
1908	Gross	Casein.
1909	Liebmann	Egg-white suspension.
1921	Michaelis	Serum proteins.
1922	Glaessner	Globin (of HB) with precipitation.
1922	Ege	Edestin modified with titration method.
1922	Loeper & Debray	Blood proteins, for blood enzymes.
1922	Euler	Rennet
to	Willstatter	Gelatin viscosity changes
1932	Northrop	Formation of soluble nitrogen Increase in formol titration.
1932	Anson & Mirsky	Hemoglobin

In essence, the earlier methodologies reported prior to 1922 could be divided into 2 groups:

1. those depending on the digestion of solid protein
2. those depending on the precipitation of proteins in solution.

1. Methods Depending On The Digestion Of Solid Proteins

a) 1861 Brucke used the clearing of suspensions of egg albumin at room temperature or fibrin flakes at 36-40^{0}C to first detect activity in the urine which he initially acidified on collection of the phosphoric acid [7]. The egg white method was subsequently utilized by Liebmann [8].

b) Grutzner (1874) digested solid fibrin [9] and subsequently

c) with Sahli [10] and Gehrig [11] (1895 & 1886) modified this methodology by combining fibrin with carmine and measuring the amount of dye released after peptic digestion. The method was not entirely sensitive since fibrin staining by carmine was not always equal and the

sensitivity was somewhat lacking.

d) In 1902 Nierensteiner and Schiff [12] modified the original method of Mett (1894) [13] in which glass tubes were filled with coagulated egg white and suspended in the acidified test solution. This method was initially used for clinical observation but its vague endpoint and crude results precluded its use in accurate studies.

e) Jacoby and Solms (1907) utilized the principle of the clearing of a fine suspension of ricin and egg white [14]. The difficulty with this methodology was the preparation of a reproducible substance and the problems involved in measuring a loss of translucency in a suspension of particles which was not entirely uniform.

f) Subsequently in 1920 Michaelis used suspensions of diluted human or sheep serum precipitated with 10% sulpho-salicylic acid to produce a milky suspension [15]. This methodology suffered from the same problems as that experienced in measuring the loss of translucency in the ricin egg white mixture of Jacoby and Solms.

The methodologies reported above were inaccurate and tedious to the extent that they were not able to be used for scientific investigation of an accurate nature. One of the main problems was the difficulty in preparing reproducible substrates and the inability to accurately measure endpoints. Of particular concern was the failure to accurately pH the digest and the result that a digestion methodology might thus be occurring at a non-optimal pH.

2. Methods Depending on Change in Precipitation of Dissolved Proteins

a) Hammerschlag (1894) used the principle that egg white in solution is precipitated by Esbach's reagent and that this property is lost on digestion by pepsin [16]. Unfortunately the measurement took a considerable time, the endpoint was vague and a large amount of gastric juice was required.

b) Fuld and Levison (1907) utilized the precipitation method in which edestin in hydrochloric acid was digested and the undigested portion precipitated with NaCl [17]. The undigested proportion was then titrated with alkaline (NaOH) and the amount of NaOH required to dissolve the precipitated edestin was taken as a measure of the amount that had been digested. Again, the difficulties in substrate preparation, inequality of samples and the indeterminate endpoint of solution of undigested edestin rendered this methodology inadequate.

c) In 1908 Gross, utilizing a similar technique to Fuld and Levison, employed casein in hydrochloric acid as a substrate [18]. He utilized sodium acetate to precipitate the undigested casein and the amount remaining was used as an index of the peptic activity by subtraction. Again, the difficulty with the methodoloy related to the fact that pure casein was not easy to store and that impure casein did not give a clear solution with a sharp endpoint. Gross made an important contribution in stating that peptic activity was mathematically proportional to the amount digested. This conclusion had been derived by an extrapolation of the Schutz-Borrisow law which stated that pepsin activity was proportional to the square of the protein digested [19].

d) Glaessner in 1922 employed the globin of hemoglobin which he precipitated with ammonia and redissolved with NH_4Cl as a measure of the amount left over after digestion [20].

e) Ege in 1923 improved the method of Fuld and was the first to utilize the measurement of the pH of each digest [21]. He used edestin in acetic acid at a pH of 1.5-1.6 precipitated with 20% NaCl followed by 70% $(NH_4)_2SO_4$ and compared this to a standard 0.5% gum arabic suspension. The endpoint was extremely reproducible to within 1-3 drops of the precipitant. The method was subsequently further refined by comparison of the unknown with standard Armour pepsin. In 1924 Gottlieb claimed that he was able to work to within an accuracy of 95% with this method and it remained the methodology of choice for scientific work until the hemoglobin method of Anson and Mirsky replaced it [6].

INITIAL PRINCIPLES OF PEPTIC ASSAY

In 1932 Northrop discussed the various principles and techniques involved in peptic assay:[22]

1. the measurement of change in viscosity of protein induced by pepsin
2. the production of non-protein nitrogen by pepsin and the determination of activity by the increase in formal titration
3. the production of amino (carboxyl) groups.

The first effect of pepsin on a protein solution is the rapid decrease in viscosity of the solution followed by a second and slower decrease in substrate protein nitrogen. The third change which is the slowest of all is an increase in the number of titratable carboxyl (amino) groups. Although other changes which include alterations in optical activity or conductivity occur they are less specific.

1. **Viscosity Changes**

Northrop used a number of different changes for estimating viscosity including gelatin and edestin, casein and dried milk. He claimed that the viscosity changes might be measured accurately and rapidly with less than 3% error (Northrop and Hussey 1923). However the viscosity changes and their relation to the chemical changes induced by acid protease were not clear. Arbitrary units were required for the measurement of specific viscosity and depended heavily on temperature and time measurement.

2. **The Production of Non-protein Nitrogen**

This method utilized casein or edestin and was based upon the measurement of increased non-protein nitrogen, either after increasing time intervals with a fixed concentration of enzyme or after a fixed time using different concentrations of enzyme. The exact significance of the changes in relation to the chemical changes was not clear and the further disadvantage was that it was somewhat slower than the viscosity change measurements. Furthermore there was no simple relation between the amount of non-protein nitrogen produced and the amount of active enzyme, except when the latter was present in extremely small quantities. Under the latter circumstances, however, the determination was somewhat inaccurate. The method was complicated overall and required interpretation by interpolation of different concentrations of an unknown enzyme solution with the result that the calculations varied considerably with the purity of the solution. As with the previous methodology, the development of arbitrary units of activity were required which defined the liberation of non-protein nitrogen under extremely specific conditions.

3. **Production of Amino (Carboxyl) Groups**

The advantage of this methodology was the fact that it had a clear chemical relation to enzyme reaction. Unfortunately the amount produced was small and not directly proportional to the amount of enzyme present. The methodology was difficult and the end result somewhat inaccurate. Gelatin, casein and edestin were used to generate activity curves and arbitrary units of activity defined.

In 1932 Anson and Mirsky developed the hemoglobin digestion method which has remained the basis for most subsequent methodologies utilized in the measurement of peptic activity [6]. It was accurate since it measured end products; was based upon rapid digestion by pepsin giving proportionality of results for both the concentration of enzyme and time; and utilized a

substrate of considerable reproducibility. Furthermore the substrate could be stored unchanged over long periods of time and small variations in the methodology of the measurement did not significantly influence the final result. As a result, the methodology of Anson and Mirsky has formed the keystone for most of the subsequent 50 years of peptic activity measurement. It has been variously upgraded by altering certain of the test conditions, altering the substrates and labelling the protein substrate with radio-active isotopes. In the subsequent section, I will detail the final form of the ^{125}I-labelled albumin assay that was developed in my laboratory to measure the peptic activity of secreting guinea pig gastric mucosal membrane mounted in Ussing chambers. In initial studies (isolated gastric glands) I utilized the standard Anson Mirsky methodology. Subsequently, when further accuracy and sensitivity was required I switched to ^{14}carbon labelled hemoglobin as the method of choice. Unfortunately the expense of this method necessitated the development of a less costly technique. I thus spent some time modifying the methodology to a certain extent and developing the use of ^{125}I-labelled albumin as a basic substrate. The exact details of this latter methodology will be presented subsequently.

B) OVERVIEW

Pepsinogen is the inactive precursor of pepsin and itself possesses no catalytic activity and can thus only be measured using an immunoassay. Specific radio-immunoassays for PG I and PG II have been developed and employed for measurements of pepsinogen in tissues, serum and urine [23,24]. This methodology has been adequately demonstrated to be highly sensitive and extremely specific. It is, however, only of clear advantage if the sample size is small or a specific distinction between PG I or PG II is necessary for the investigation or study under progress.

In general terms, however, to assay pepsinogen one has to express the result in terms of pepsin since pepsinogen must be hydrolyzed into pepsin before demonstrating any activity. This usually results in a somewhat less sensitive assay but is adequate since it allows the new bioactive pepsin to be measured using the resulting acid protease activity as an indicator. The first formal assay performed using this principle was the classical methodology developed by Anson and Mirsky who used a hemoglobin substrate at a pH of 2 [6]. Subsequently this assay has been considerably modified for large scale use in an auto-analyzer [25]. Alternatively, the sensitivity of the assay may be enhanced by measurement of the proteolytic end products by a protein assay procedure [26]. A further refinement of this technique which allowed for increased sensitivity was the usage of radiolabelled hemoglobin assay substrate [27]. There are a large number of different methodologies which have been utilized to measure

the proteolytic activity of the activated acid protease. Mostly these have employed different pH's, different substrates and radiolabelling techniques. In many cases, they have been specific to the investigative group which first described them and have not attained general usage. In the subsequent paragraphs of this chapter I will describe the modification which we have utilized to measure the small amounts of pepsinogen secreted by mammalian guinea pig mucosa.

A particular concern in the assay of pepsinogen which has been pointed out by a number of different authors is the use of different units for expressing the result. The original definition of one peptic unit [6] is the activity of pepsin that releases 0.1 umol of tyrosine from 5.0 ml of 2% hemoglobin at pH 1.7 in 10 minutes at 37°C. Unfortunately, most assays suffer from sufficient deviation in standard conditions to prohibit direct expression of the values in peptic units. As a result most assay values in more recent studies have been compared to standards of crystalline pepsinogen which have a defined activity in terms of peptic units. Until better and more precise methodology is available this will probably remain the standard methodology for expressing peptic activity [28].

C) Contemporary Measurement Of Pepsin

The measurement of pepsinogen and pepsin in biological fluids has suffered from several major deficiencies. These have included the absence of an acceptable and available reference standard, the potential presence of other proteolytic enzyme activity at acid pH and the use of protein substrates of differing compositions. In more recent times the estimation of proteolytic activity at acid pH have included a nephelometric assay using bovine albumin as a substrate [29], a colorimetric method using an insoluble substrate covalently labelled with Remazolbrilliant Blue [30], a method using autologous serum as a substrate [31], a radial diffusion assay in which the hemoglobin substrate is incorporated into agar gel [32], a microassay based on the detection of amino groups liberated from N,N-dimethylhemoglobin [33], a titrimetric assay based on the consumption of hydrogen ions during the hydrolysis of peptide bonds [34], and a method using human hemoglobin as a substrate [35]. In addition, a semi-automated procedure for serum pepsinogen has also been reported [36]. A number of recent studies have indicated that pepsin hydrolyzes ester as well as peptide bonds. Pepsin has been shown to hydrolyze selected esters of b-phenyl-L-lactic acid [37,38,39] and certain sulfite esters [40,41]. Robinson and White have reported that p-nitrophenyl sulfite is a suitable substrate for the spectro-photometric assay of peptic activity in canine gastric juice [42]. The relative specificities of the human group I and group II pepsins for these substrates is however not known. Furthermore, considerable investigation has been undertaken regarding the hydrolysis of various synthetic peptides by hog

pepsin [43,44,45,46]. Although these studies have been concerned with delineating the mechanism of pepsin action and determining the active site of the enzyme it is possible that this methodology may be of use in a analytic technique.

Whilst most methodology for the measurement of peptic activity has concentrated on overall measurement, recent studies have attempted to develop assays which are capable of differentiating group I from group II pepsinogens and pepsins. Chiang et al. [47] have reported that the group I pepsins, but not the group II pepsins (gastricsin), hydrolyze N-acetyl-L- phenylalanyl-L-diiodotryosine (APDT). The activity of the group II pepsins in gastric juice can thus be determined by subtracting activity obtained against APDT from activity obtained against hemoglobin. Similarly, Huang and Tang [48] have recently discovered a series of synthetic substrates apparently specific for the group II pepsins. The relative insolubility of these substrates and the prolonged incubation time required for measurement has limited the usefulness of this test.

A second approach has been based on the differential heat lability of the two groups of pepsins. At pH 7.25 and 25°C for 2 minutes or, at pH 7.1 and 20°C for 90 minutes the group II pepsins are stable but the group I pepsins are inactivated [49].

A third potentially useful approach is based on the finding that the group I and group II pepsinogens are immunochemically distinguishable. This has not been definitively shown for the respective pepsins. Preliminary results with an electrophoretic immunoprecipitation technique using antiserum apparently specific for the group I pepsinogens and pepsins have been reported [50]. Considerably more work must be done in this area since proteolytically inactive hydrolysis products of pepsin may retain immunological activity [51].

REFERENCES

1. Gottlieb E: Untersuchungen Uber die Propepsinmengen im Blut und Harn., Skand. Arch. Physiol.46:1,1924.

2. Ege R, and Menck-Thygesen P: Uber die Aktivierung des Propepsins. Biochem Z, 264,13,1933.

3. Sumner JB, & Somers GF: Chemistry and methods of enzymes. New York: Academic Press, 1943.

4. Herriott RM: Isolation, crystallization and properties of swine pepsinogen. J. Gen. Physiol 21:501,1938.

5. Hirschowitz BI: A study of the Physiology of Pepsin in the Human. M.D.Thesis. Univ. of the Witwatersrand 1953.

6. Anson ML, and Mirsky AE: The estimation of pepsin with hemoglobin. J Gen Physiol 16.89 1932.

7. Brucke E: Beitrage zur Lehre von der Verdaaung.V.Die verdauende Substanz im Urin. S B Akad Wiss Wien 43:618,1861.

8. Liebmann P: Eine neue methode klinische Pepsinbestimmung. Med Klinik 5:1784,1909.

9. Grutzner P: Ueber eine neue methode Pepsinmengen colorimentrisch zu bestimmen. Pflug Arch ges Physiol 8:452,1874.

10. Sahli W: Ueber das Vorkommen von Pepsin and Trypsin im normalen menschlichen Harn. Pflug Arch ges Physiol 36:209,1885.

11. Gehrig P: Ueber Fermente im Harn. Pflug Arch ges Physiol 38:35,1886.

12. Nirenstein E, & Schiff A: Ueber die Pepsinbestimmung nach Mette und die Notwendigkeit ihrer Modifikation fur klinische Zwecke. Arch Verdaukr 8:559,1902.

13. Mett SG: Beitrage zur Physiologie der Absonderungen; zweite Mittheilung; weitere Mittheilung zur Innervation der Bauchspeicheldruse. Arch Anat Physiol p58, 1894.

14. Solms E: Ueber eine neue Methode der quantitativen Pepsinbestimmung und ihre klinische Verwendung. Z klin Med 64:158,1907.

15. Michaelis L, & Rothstein M: Die Zerstorung von Lab und Pepsin durch Alkali. Biochem Z 105:60,1920.

16. Hammrschlag A: Ueber eine neu Methode zur quantitativen Pepsinbestimmung. Int Klin Rundschau 8:1393,1894.

17. Fuld E and Levison LA: Die Pepsinbestimmung mitte der Edestinprobe. Biochem Z 6:473,1907.

18. Gross O: Die Wirksamkeit des Pepsins und eine einfache Methode zu ihrer Bestimmung. Berl klin Wschr 45:643,1908.

19. Schutz J: Zur Kenntniss der quantitativen Pepsinwirkung. Hoppe-Seyl Z 30:1,1900.

20. Glaessner K: Eine neue Pepsinbestimmungsmethode. Biochem Z 127:312,1922.

21. Ege R: Eine Modifikation des Fuldschen Verfahrens zur Bestimmung von Pepsin. Hoppe-Seyl Z 127:125,1923.

22. Northrop JH: Pepsin activity units and methods for determining peptic activity. J Gen Physiol 16:41,1932.

23. Samloff IM: Pepsinogens I and II: Purification from gastric mucosa and radioimmunoassay in serum. Gastroenterology,82:26-33,1962.

24. Samloff IM, and Liebman WM: Radioimmunoassay of group I pepsinogens in serum. Gastroenterology 66:494-502,1984.

25. Vatier MM, Cheret AM, and Bonfils S: Le dosage automatique de l'activite proteolytique du suc gastrique. Biol Gastroenterol 1:15-29,1968.

26. Hersey SJ, May D, and Schyberg D: Stimulation of pepsinogen release from isolated gastric glands by cholecystokininlike peptides. Am J Physiol 244:G192-G197,1983.

27. Kasbekar DK, Jensen RT, and Gardner JD: Pepsinogen secretion from dispersed glands from rabbit stomach. Am J Physiol. 244:G392-G396,1983.

28. Hersey SJ: Pepsinogen Secretion. In Physiol of the G I Tract Ed L R Johnson Raven Press NY,944-957,1987

29. Gerring EL, Allen EA: A nephelometric pepsin method. Clin Chim Acta 24:437-443,1969.

30. Rinderknecht H, Geokas MC, Silverman P, et al: A new ultrasensitive method for the determination of proteolytic activity. Clin Chim Acta 21:197-203,1968.

31. Uete T, Wasa M, Shimogami A: A simplified method for the determination of pepsinogen in blood and urine. Clin Chem 15:42-55,1969.

32. Samloff IM, Kleinman MS: A radial diffusion assay for pepsinogen and pepsin. Gastroenterology 56:30-34,1969.

33. Lin Y, Means GE, Feeney RE: The action of proteolytic enzyme on N, N-dimethyl proteins. Basis for a microassay for proteolytic enzymes. J Biol Chem 244:789-793,1969.

34. Martin F, Vuez JL, Berard A, et al: A study of the mechanisms of inhibition of peptic proteolysis by a sulphated polysaccharide. Digest 1:165-174,1968.

35. Berstad A: A modified hemoglobin substrate method for the estimation of pepsin in gastric juice. Scand J Gastroent 5:343-348,1970.

36. Wenger J, Munro M: Semiautomated method of serum pepsinogen determination. Clin Chem 16:207-211, 1970.

37. Lokshina LA, Orekhovich VN, Sklyankina VA. Eesterase activity of pepsin. Nature (London) 204:580,1964.

38. Inouye K, Fruton JS: Pepsin as an esterase. J Amer Chem Soc 89:187-188,1967.

39. Inouye K, Fruton JS: Studies on the specificity of pepsin. Biochemistry (Wash) 6:1765-1777,1967.

40. Reid TW, Fahrney D: The pepsin-catalysed hydrolysis of sulfite esters. J Amer Chem Soc 89:3941-3943,1967.

41. Reid TW, Stein TP, Fahrney D: The pepsin catalyzed hydrolysis of sulfite esters. II. Resolution of alkyl phenyl sulfites. J Amer Chem Soc 89:7125-7126,1967.

42. Robinson LA, White TT: A spectrophotometric method for the analysis of pepsin. Gastroenterology 58:798-800,1970.

43. Hollands TR, Fruton JS: Kinetics of the hydrolysis of synthetic substrates by pepsin and by acetyl-pepsin. Biochemistry (Wash) 7:2045-2053,1968.

44. Clement GE, Snyder SL, Price H, et al: The pH dependence of the pepsin-catalyzed hydrolysis of neutral dipeptides. J Amer Chem Soc 90;5603-5610,1968.

45. Jackson WT, Schlamowitz M, Shaw A, et al: The effect of pH on the kinetic constants of peptic substrates and inhibitors. Arch Biochem 131:374-385,1969.

46. Hollands TR, Voynick IM, Fruton JS: Action of pepsin on cationic synthetic substrates. Biochemistry (Wash) 8:575-585,1969.

47. Chiang L, Sanchez-Chiang L, Wolf S, et al: The separate determination of human pepsin and gastricsin. Proc Soc Exp Biol Med 122:700-704,1966.

48. Huang WY, Tang J: On the specificity of human gastricsin and pepsin. J Biol Chem 244:1085-1091,1969.

49. Sjeiffers MJ, Tkatch R: Assay of two pepsin factions in human gastric juice by alkaline inactivation. Gastro. 59.528-533,1970.

50. Hirsch-Marie H: Dosage immunochimique de pepsinogene et e la pepsine gastrique et urinaire par la methode de Laurell. Clin Chem Acta 24:411-416,1969.

51. Samloff MI. Pepsinogens, Pepsins and Pepsin inhibitors. Gastro. 60.4.586-599,1971.

CHAPTER 6

Ussing Chamber

This apparatus was originally developed in the late 1940's to measure ion flux in amphibian epithelium [1]. It has since been modified to maintain gastric mucosa [2,3,4,5]. Through improved tissue handling techniques, refinement of the tissue environment, and development of a sensitive pepsin assay, I have been able to study mammalian gastric mucosa instead of the more commonly utilized amphibian (frog) tissues. Hartley guinea pigs from 150-200 grams were anesthetized by Metofane inhalant anesthesia in an ether jar and sacrificed by cervical decapitation. Their peritoneal cavities were entered through a vertical midline incision and their antrectomized stomachs removed. The stomachs were opened and rinsed of their contents using deionized water. The serosal and submucosal layers were dissected from the mucosa. The intact mucosal sheets were then mounted between two lucite half-chambers. The submucosal solution was bathed in mammalian Ringers solution (122 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCL, 1.3 mM $MgSO_4$, 2.0 mM $CaCl_2$, 1.0 mM KH_2PO_4, and 20 mM glucose) and gassed with 95% O_2 and 5% CO_2. The osmolality of the solution was 293 mosm/l. The pH of the media was adjusted to 7.30-7.40 with 14 N HCL. The luminal side of the mucosal sheet was gassed with pure oxygen and bathed in 154 mM NaCl (308 mosm/l). Casein hydrolysate 0.03% w/v (Polypep, Sigma, St. Louis) was added to saturate protein binding sites in the chamber and improve pepsinogen recovery. Luminal solution pH was maintained at 5.0 by titration with 5mM NaOH using the pH stat technique with an autotitrator (Radiometer Copenhagen). Acid secretion was calculated from the amount of alkali added to maintain constant pH. The luminal solution was sampled in triplicate (100 ul each) at twenty minute intervals for measurement of pepsinogen concentrations. All fluids were warmed to 39-40 ^{o}C and maintained at this level by a circulating water bath.

The pH of 5 chosen for the luminal solution represents a compromise between the instability of pepsin at higher pH [6] and the inaccuracies of pH stat technique at lower pH [7]. Pepsin is relatively unstable at alkaline or neutral pH because of the relatively low number of basic amino acids in its structure. The pH stat methodology loses some accuracy at increased hydrogen ion concentrations because of added hydration of CO_2 and dissociation of H_2CO_3. A further inaccuracy results from the higher volume of titrant utilized to keep the pH relatively low. By convention, for titration of H^+ with an OH^- titrant it is assumed that $V_T = A_H/[OH^-]_T$ where V_T is the volume of titrant added to bring the solution to the desired end point; A_H the amount of H^+ (hydrogen ion concentration of sample multiplied by the volume of the sample); and $[OH^-]_T$ is the OH^- concentration of the titrant. The equation for pH stat, however, is $V_T = A_H/[H^+]_O + [OH^-]_T$ where $[H^+]_O$ is the initial and final H^+ concentration. Thus, the pH of 5 utilized in these experiments causes a difference of the calculated from actual hydrogen ion secretion of less than 2%.

A voltage clamp apparatus (DVC 1000 World Precision Instruments, New Haven) connected to the chamber by silver chloride electrodes in series with 4 M KCl half cells and 4 M KCl 5% agar bridges is used to continually ascertain tissue viability by measurement of the potential difference across the mucosa. By placing a 25 microamp current across the membrane, tissue resistance can be calculated utilizing Ohm's law, $V=I \cdot R$. Increased tissue resistance represents decreased cell function and poor tissue viability. Attainment of stable baseline acid secretion is another indicator of tissue viability.

After the initial equilibration period of 150 minutes is completed, the mean potential difference and the mean electrical resistance remain stable. Figure 1 is a representative graph of potential difference and tissue resistance of a typical mucosa over the final 150 minutes of a mounting period. Acid and pepsinogen secretion of this same tissue remained constant at 1.44 ueq/hr and 0.024 units/hr respectively. Figure 2 depicts the total amount of acid and pepsin that an unstimulated mucosal sheet secretes into the luminal solution over a 2.5 hour experimental period.

Mucosal viability is further supported by morphological studies. Figure 3 is a light micrograph of a deserosalized gastric mucosal sheet at the time of mounting. It is stained with toluidine blue, which is relatively selective for chief cells and periodic acid Schiff (PAS), which is selective for mucous cells. The secretory product from the mucous cells is distributed exclusively at the cell apex. The zymogen granules from the chief cells are relatively heterogenous in their distribution, although their general orientation is apical. Figure 4 is an electron micrograph of a glandular unit from a mucosal sheet that has been maintained in the Ussing chamber for 5 hours. There is no disruption of glandular or cellular architecture as demonstrated by minimal tissue edema, normal nuclear chromatin, abundant microvilli lining the gland lumen and maintenance of tight junctions and glandular architecture.

REFERENCES

1. Ussing, H.H. "Transport of Ions Across Cellular Membranes", Physiological Reviews, 29:127-155, 1949.

2. Rutten, M.J., Ito, S. "Morphology and Electrophysiology of Guinea Pig Gastric Mucosal Repair in Vitro", American Journal of Physiology, 244:G171-182, 1983.

3. Inoue, M., Fong, J., Shah, G., Hirschowitz, B. "Mediation of Muscarinic Stimulation of Pepsinogen Secretion in the Frog", American Journal of Physiology, 248:G79-86, 1985.

4. Shirakawa, T., Hirschowitz, B. "Cellular Messengers of Stimulants of Pepsinogen Secretion from Isolated Frog Esophageal Mucosa", American Journal of Physiology, 250:G361-368, 1986.

5. Basson, M., Adrian, T., Modlin, I. "A New Model for the Study of Mammalian Pepsinogen Secretion", Gastroenterology, 92:1310, 1987.

6. Herriott, R. M. "Pepsinogen and Pepsin", Journal of General Physiology, 45:47, 1962.

7. Sanders, S., Hayne, V., Rehm, W. "Normal H^+ Rates in Frog Stomach in Absence of Exogenous CO_2 and a Note on pH Stat Method", American Journal of Physiology, 225:1311-1321, 1973.

CHAPTER 7

Pepsinogen Assay

In 1930 Northrup assayed pepsin by the digestion of egg white, casein and gelatin [1]. A few years later, Anson and Mirsky [2] developed an assay based on the ability of pepsin to degrade the hemoglobin protein. This technique, however, is not sensitive enough to measure the small quantities of pepsinogen present in mammalian Ussing chamber samples. Later, ^{14}C-labelled hemoglobin was used as a protein substrate to automate pepsinogen assaying [3]. Although the ^{14}C-hemoglobin technique is sensitive, it is quite expensive and requires amplification of beta particles with scintillation fluid. In addition, the trichloracetic acid-precipitated hemoglobin is difficult to separate.

The pepsinogen content of guinea pig gastric mucosa is 700 $\pm$ 50 units/gram wet weight (n=6, mean $\pm$ SEM) or 21.6 $\pm$ 1.6 units/cm^2 (mean $\pm$ SEM) after stripping the serosa and extraction by freeze-thawing in 0.1% Triton X dissolved in 0.01 M HCl with a pH of 2.0. By comparison, the mucosa of the frog esophagus has been reported to contain 0.52-2.32 mg/cm^2 of pepsinogen, depending on the season [4]. Thus, the guinea pig gastric mucosas used in these experiments have less than one hundredth of the pepsinogen present in the frog esophagus. Similarly, the basal secretory rate measured for guinea pig mucosal sheets in the Ussing chamber preparation was 0.07 units/cm^2/hr, significantly less than the (8.7-43.3 ug/cm^2/hr reported for frog esophagus preparations by Hirschowitz. The pepsinogen assay used for these experiments was therefore adapted for the detection of the small quantities of pepsin secreted by guinea pig mucosa.

First, I undertook some modification of the classical Anson-Mirsky assay to increase sensitivity. Initially I substituted ^{14}C-methemoglobin for non-radioactive hemoglobin in order to automate the assay counting and to increase precision. Finally, I changed the substrate for the assay to ^{125}I-labelled albumin. As in the Anson-Mirsky methodology, pepsin is allowed to incubate with the protein. Non-digested protein is precipitated with trichloracetic acid, and the pepsin content calculated from the quantity of peptides in the supernatant fluid. The ^{125}-albumin assay measures the gamma emission from the labeled peptides and is sensitive to a level of five micrograms of pepsinogen. The brief description of this assay is followed by detailed methodology on page 85.

Radioactive protein substrate is prepared by the incorporation of ^{125}I to bovine serum albumin using chloramine T as an oxidant. The reaction is terminated by addition of sodium metabisulfite. Iodinated albumin is separated from free iodine by gel permeation chromatography using Sephadex G-50. Figure 5 shows the radioactivity of 0.3 ml (two minute) aliquots collected off the column. The first peak, just after the void volume of the column, represents iodinated albumin. The second peak represents unbound iodine and small protein fragments. Aliquots from the first peak are diluted and used to generate a standard curve of known pepsin

activity for each assay performed (Figure 6).

To determine the optimum time of assay incubation, albumin-pepsin standards were incubated for 24, 48, and 72 hours, respectively (Figure 7). These data demonstrate stability of albumin in an acid environment and no significant advantages between one, two or three day incubations. This technique has an inter-assay variation of 4-9% and an intra-assay variation of 3-7%.

Specific details of all three assays used follow.

COLORIMETRIC ASSAY TECHNIQUES:

The first assay utilized in the laboratory was spectrophotometric using a modified version of the classical Anson-Mirsky technique. Pepsinogen in the medium was assayed by an acid protease method in which a fixed aliquot of medium was incubated with hemoglobin solution at pH 2.0 for a fixed time interval; the enzyme reaction was terminated by adding trichloroacetic acid. The hemoglobin reagent contained 20 mg/ml hemoglobin titrated with HCl to a final pH of 2.0. After a preliminary 10 min activation with 0.3 ml of 0.04 N HCl, 100 ul of medium was incubated with 0.8 ml of hemoglobin reagent for 20 min at 37 ^{o}C. Undigested proteins were precipitated with 1.8 ml of 5% trichloracetic acid and allowed to react for 10 min. The contents were filtered through Whatman No. 4 filter paper. The extent of hemoglobin digestion was assessed in 1 ml of filtrate by adding 5.0 ml of 0.2 N NaOH and, after 10 min, 0.5 ml of 1 N phenol (Folin-Ciocalteu) reagent. Spectrophotometry was performed at 760 nm after 30 min. Pepsinogen activity was expressed in terms of peptic units (PU) released per 2 ml final sample. One PU corresponded to the amount of enzyme yielding an absorbance change of 0.001 in one min under the assay conditions.

^{14}C-METHEMOGLOBIN TECHNIQUE:

The Anson-Mirsky assay was later modified to measure the digestion of ^{14}C-methemoglobin. To each milliliter of assay buffer (pH 2.0), 10^{5} cpm of ^{14}C-methemoglobin (New England Nuclear Research Products, Dupont Company, Wilmington, DE) was added. All samples were incubated for thirty minutes in a shaking water bath at 37 ^{o}C until the reaction was terminated by the addition of 1.8 ml of trichloracetic acid (5% w/v) with rapid mixing for an additional ten minutes. To separate the intact hemoglobin molecules from the digested peptide fragments, each 10 X 75 mm tube was centrifuged at 1500 g (Beckman J-6M) for 60 minutes. The supernatant was added to 5.0 ml of scintillation fluid (Safety Solve, Research Products International Corporation, Mount Prospect IL) in a 20 ml scintillation vial. Then, gamma emission was measured in an automated counter (Tricarb 1500. Packard Instrument Co., Sterling VA). Total gland pepsinogen content was determined

by freezing gland suspensions in liquid nitrogen for 5 minutes, thawing at 23 ^{o}C for 30 minutes, centrifuging the fluid for 30 minutes at 1500 g and assaying the supernatant for pepsinogen as described above.

^{125}I-ALBUMIN TECHNIQUE:

To measure pepsinogen in Ussing chamber samples the assay was modified to use 125-iodinated albumen as a substrate. Because of improved sensitivity and relative simplicity, this assay is now also utilized for pepsinogen determination in all non-Ussing chamber pepsinogen experiments in my laboratory. One and one-half nmol RIA grade bovine serum albumin (Sigma, St. Louis) was added to 4.0 nmol Na^{125}I (Amersham IMS 30, Amersham Corp. Arlington Heights, IL). Chloramine T (70 nmol) was added to the solution and mixed for 15 seconds. The reaction was terminated by the addition of 200 nmol of sodium metabisulfite. The solution was then diluted with 300 ul 0.06 M phosphate buffer (pH 7.4). The iodinated albumin was then separated from the free iodine by running the solution through a 6 x 0.5 cm column of Sephadex G-50 Superfine (Pharmacia, Piscataway NJ) eluted with 0.06 M phosphate buffer (ph 7.4).

Fractions of 0.3 ml were collected and their radioactivity measured (Figure 5). Iodinated albumin was the sample collected just after the void volume. Incorporation of ^{125}I with this method was 75%, giving an expected specific activity of 0.43 mCi/nmol (16 Bq/fmol). The first collection of labeled albumin was then diluted 1:25 with 0.5 M acetic acid and stored frozen in 1.0 ml aliquots for later use. No significant autodigestion of the protein was observed for up to four weeks of storage.

For each pepsinogen assay one aliquot of ^{125}I-labelled albumin was thawed and added to 50 ml of a 0.031 M HCl / 0.05 M KCl buffer, pH 1.8. To each 100 ul sample, 250 ul of the acid-albumin solution was added. The pepsinogen in each sample was, thus, converted to pepsin. The samples were incubated for 18 hours. Nine hundred (900) ul of 5% w/v trichloroacetic acid was added to each tube to precipitate the undigested albumin. Fifty ul of 1% (w/v) unlabelled albumin was then added to each tube to facilitate the formation of a visible pellet following the subsequent centrifugation at 1500 g for 60 minutes. The digested fragments of ^{125}I-albumin remaining in the supernatant were quantitated by automated gamma scintigraphy (Packard Auto-Gamma 5780, Sterling, VA).

Each assay was standardized using purified porcine pepsinogen (Sigma) of known activity. One unit of peptic activity is conventionally defined as the amount needed to produce a change in absorbance at 280 nm of 0.001 per minute at pH 2.0 at 37 ^{o}C, measured as trichloroacetic acid-soluble products, using hemoglobin as a substrate.

MORPHOLOGICAL STUDIES

Gastric mucosal sheets were examined at both the light and electron microscopic levels. Samples for light microscopy were placed in buffered formalin, embedded in paraffin and stained with hematoxylin and eosin. Additional samples were treated with toluidine blue and Biebrich scarlet which have stronger affinity for chief cells.

Samples for electron microscopy were placed in a fixative of 3% glutaraldehyde, oriented with a dissecting microscope, embedded in epoxy 812 (E.F. Fullam Inc., Latham NY) and sectioned to one micron thickness. Plastic embedded sections were stained with basic fuschin and examined under the light microscope for orientation and subselection of representative areas for ultrathin section. The ultrathin sections were placed on copper grids, stained with osmium tetroxide and uranyl acetate and examined using a Phillips 300 electron microscope.

Criteria for tissue injury included evidence of edema, vacuolization, diminished numbers of microvilli, and disruption of tight junctions and cellular membranes.

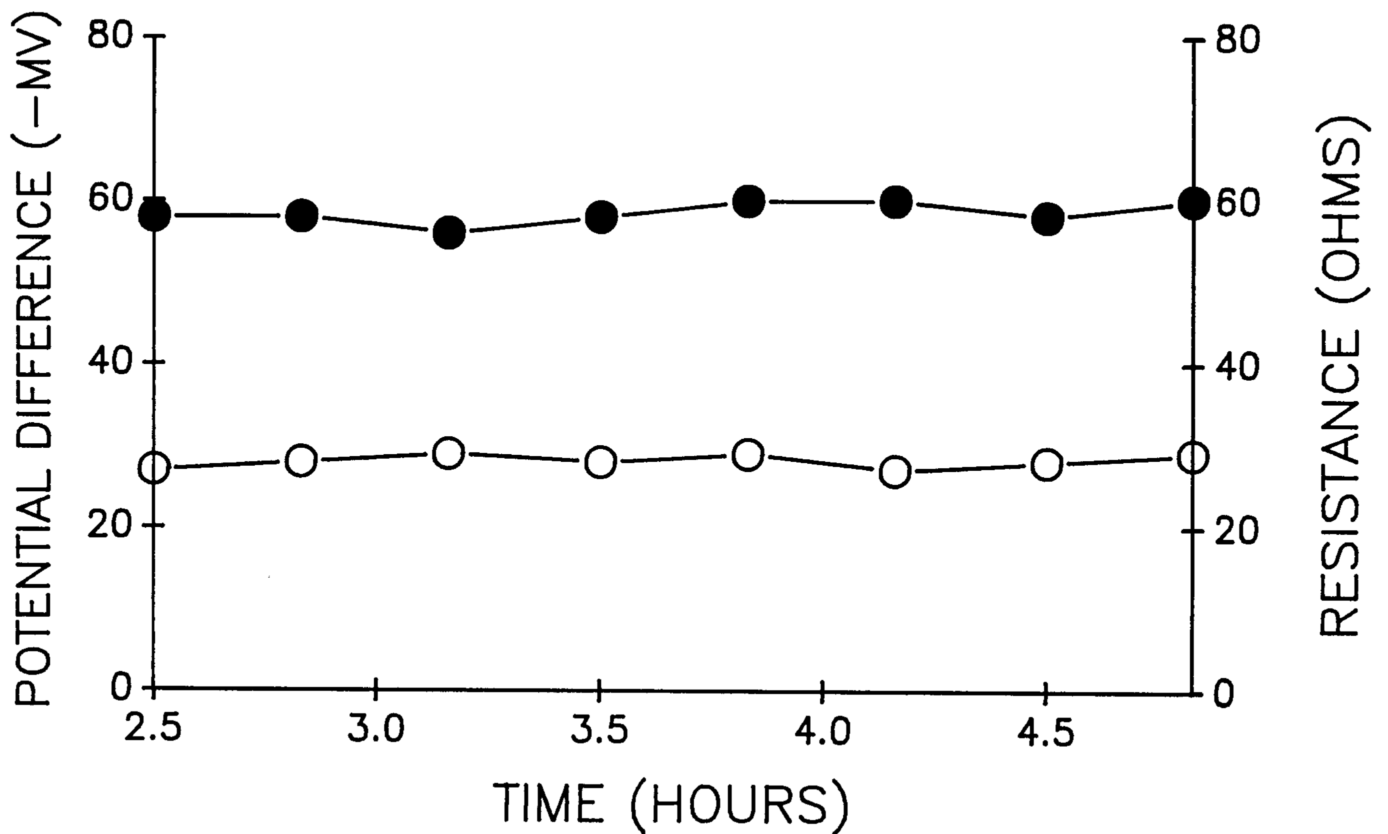

Figure 1. Graph of potential difference expressed in -millivolts (open circles) and resistance expressed in ohms (filled circles) vs. time (hours) for a typical gastric mucosal preparation after an initial 150 minute equilibration period. The relative stability of these electrical parameters suggests maintenance of tissue viability.

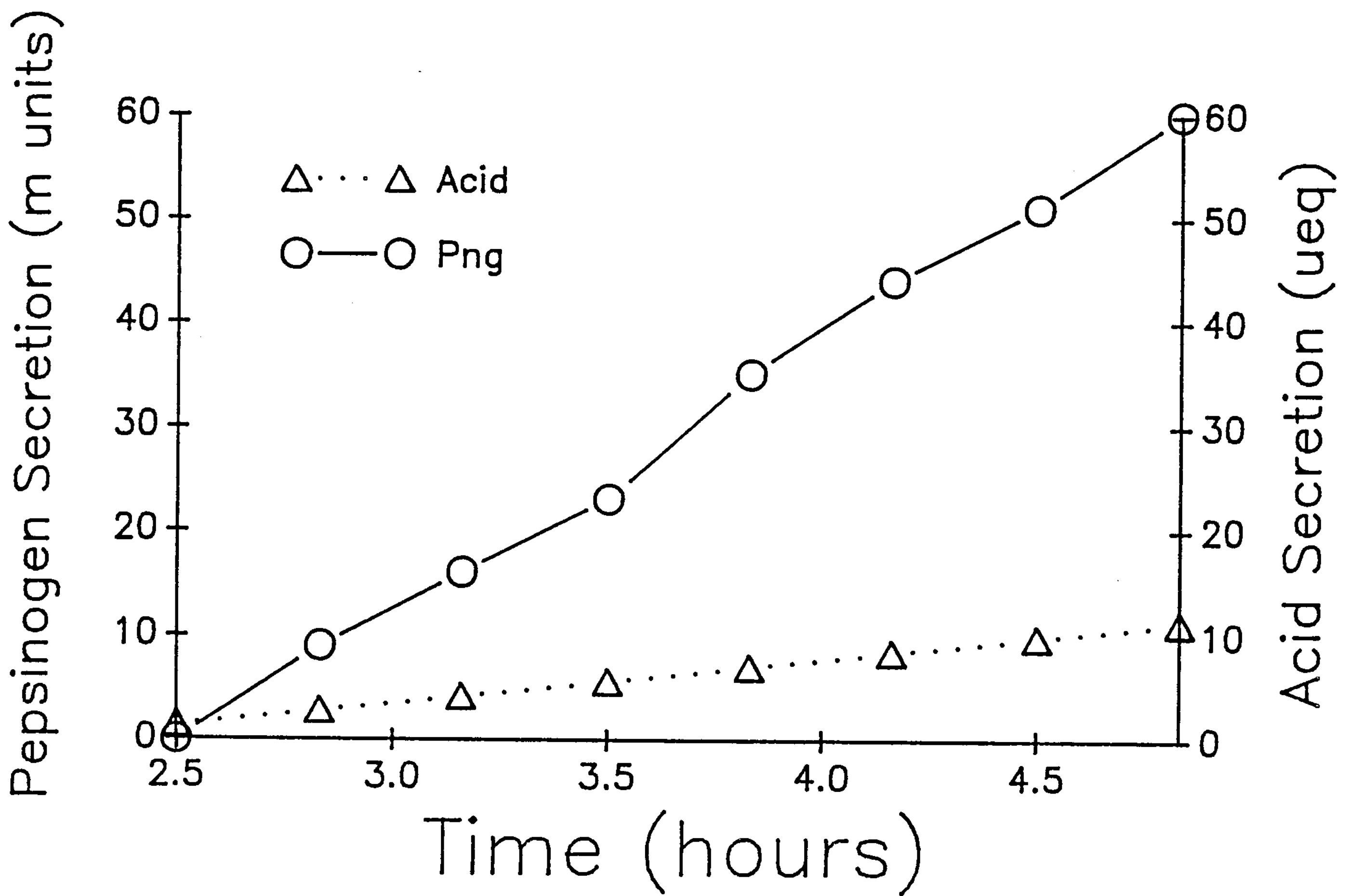

Figure 2. Graph of total acid (microequivalents/ml) and pepsin (milliunits/ml) vs. time (hours) secreted into the luminal solution by an unstimulated gastric mucosal sheet after a 2.5 hour equilibration period. Acid and pepsin secretion remain stable.

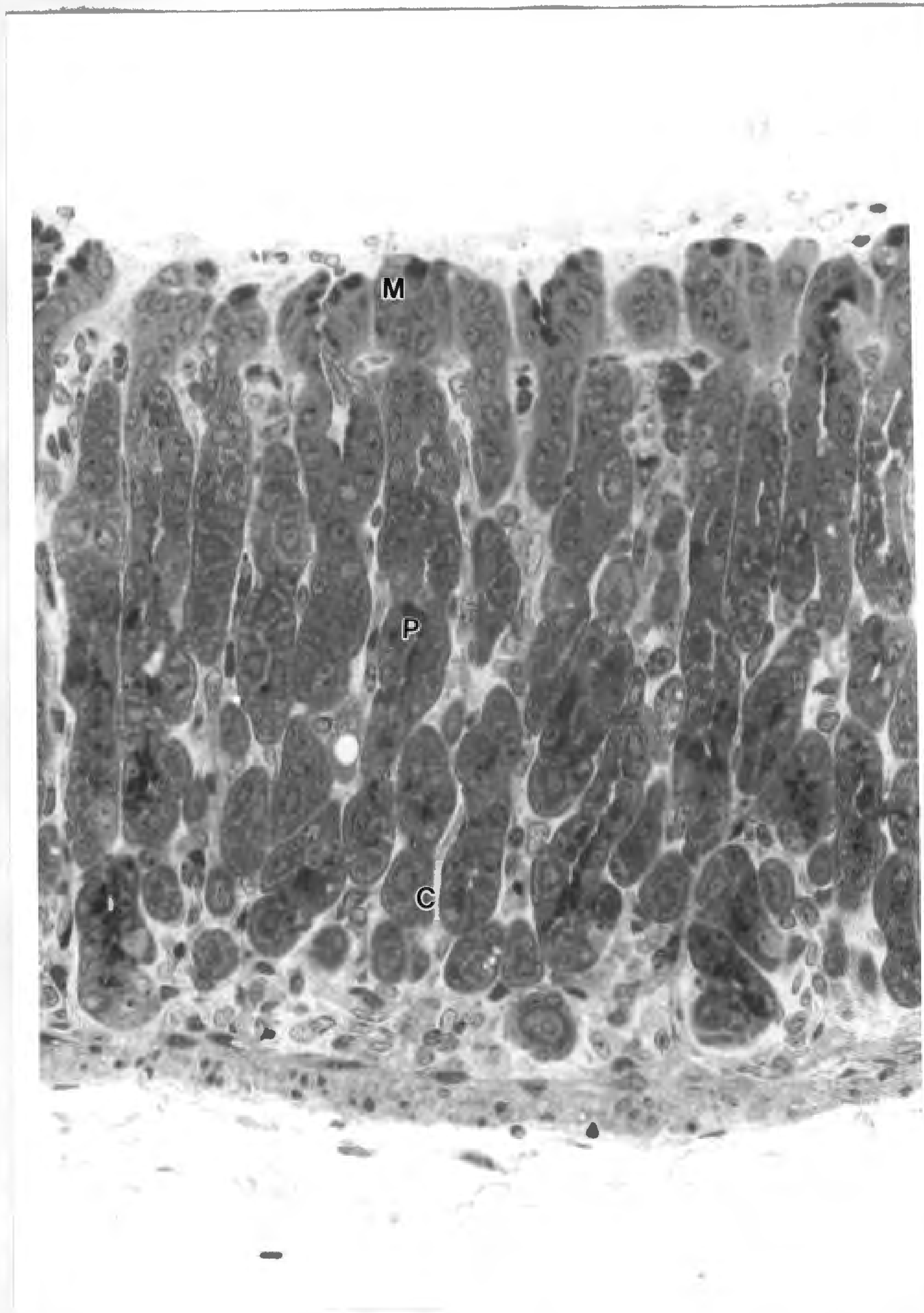

Figure 3. Light micrograph (400 X magnification) of guinea pig gastric mucosa stained with toluidine blue and periodic acid Schiff. The serosa is removed from the mucosa by blunt dissection at the level of the muscularis mucosa. No disruption of glandular architecture is observed. Mucus cells (M) are located near the glandular neck, parietal cells (P) are situated in the middle of the gland, and chief cells (C) are found at the base of the gland.

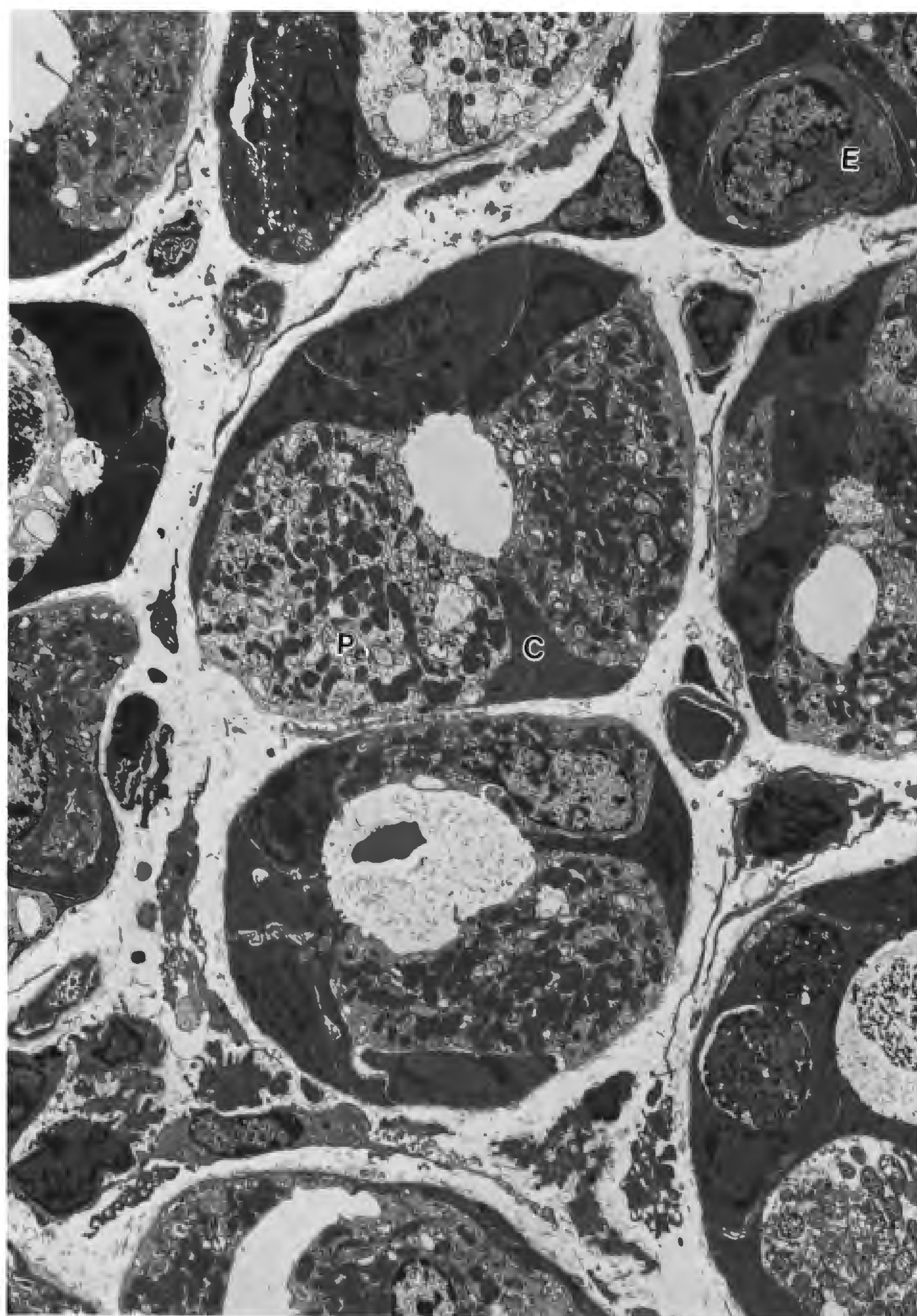

Figure 4. Electron micrograph (3000 X magnification) of a glandular unit from an unstimulated guinea pig gastric mucosal sheet maintained for five hours in an Ussing chamber apparatus. A gastric gland has been transected revealing chief (C), parietal (P), and endocrine cells (E). The parietal cells are relatively pale and contain numerous mitochondria and endoplasmic reticulum. The zymogen granules are oriented apically are readily visible in the dark staining chief cells. Chief cells can be differentiated from mucous cells by their relative heterogeneity of orientation of secretory product. The endocrine cells have secretory granules located at the base of the cell. The micrograph reveals no sign of damage to the gastric gland cells despite over 5 hours of in vitro nourishment.

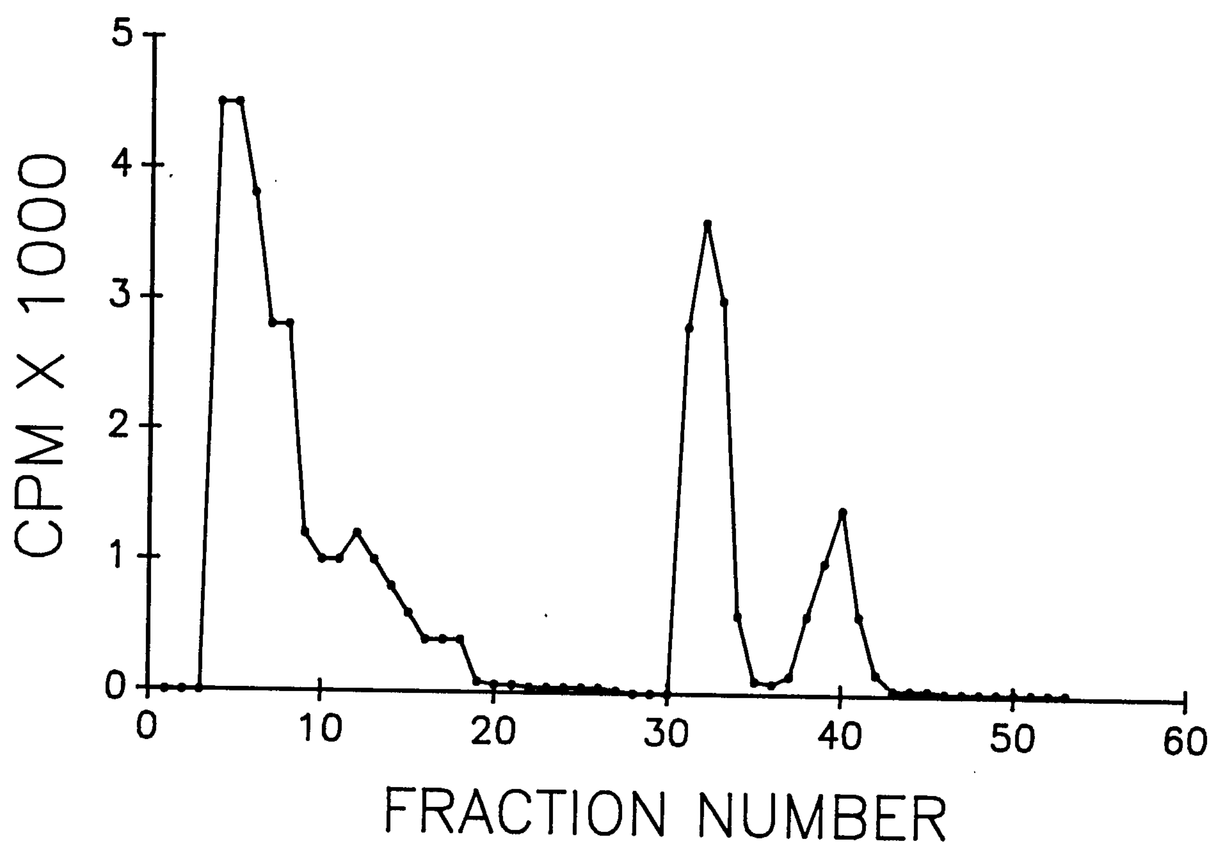

Figure 5. Graph of counts per minute (CPM) vs. fraction number of 0.3 ml aliquots for ^{125}I-labelled albumin collected from a 6.0 cm gel permeation chromatography column of Sephadex G-50. The column rate was 0.15 ml/min. The first peak represents intact molecules of labeled albumin. Subsequent peaks represent labelled protein fragments and free ^{125}iodine. Fraction 4 is diluted in 0.5 M acetic acid and used for the pepsinogen assay.

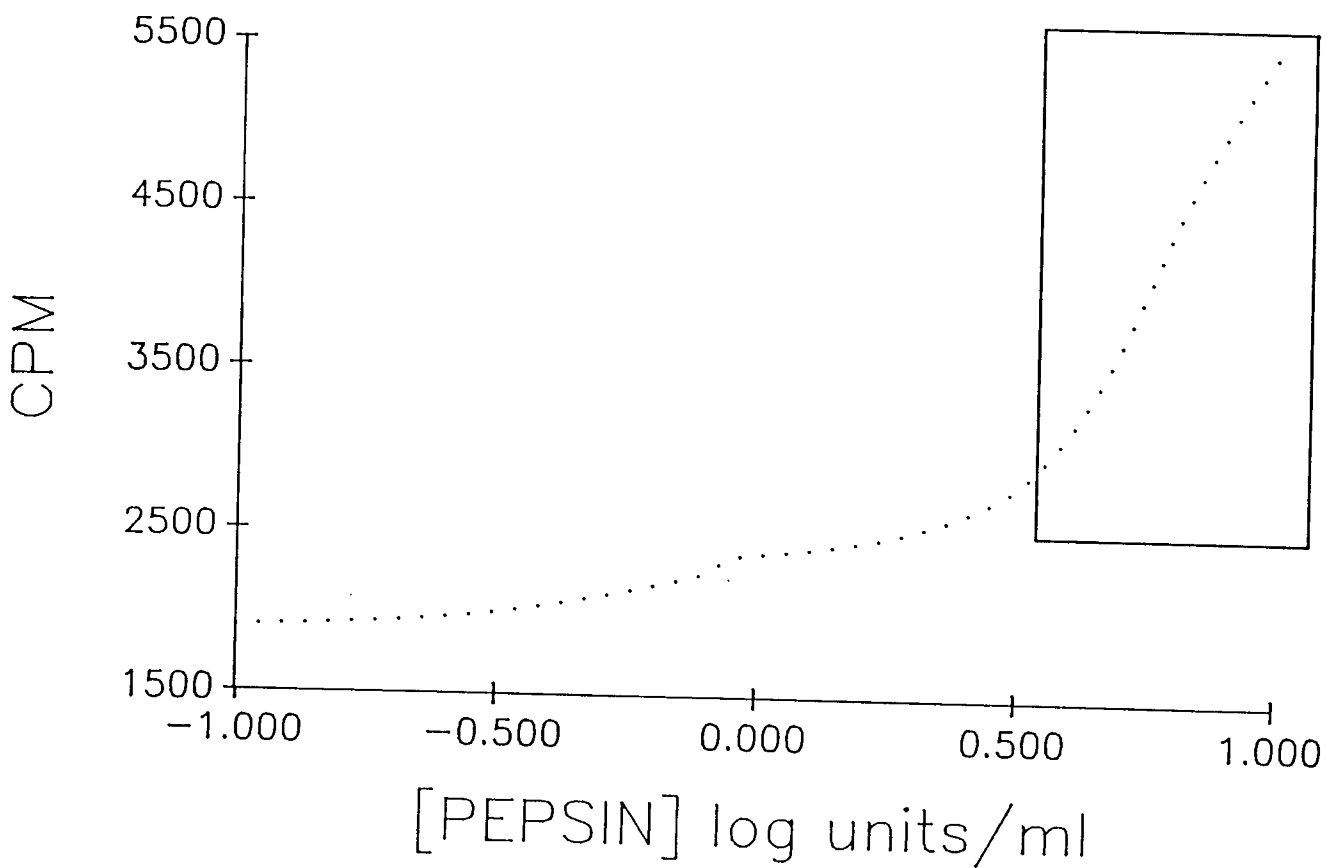

Figure 6. Typical standard curve for pepsinogen assay of counts per minute (CPM) vs log units/ml pepsin concentration. Pepsinogen concentrations in the experimental conditions range from 0.5 to +1.0 log units/ml (boxed portion of the graph).

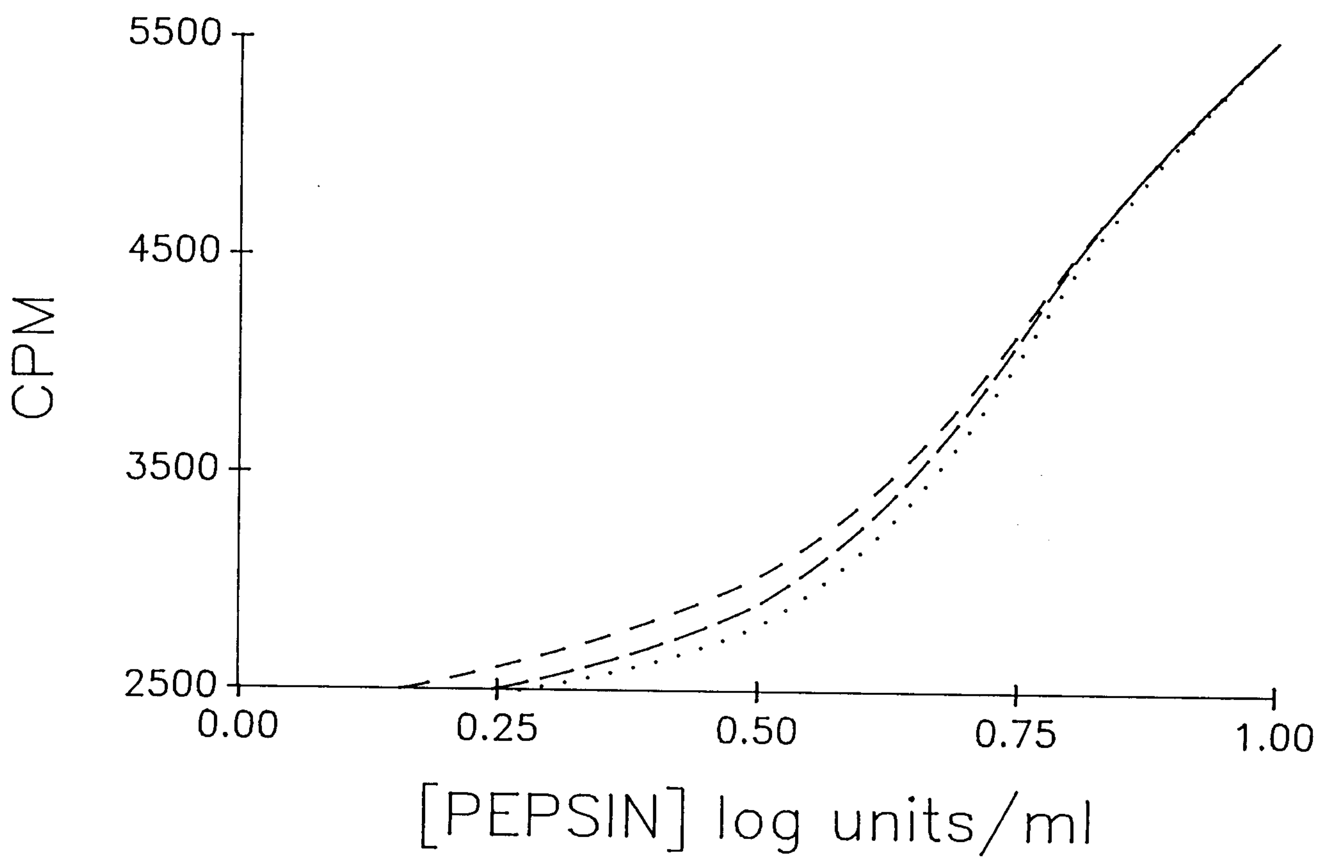

Figure 7. Standard curves of pepsinogen assay of 24, 48, and 72 hour incubation times. Stability of albumin in an acid environment is demonstrated.

REFERENCES

1. Northrup, J.H. "Crystalline Pepsin. I. Isolation and Tests of Purity", Journal of General Physiology, 13:739, 1930.

2. Anson, M., Mirsky, A. "The Estimation of Pepsin with Hemoglobin", Journal of General Physiology, 16:59-63, 1932.

3. Kasbekar, D., Jensen, R., Gardner, J. "Pepsinogen Secretion from Dispersed Glands from Rabbit Stomach", American Journal of Physiology, 244:G392, 1983.

4. Shirakawa, T., Hirschowitz, B. "Seasonal fluctuations in pepsinogen secretion from frog esophageal peptic glands", American Journal of Physiology, 250:G484-488, 1986.

CHAPTER 8

A Micro-Method for the Assay of Cellular Secretory Physiology

INTRODUCTION

Isolated cell systems are critical for investigations of cellular physiology and intracellular mechanisms regulating stimulus-secretion coupling. These studies require reliable methods for rapid processing of large numbers of replicate samples in parallel studies investigating both secretory product and intracellular messengers. Isolated enriched parietal cells have proven to be useful models in the study of the mechanisms of gastric secretion [1]. Rabbit parietal cells secrete both acid and intrinsic factor. While acid secretion can not be assessed directly in isolated cells, the accumulation of the weak base aminopyrine into cells has been adopted as a semi-quantitative method which reflects the general pattern of parietal cell acid secretion [2,3]. Intrinsic factor secretion into the media can be determined directly by its binding to cyanocobalamin [4]. Traditionally, parietal cell secretion has been measured through the use of multiple incubation flasks or tubes under conditions of intermittent oxygenation. These methods can often be cumbersome, especially when large numbers of conditions or time course studies are involved. We present here an simplified method for carrying out secretory assays using an incubation procedure miniaturized into microtiter plates and combined with continuous oxygenation and rapid separation of cells from media by vacuum filtration. The data presented here demonstrate that intracellular accumulation, secretory product and intracellular second messengers can all be measured in parallel with easier handling of replicate specimens. A preliminary report of this work has been presented [5].

METHODS

MATERIALS

New Zealand White Rabbits (3.5-4.0 kg) were obtained from Millbrook. [^{14}C]-dimethylamine-aminopyrine (4,366 Bq/nmol) and [^{57}Co]-cyanocobalamin (4000 Bq/pmol) were from Amersham. Cyclic AMP assay kits were from Diagnostic Products. Safety-Solve scintillation fluid was obtained from Research Products. Collagenase (type I), Lactate Assay kits and all other chemicals were purchased from Sigma.

ISOLATED PARIETAL CELL PREPARATION

Parietal cells were isolated from rabbit fundic mucosa using collagenase digestion and separation through counterflow elutriation, as previously described [6-8]. The resulting enriched fraction contained 80±5% cells with a yield of at least 10^8 cells. These cells were then resuspended in incubation media (134 mM NaCl, 5.4 mM KCl, 5.0 mM Na_2HPO_4, 1.0 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 10 mg/L phenol red, 2 mg/ml bovine serum albumin (Fraction V) and 2 mg/ml glucose, pH 7.4) for subsequent studies. Cells were counted in an improved Neubauer chamber

and purity of the elutriated cells was assessed by examination of approximately 500 cells stained with hematoxylin and eosin in randomly selected fields. Cell viability was assessed by trypan blue exclusion and lactate concentration in the entire contents (cells plus media) of a single well.

INCUBATION CHAMBER

A microtiter plate incubation chamber was designed and constructed completely from polycarbonate (Yale Medical Instrumentation Facility, New Haven, CT). The chamber (Figure 1) holds a single 96-well microtiter plate which is oxygenated through the continuous flow of humidified gas over the plate. The chamber is sealed through a circumferential silicone rubber rim attached to the chamber top. The top is held in place with four clamps which can be rapidly released and latched for access to the plate during time course experiments. The entire chamber is completely immersible in a heated shaking water bath for maintenance of an even incubation temperature. The rate of gas flow through the chamber can be monitored and regulated at the chamber outlet.

AMINOPYRINE UPTAKE

Enriched parietal cells from elutriation were suspended in a incubation media at 2.5 x 10^6 cells/ml and preincubated with [^{14}C]-aminopyrine at 2.12 nmol/ml for 30 min with humidified 100% O_2 at 37^oC in a shaking water bath. The incubation of parietal cells with secretagogues was carried out in the incubation chamber utilizing a 96-well millititer filter plate (Millipore, Millititer SV) possessing a Durapore membrane filter with a 5 um pore size. Incubation media was added to each well of the millititer plate (170 ul for a single secretagogue). 100 ul of the cell suspension containing 250,000 cells preincubated with [^{14}C]-aminopyrine was then added to each well. Concentrations of greater than 250,000 cells per well were not utilized because of impaired filtration in subsequent steps. Secretagogue was added to each well in a volume of 30 ul (with 10-fold higher than the desired final concentration) making a total incubation volume of 300 ul. In the time course studies, the plate was incubated for a two hour period and secretagogue was added at 0, 30, 60, 70, 80, 90, 100, 105, 110, 115, and 117.5 min after beginning of incubation, thus giving a time course in the presence of secretagogue from between 2.5 and 120 min. During incubation, the incubation chamber was immersed in a shaking water bath at 37^oC with 100% humidified oxygen at 2 p.s.i. At the end of the incubation period the plate was immediately removed and placed on a vacuum filtration chamber (Millipore) and rapidly filtered under vacuum. The filtrate was collected into the respective wells of a standard tissue culture microtiter plate using a transfer guide (Millipore). These supernatants were retained for the measurement of intrinsic factor and scintillation counting for [^{14}C]-aminopyrine

when ratios were determined. The filtrate microtiter plates could be stored at -20° C for assay of intrinsic factor at a later date, usually within 2 weeks. The Durapore membrane filters were dried on the vacuum system, a process that took approximately 4 min. Dried filters were then punched out using a filter punch (Millipore) into scintillation vials, for measurement of [^{14}C]-aminopyrine uptake into cells retained on the membrane filter. Scintillation fluid (3 ml) was added directly to the vials for beta counting. The accumulation of [^{14}C]-aminopyrine within each fraction was expressed as pmol aminopyrine uptake per million cells, after subtraction of the background radioactivity adherent to the membrane filter assessed by measuring the binding of [^{14}C]-aminopyrine in the absence of parietal cells.

MEASUREMENT OF WHOLE LACTATE CONCENTRATION

For evaluation of the respiratory status of cells incubated in the microtiter plate chamber versus flasks, cells were incubated either under standard conditions in the microtiter plate chamber with continuous oxygenation or in a 50 ml Erlenmeyer flask with intermittent oxygenation for 60 seconds every 15 minutes. In both cases, the concentration of cells was maintained at 750,000 cells/ml. After 60 minutes of incubation, the contents of two wells from the microtiter plate or 600 ul of cell suspension from the flask were withdrawn and added to an equal volume of 10% trichloracetic acid. Duplicate samples were utilized for each condition. Lactate was then determined in the deproteinized supernatant utilizing a standard enzymatic/ spectrophotometric assay [Sigma Lactate Kit; 9]. Results were expressed as mM lactate for the entire sample (cells plus media).

MEASUREMENT OF CELLULAR cAMP CONTENT

Intracellular cyclic AMP concentrations were measured in parallel to the secretion studies described above except that parietal cells were preincubated in incubation media without [^{14}C]-aminopyrine. Measurements were made in triplicate wells incubated on the same plate as the secretory studies described above. At the end of the incubation period the plate was rapidly dried and filters punched into vials containing 0.5 ml of ice cold 3% perchloric acid in an ice bath. The samples were extracted by vortex mixing and were then neutralized to pH 6.5 by the addition of 30% potassium bicarbonate. Cyclic AMP concentrations were then measured on these supernatants by the method of Tovey, et al [10], using a commercially available kit (Diagnostic Products Corp). This method utilizes competitive binding between [^{3}H]-cyclic AMP and endogenous cyclic AMP for the specific binding protein. Separation of free from bound cAMP is accomplished by absorption onto dextran coated charcoal, followed by beta counting of the resulting supernatant. This assay shows a cross reaction with cyclic GMP of less than 0.1% The method detects changes between the adjacent samples of 40 fmol/tube with 95% confidence (equivalent to 0.64 pmol

cAMP/million cells).

INTRINSIC FACTOR SECRETION

Intrinsic factor secreted into the media was measured by assessing the binding of [^{57}Co]-cyanocobalamin in cell supernatants by a modification of the method of Gottlieb, et al. [11]. Fifty ul of filtrate were incubated with 100 ul [^{57}Co]-cyanocobalamin (300 fmol, specific activity 0.4 Bq/fmol) in 1 ml of 150 mM NaCl for 40 min at 24°C. Bound label was then separated from free by the addition of 0.5 ml of a hemoglobin (25 mg/ml) coated, activated charcoal (Norit A, 250 mg/ml) suspension. After 20 minutes, the suspension was centrifuged at 1700 g for 15 min. The supernate was then decanted for gamma counting. Results were expressed as pmol intrinsic factor secreted per million cells. All cyanocobalamin binding capacity was assumed to be intrinsic factor.

STATISTICAL ANALYSIS

Between animal precisions for aminopyrine uptake, intrinsic factor secretion and intracellular cAMP were calculated as a coefficient of variation. Statistical significance was assessed using the Student's t-test for paired data using the mean data from triplicate wells for each data point in the experiments, where n=number of rabbits used in each procedure.

RESULTS

Cells incubated in the microtiter plate chamber demonstrated greater than 95% viability for up to six hours after preparation as assessed by trypan blue exclusion. Similarly whole lactate concentrations (media plus cells) were not significantly changed during the first 60 min of incubation (0.28 $\pm$ 0.09 mM at time zero versus 0.35 $\pm$ 0.11 mM at 60 min). In contrast, cells incubated in flasks with intermittent oxygenation showed a greater than two-fold higher lactate level (0.81 $\pm$ 0.11 mM; $p<0.05$) in after 60 min of incubation.

Uptake of [^{14}C]-aminopyrine in unstimulated cells was 10.2$\pm$1.5 pmol/million cells which corresponds to 1.25$\pm$0.18% of the total radioactivity added. Dose-response curves for histamine and carbachol stimulation (60 min incubation) of both aminopyrine uptake and intrinsic factor secretion are shown in Figures 2 and 3, respectively. Aminopyrine uptake and intrinsic factor secretion were significantly stimulated by 10^{-7}M histamine ($p<0.05$) and the maximal effect was seen at 10^{-5}M. Half maximal effective dose of histamine (ED_{50}) was 10^{-6}M. The effect of 10^{-4}M histamine was completely abolished by cimetidine 10^{-4}M. In comparison forskolin (10^{-6}M) elicited an almost two-fold higher response than the maximal histamine dose. Carbachol at 3 x 10^{-6}M caused a small, but significant increase in

aminopyrine uptake ($p<0.05$) and intrinsic factor secretion ($p<0.05$) with a maximal effect seen at $3x10^{-5}$M. The half maximal effective dose of carbachol was 10^{-6}M.

The time course for histamine (10^{-5}) stimulation of aminopyrine uptake, intrinsic factor secretion and cellular cAMP concentrations are shown in Figure 4. There was a rapid three-fold increase in cytosolic cAMP concentrations in the first 5 minutes of stimulation with histamine ($p<0.005$). This increase was sustained throughout the two hour period of study. Histamine elicited a continuing increase in aminopyrine uptake ($p<0.001$) and intrinsic factor secretion ($p<0.002$ from 15 min on) throughout the two hour period of study. Basal aminopyrine accumulation into unstimulated cells incubated for 30, 60 or 120 min in the absence of secretagogue did not significantly change. Similarly, intrinsic factor secretion from unstimulated cells did not significantly change during a two hour incubation period.

The time course of aminopyrine uptake, intrinsic factor secretion and cellular cAMP content in carbachol stimulated parietal cells is shown in Figure 5. Aminopyrine uptake increased rapidly in response to carbachol within the first ten minutes of stimulation ($p<0.01$). No significant further accumulation occurred after 30 minutes. Intrinsic factor secretion followed a similar pattern with significant initial release (by 10 min, $p<0.05$) and hardly any secretion after the first 30 min. There was no significant change in cellular cAMP content during carbachol stimulation. In contrast, however, the forskolin control (10^{-6}M) caused a 20-fold increase in intracellular cAMP content from 5.6 ± 1.2 pmol/million cells to 49.6 ± 5.8 pmol/million cells ($p<0.001$).

The coefficients of variation between animals for histamine (10^{-5}M) and carbachol (10^{-5}M) on aminopyrine uptake, intrinsic factor secretion and cellular cAMP are shown in Table 1. Coefficient of variation for all parameters was below 30%.

TABLE 1

Between animal coefficient of variation of aminopyrine uptake, intrinsic factor secretion and cellular cAMP content in unstimulated parietal cells and following incubation with histamine, carbachol and forskolin (n=12).

	Aminopyrine Uptake	Intrinsic Factor	Cellular cAMP
Unstimulated	22.0%	23.7%	14.0%
Histamine (10^{-5}M)	11.7%	28.4%	26.1%
Carbachol (10^{-5}M)	27.5%	28.5%	24.2%
Forskolin (10^{-5}M)	9.5%	24.9%	28.7%

Figure 1. Custom built polycarbonate incubation chamber for parietal cell incubation. This chamber allows cells incubated in a microtiter plate to be sealed (with lid and gasket shown), and immersed in a shaking water bath at 37°C. The design of the chamber allows continuous gassing with humidified oxygen which flows in one end, over the microtiter plate and out of the opposite end of the chamber. Clamps on the sides of the chamber ensure a watertight seal but can be released rapidly for addition of secretagogues during time course studies.

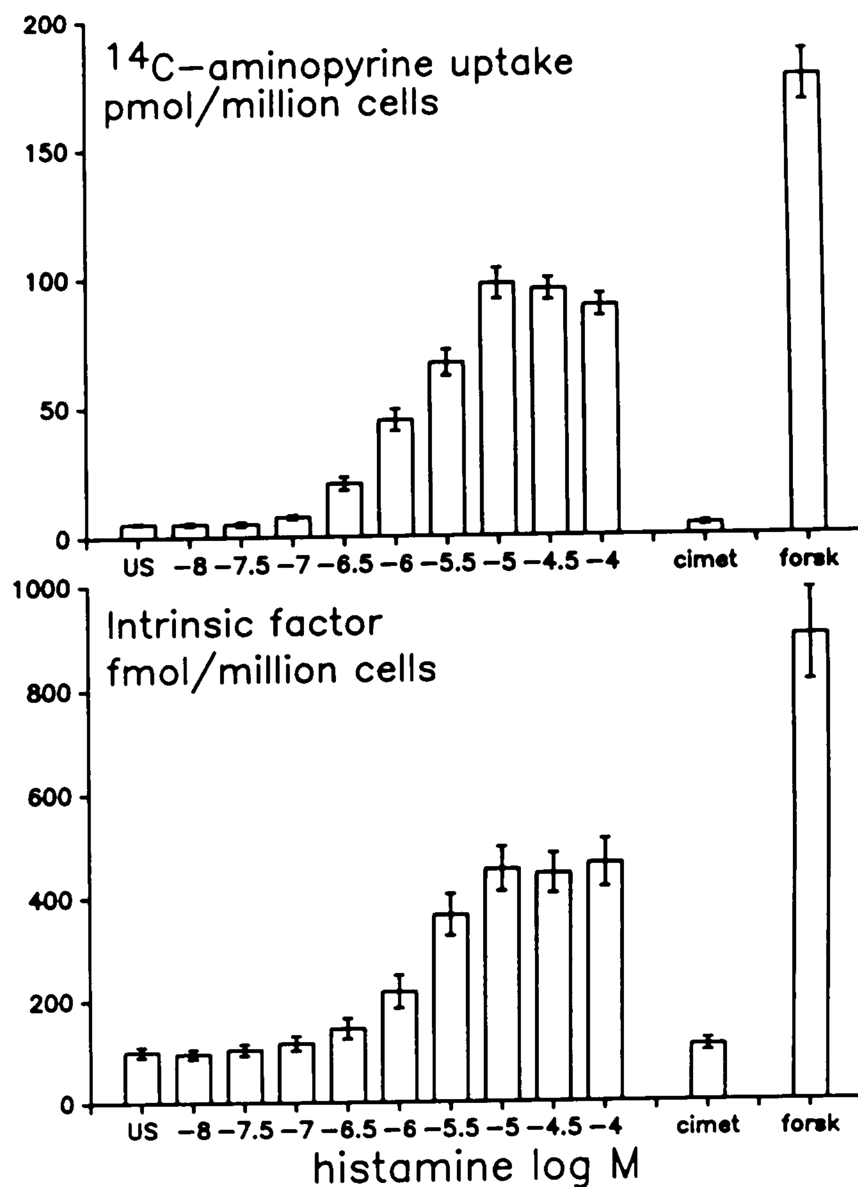

Figure 2. Dose-response effect of histamine on [^{14}C]-aminopyrine uptake (an indirect measure of acid secretion) and intrinsic factor secretion from isolated rabbit parietal cells. Effect of histamine 10^{-4}M with cimetidine 10^{-4}M (Cimet) and forskolin 10^{-5}M are added for comparison. Bars show mean and SEM from 6 separate experiments.

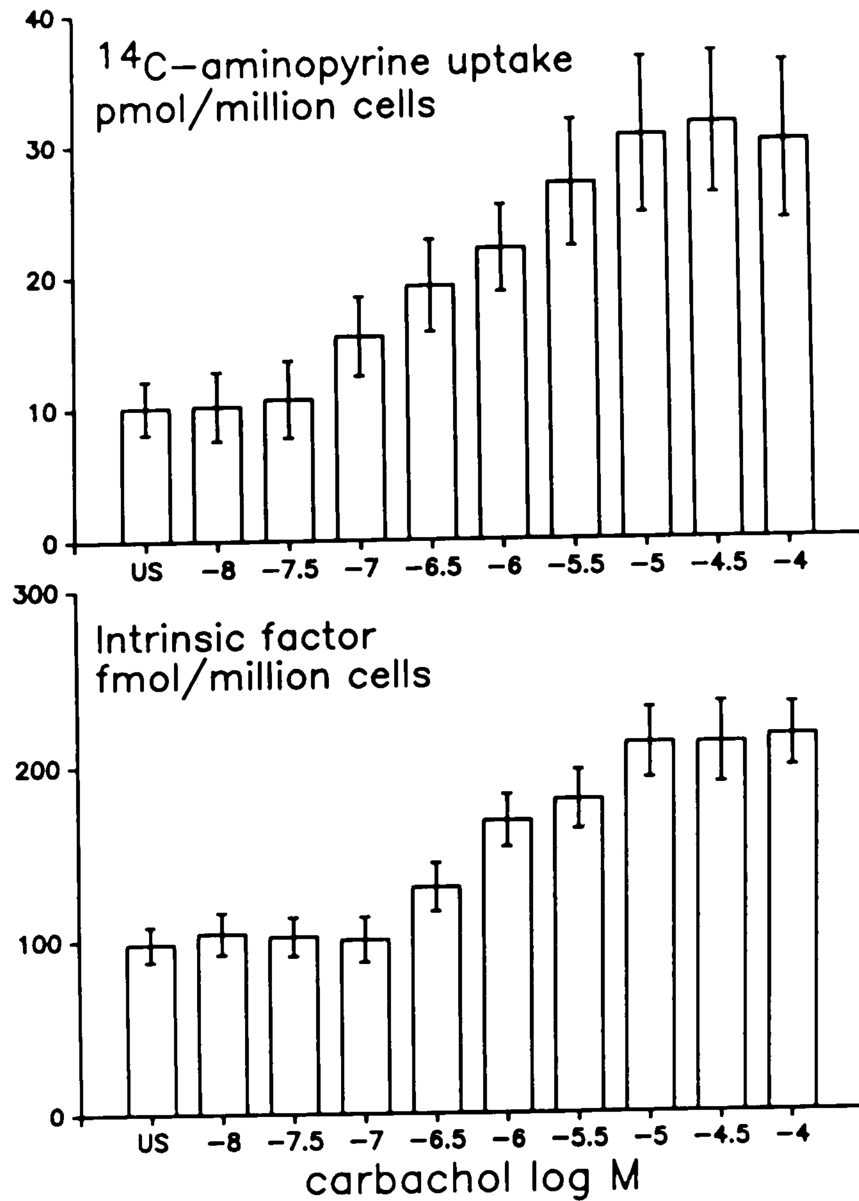

Figure 3. Dose-response effect of carbamylcholine (carbachol) on [^{14}C]-aminopyrine uptake and intrinsic factor secretion from isolated rabbit parietal cells. Bars show mean and SEM from 6 separate experiments.

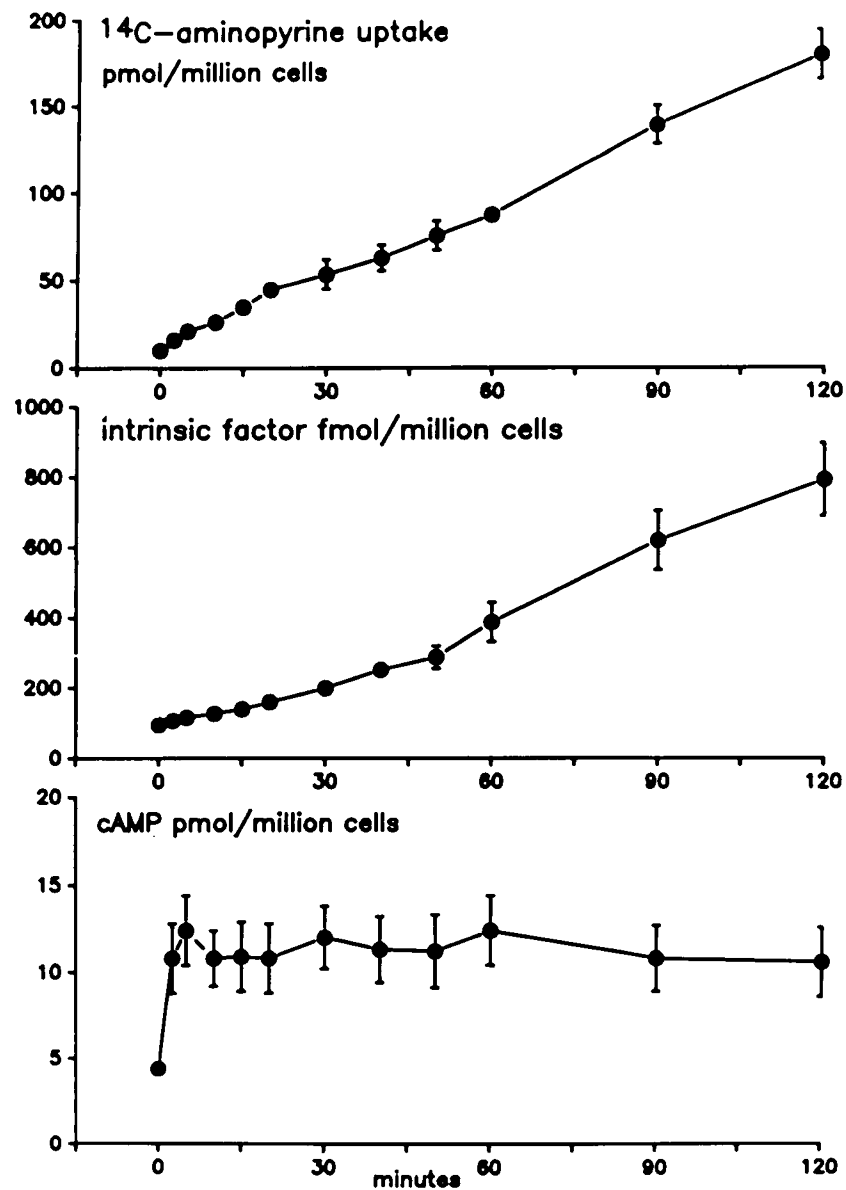

Figure 4. Time course effect of histamine stimulation of rabbit isolated parietal cells on: top panel: [^{14}C]-aminopyrine uptake, middle panel: intrinsic factor secretion and bottom panel: cellular cAMP content. Graphs show mean and SEM from 6 separate experiments.

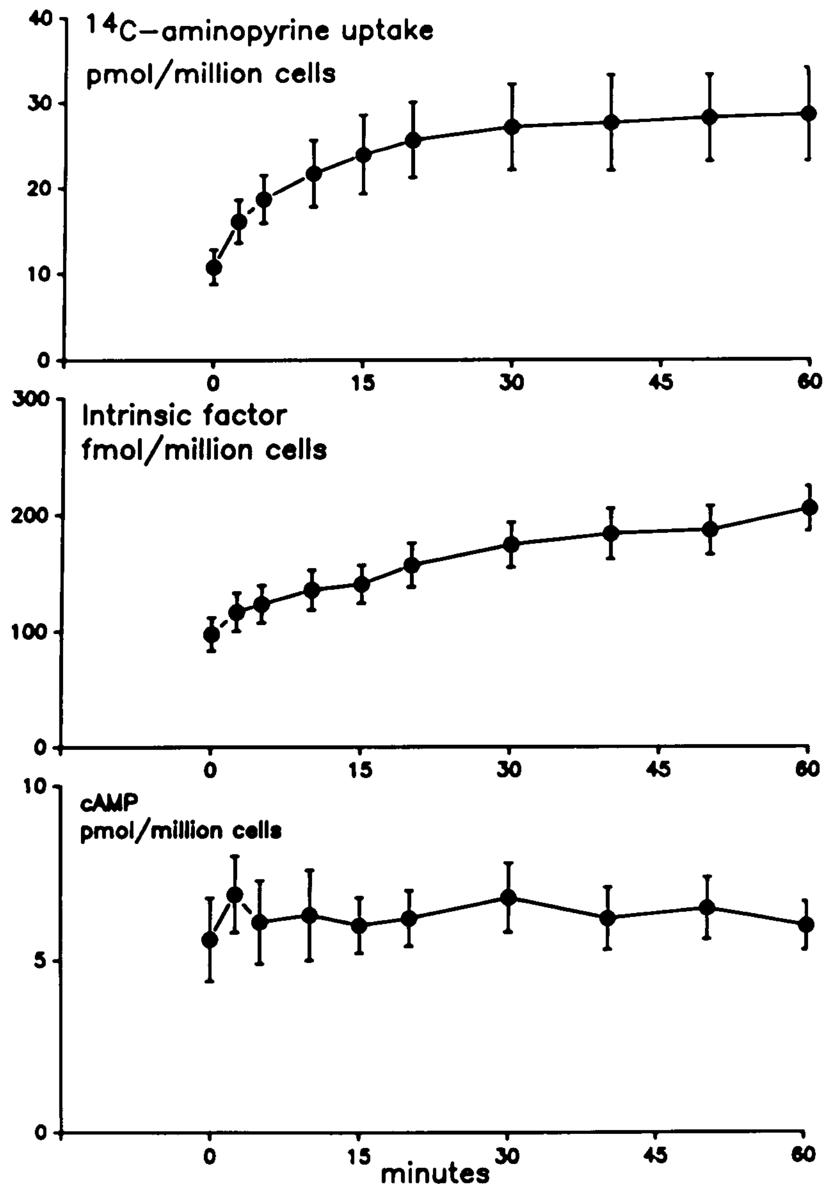

Figure 5. Time course effect of carbamylcholine (carbachol) stimulation of isolated rabbit parietal cells on: top panel: [^{14}C]-aminopyrine uptake, middle panel: intrinsic factor secretion and bottom panel: cellular cAMP content. Graphs show mean and SEM from 6 separate experiments.

DISCUSSION

The micro-method of incubation for rabbit isolated parietal cells has proven to be simple, reliable and very flexible. Using four incubation chambers and two shaking water baths it is possible to process nearly 400 replicates (4 x 96) simultaneously from a single rabbit stomach. This enables the investigator to undertake very complex experiments with several dose response curves or multiple time points. Secretagogue can be added to multiple wells simultaneously using a multiple channel micropipette thus making time course study particularly fast. The method is not subject to the large variability seen with conventional flask incubations. This is evidenced by the small coefficients of variation within and between groups. Replicates within a study are represented by different wells in the microtiter plate and are, therefore, "true" replicates as opposed to serial samples taken from a single incubation flask. Indeed the conditions within each well remain identical, except for dose of secretagogue (dose responses), or time of contact with agent (time courses). The cells are always incubated for the same total period and the incubation volume does not change, as it does with sequential sampling. Above all the method is quick and simple. With practice and automatic pipettes, a whole plate can be set up in 4-5 minutes.

Another micro-method for the study of oxyntic cell function has previously been described using isolated gastric glands from human gastroscopic biopsies [12]. This method utilizes microtubes for the incubation of gastric glands and thus overcomes the problem of individual replicates [12]. The microtube method still has only a limited oxygen supply compared with continuous oxygenation in the microtiter plate chamber. In addition, if this method were applied to handling several hundred replicates, in individual small tubes, it would prove to be rather cumbersome.

The time course of histamine stimulation shows a sharp rise in cellular cAMP concentrations which is maintained throughout the 2 hour time course. This is accompanied by a marked increase in both aminopyrine uptake and intrinsic factor secretion which occurs in linear fashion throughout the two hour incubation period. In contrast the secretory response to carbachol is transient and less pronounced. Aminopyrine uptake and intrinsic factor secretion during carbachol stimulation increase rapidly during the first 5 minutes, and then plateau by 30 minutes. Basal aminopyrine uptake measured in cells incubated for 30, 60 and 120 minutes showed no significant change (data not shown). As would be expected with a secretagogue acting via a calcium-dependent mechanism there was no change in cellular cAMP content during carbachol stimulation.

These observations in cells incubated in the microtiter plate chamber are similar to the results obtained in previous studies with the exception of the time course for both

aminopyrine uptake and intrinsic factor secretion stimulated by histamine. The histamine time course is rather different from that described using previous methods [1,8,13,14]. Other studies, including our own employing flask incubation methods, have demonstrated aminopyrine uptake in response to histamine over a period of only 30 or 40 minutes. Nevertheless, a similar linear time course of aminopyrine uptake has been seen recently in cultured rabbit parietal cells [15]. The only major difference between the present work and previous studies lies in the incubation method. Previous investigators have demonstrated that aminopyrine uptake is independent of the media aminopyrine concentration [16]. Similarly, the results are not biased by our expression of the data as pmol aminopyrine accumulated rather than ratios. The expression of the data as a ratio was based on the need to adjust the data to consider the intracellular volume in gastric glands [3]. In the case of the data presented here, where a fixed number of isolated cells are added to each well in each experiment, the correction for intracellular volume becomes a constant [1,7]. Calculated mean aminopyrine ratios at 60 and 120 min of stimulation with histamine were 150 and 300, respectively. If the parietal cell acid space is assumed to be 10% of the cell volume, this would yield an approximate acid space pH of 3 [3]. The representation of the data was unchanged when ratios were used and so we have represented the data as accumulation into a specific number of cells for ease of calculation. In any case, the continued release of intrinsic factor into the media throughout the two hours of histamine stimulation suggests that secretion from parietal cell continued throughout the period. Intrinsic factor release was not due to dying cells, since no significant release was observed in unstimulated cells. Indeed, total intrinsic factor content was unchanged throughout the two hour incubation (Adrian, et al., Unpublished Results). Finally, the results do not represent an artifact encountered through the absence of a bicarbonate buffer system, since similar patterns of histamine stimulated aminopyrine accumulation and intrinsic factor secretion were obtained using a bicarbonate buffer system and 95% O_2/5% CO_2 as the chamber gassing medium (Lewis and Goldenring, Unpublished Results).

The lower lactate concentration in cells incubated in the microtiter plate chamber compared to those incubated in flasks with intermittent oxygenation suggests that the chamber maintains the oxidative respiratory status of the cells during long incubations. The linear course of aminopyrine accumulation and intrinsic factor release stimulated by histamine may therefore reflect an improved ability of the cell to continue secretory activity. The parietal cell is extremely rich in mitochondria, and on stimulation, oxygen consumption is increased in direct proportion to the rate of acid secretion [7, 17]. The absence of sustained secretion seen in other models could reflect inadequate oxygen tension resulting in a reduction of secretion with time. The increased levels of lactate seen in

cells incubated in flasks as opposed to the microtiter plate chamber suggest that the chamber may improve the level of aerobic respiration in these cells.

The results demonstrate that the present method easily allows accurate simultaneous determination of cellular accumulation, secretory products and intracellular messengers. The method should also find broader application to other second messenger measurements including inositol trisphosphate levels and ^{45}Ca flux. The time course experiments demonstrate the capability of this new method of studying secretion from the isolated rabbit parietal cell. The versatility and ease of handling large numbers of replicates makes this technique valuable for the investigation of a wide range of tissues and cell preparations.

SUMMARY

A new micro-method for investigating secretory physiology in isolated cells was developed and evaluated. The method utilized a specially designed polycarbonate incubation chamber to provide constant oxygenation to cells incubating in a 96 well microtiter plate. Cells were rapidly separated from media by vacuum filtration. Isolated parietal cells wee utilized to demonstrate the versatility of the method for assay of intracellular accumulation of [^{14}C]-aminopyrine, secretion of intrinsic factor into the medium as well as assay of intracellular cAMP. Histamine stimulated the uptake of [^{14}C]-aminopyrine and intrinsic factor secretion in a sustained and linear fashion. At the end of the two hour period uptake of aminopyrine and secretion of intrinsic factor were increased 17 and 5-fold, respectively. This response to histamine was accompanied by a rapid and sustained 3-fold rise in intracellular cyclic AMP. In contrast, carbamylcholine caused a transient increase in [^{14}C]-aminopyrine accumulation and intrinsic factor secretion which was most pronounced during the first 10 minutes and had almost ceased by 30 minutes. Carbamylcholine had no effect on intracellular cAMP levels. This method, which can handle 400 replicates using parietal cells from the fundic mucosa of a single rabbit, is suitable for studying the time course of intracellular events which accompany general secretory processes.

REFERENCES

1. Soll, A. H. (1980) Secretagogue stimulation of ^{14}C-aminopyrine accumulation by isolated canine parietal cells. Am. J. Physiol. 238, G366-375.

2. Berglindh, T. (1977) Effects of common inhibitors of gastric secretion on secretagogue induced respiration and aminopyrine accumulation in isolated gastric glands. Biochim. Biophs. Acta. 464, 217-233.

3. Berglindh, T., Helander, H. F., and Obrink, K. J. (1976) Effects of secretogogues on oxygen consumption, aminopyrine accumulation and morphology in isolated gastric glands. Acta Physiol. Scand. 97, 401-414.

4. Kapadia, C. R., Shafer, D. E., Donaldson, R. M. and Ebersole, E. R. (1979) Evidence for th involvement of cyclic nucleotides in intrinsic factor secretion by isolated rabbit gastric mucosa. J. Clin. Invest. 63, 1044-1049, 1979.

5. Adrian, T. E., Oddsdottir, M., Zdon, M. J., Zucker, K. A., Goldenring, J. R. and Modlin, I. M. (1987) Sustained increase in intracellular cAMP and secretion in histamine stimulated isolated parietal cells. Gastroenterology. 92, 1288.

6. Berglindh, T. and Obrink, K. J. (1976) Acta Physiol. Scand. A method for preparing isolated glands from the rabbit gastric mucosa. 96, 150-159.

7. Soll, A. H. (1978) Extracellular calcium and cholinergic stimulation of isolated canine parietal cells. J. Clin. Invest. 61, 370-380, 1978.

8. Zdon M. J., Ballantyne, G. H., Schafer, D. E., Tyshkov, M., Cambria, R. P. and Modlin, I. M. (1986) Evidence for non-gastrin mediated somatostatin inhibition of parietal cell function. Surgery 100, 954-961, 1986.

9. Schon, R. (1965) A simple and sensitive enzymatic assay for determination of L(+)-lactic acid. Anal. Biochem. 12, 413-416.

10. Tovey, K. C., Oldham, K. G. and Whelan, J. A. M. (1974) A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. Clinica Chimica Acta. 56, 221-234.

11. Gottlieb, C., Kam-Seng, L., Wasserman, L. R. and Herbert, V. (1965) Rapid charcoal assay for intrinsic factor, gastric juice unsaturated B_{12} binding capacity, antibody to IF and serum unsaturated B_{12} binding capacity. Blood. 25, 875-884.

12. Fellenius, E., Elander, B., Wallmark, B., Hagland, H., Helander , H. and Olbe, L. (1983) A micro-method for the study of acid secretory function in isolated oxyntic glands from gastroscopic biopsies. Clin. Sci. 64, 423-431.

13. Chew, C. S., Hersey, S. J., Sachs, G. and Berglindh, T. (1980) Adenylate cyclase stimulation by histamine is coupled to acid secretion. Am. J. Physiol. 238, G312-G320.

14. Soll, A. H. and Wollin, A. (1979) Histamine and cyclic AMP in isolated canine parietal cells. Am. J. Physiol. 237, E444-E450.

15. Chew, C. S., Ljungstrom, M., Smolka, A. and Brown, M. R. (1989) Primary culture of secretagogue-responsive parietal cells from rabbit gastric mucosa. Am. J. Physiol. 256, G254-G263.

16. Sack, J. and Spenney, J. G. (1982) Aminopyrine accumulation by mammalian gastric glands: an analysis of the technique. Am. J. Physiol. 243, G313-G319.

17. Holian, O., Ruiz, C., Bombeck, C. T. and Nyhus, L. M. (1983) Comparison of cyclic AMP systems in parietal cells from rat and guinea pig. Scand. J. Gastroenterol. 18, 819-824.

CHAPTER 9

Isolated Gastric Glands in the Study of Gastric Secretory Relationships

INTRODUCTION

The physiological and medical importance of gastric secretion and its regulation needs no explanation. The subject has long occupied a prominent position in surgical research. In the past, particular attention was paid to whole-animal models. Studies in intact animals have an understandable appeal because they seem to approximate "real" situations. At the same time, the results of experiments on whole-animal models have often been difficult to interpret, since causes and their effects may both be obscured in such experiments. For example, the actions of stimulatory or inhibitory agents may be profoundly altered by interference from vagal or sympathetic neural influences, by the release in vivo of gastrointestinal hormones, or by paracrine modulatory actions. Similarly, quantitative estimation of the products of gastric secretion is complicated in some whole-animal preparations by such problems as partial retention of luminal contents, admixture of extraneous secretions, including mucus, bile, and pancreatic juice, and mucosal ulceration and even bleeding. There is an important place in gastric secretory research, therefore, for an in vitro mucosal preparation that retains its normal secretory functions and relationships yet is free of many of the complications encountered in whole-animal studies. Intact mucosal preparations have been utilized for in vitro studies of gastric secretion. Isolated sheets of gastric mucosa mounted in Ussing chambers [1] have been particularly useful for studies over time of transmembrane potential differences, ionic currents under both open- and short-circuit conditions, and unidirectional transmembrane isotopic fluxes. The Ussing chamber requires a separate apparatus for each membrane, and a relatively limited number of membranes can be obtained from a single animal. Gastric mucosal biopsies incubated in organ culture [2,3] are well suited for long-term (24-hr) studies of biosynthetic and secretory activity. For investigation of net secretion of macromolecules on a short time scale, however, intact mucosal preparations are unsatisfactory. Adjacent areas of gastric mucosa typically display appreciable differences in secretory function, and intact mucosal preparations tend to reflect this variability. Some responses are considerably slower in intact mucosal preparations than in isolated cells and glands. Intrinsic mucosal reflexes may play a role in modulating responses to some agents.

Preparations of isolated parietal cells [4,5] and isolated chief cells [6] appear to retain many of the functional characteristics these cells display in vivo. The validity of the isolated chief cell technique is currently being independently confirmed in my laboratory. The process by which the cells are isolated is somewhat more time-consuming, complex, and stressful to the cells than the preparation of isolated glands, and cumulatively increase the likelihood that functional artifacts may occur. Another potential disadvantage of cell isolation procedures, whose importanceis

difficult to estimate at this time, is the loss of spatial relationships between secretory cells, between cells and secretory products, and between secretory products in the lumen of the gastric gland.

Some investigators have suggested the use of isolated gastric glands for research on gastric secretory function, and have shown that such glands can respond to a variety of stimuli [7,8,9]. From my point of view a major advantage of the isolated glands is the fact that they may be regarded as the secretory unit of the gastric fundus, the minimum structural entity for secretory interactions of chief and parietal cells. Thus, the preparation is well suited for either comparative studies of intrinsic factor and acid secretion by the parietal cell or pepsinogen secretion by the chief cell.

A basic requirement for studies of secretory interactions in vitro is that individual cell types in the preparation respond appropriately to individual physiological secretagogues. I have therefore examined secretory effects in the isolated gastric gland preparation of three classic stimuli for gastric acid secretion [4], which are a paracrine substance, a parasympathetic transmitter, and a peptide hormone, respectively: histamine, acting as an H2 agonist; carbachol (carbamoylcholine), a long-acting analog of acetylcholine, acting as a muscarinic agonist; and pentagastrin (G-5), a long-acting gastrin analog. In addition, I have examined the effects of an active fragment of cholecystokinin, cholecystokinin octapeptide (CCK-8), and the beta-adrenergic agonist isoproterenol, both of which have been implicated in pepsinogen secretion [10,11,12]. Finally, I have used the cAMP-mimetic analog 8-Br cAMP, since the effects of histamine on parietal cells and those of isoproterenol on chief cells are both believed to be mediated by cAMP [13,14].

I have measured effects of these secretagogues on principal parameters of parietal cell and chief cell secretion in isolated gastric gland suspensions. Parietal cell secretory function was tested by measurement of intrinsic factor (IF) secretion, with separate and combined studies of ^{14}C-aminopyrine accumulation, a measure of acid secretion. Changes in overall metabolic activity were tested by measurement of oxygen consumption. Chief cell secretory function was tested by measurement of pepsinogen (PPG) secretion. Chief cell secretion and parietal cell secretion were examined separately and in combination.

METHODS

Studies Performed

Dose-response curves were obtained for the release of intrinsic factor (IF) into the medium. For this purpose, IF secretion was measured in experiments of 30 min duration as a function of secretagogue concentration over a broad range. The time course of IF secretion between 5 and 60 min was also observed in response to histamine and 8-Br cAMP at appropriate concentrations. The time courses of ^{14}C-aminopyrine accumulation and oxygen consumption were similarly observed under comparable conditions. Dose-response curves for the release of pepsinogen (PPG) into the medium were obtained over a broad range of secretagogue concentrations in experiments of 30 min duration.

Gland Preparation

Non-fasted New Zealand white rabbits weighing 3.5-4.0 kg were anesthetized with sodium pentobarbital (Abbott), 40-45 mg/kg, via a lateral ear vein, and midline laparotomy was performed. The abdominal aorta was isolated and ligated distally, and a cardiac venous catheter was inserted in retrograde fashion infrarenally. Sodium heparin, 500 mg/kg, was administered through the catheter and allowed to circulate for 1-3 min. The animal was bled, a left thoracotomy was performed, and the thoracic aorta was clamped. The superior mesenteric artery was ligated, isolating the celiac axis. The portal vein was incised for rapid drainage. To expand mucosal connective tissue spaces and thus facilitate subsequent enzymatic digestion [7], warmed (37°C) and oxygenated colloid-free phosphate-buffered saline (PBS: 125 mM NaCl, 0.64 mM NaH_2PO_4, and 3.0 mM K_2HPO_4, pH 7.4) was then infused via the aortic cannula under 600 mm Hg pressure, with a cardiac roller pump (Travenol).

Following perfusion, the stomach was removed and opened along the lesser curvature. The cardia and antrum were discarded. The fundus was rinsed in PBS and the mucosa bluntly stripped off with a scalpel handle and minced into small pieces with fine dissecting scissors. The wet weight of the edematous fundic mucosa was 14-18 gm. The mucosa was placed in a balanced salt solution containing 12.0 mM $NaHCO_3$, 1 mg/ml collagenase (Sigma type I), 1 mg/ml rabbit or bovine serum albumin (Sigma), and 2 mg/ml glucose, and incubated at 37°C with 100 percent O_2 for 30 min in a shaker bath.

After incubation, glands were gravity filtered through decreasing sizes of Nylon mesh to a final size of 300 um. The resulting gland suspension was washed three times with 1 x gravity sedimentation and resuspended in fresh incubation medium ($NaCl_2$, 134 mM; KCl, 5.4 mM; Na_2HPO_4, 5.0 mM; NaH_2PO_4, 1.0 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 1.0 mM; phenol red, 10 mg/L; bovine serum albumin, 2 mg/ml; and glucose, 2 mg/ml, pH 7.4).

Trowell's T-8 medium, with 2 mg/ml each bovine serum albumin and glucose, was used in some experiments with no difference in the results.

Freshly isolated perfused fundic mucosa, mucosa digested in collagenase medium for 15 or 30 min, and washed glands were also fixed at 37° and 4°C in phosphate- or cacodylate-buffered glutaraldehyde, washed, and, after appropriatehistological preparation, examined by optical microscopy and by scanning and transmission electron microscopy. The ability of gland cells to exclude Trypan Blue was assessed by mixing equal volumes of gland suspension and Trypan Blue, 400 mg/100 ml in PBS [7]. The ability of glands to synthesize and retain cAMP was tested by incubation with forskolin 10^{-5} M [15] for 30 min, boiling to terminate the incubation, and measurement of cAMP content in the supernatant by a commercial radioimmunoassay.

Secretion Studies

Secretion of IF or PPG was assessed after incubation of gland suspensions for various times in incubation medium containing the secretagogue. IF or PPG was measured in the medium. In a typical experiment 1.8-ml quadruplicate aliquots of gland suspension were added to 0.2 ml of medium, or medium with added secretagogue, and incubated for 0-60 min at 37°C with 100% O_2. Following this incubation, glands and supernatants were separated by filtration (Millipore Type AP40 fiberglass filters) or centrifugation in a Sorvall centrifuge (Dupont Instruments, Newtown, CT) at 10,000 x g for 1 min. In each study initial and final unincubated controls and incubated but unstimulated control tubes (medium alone) were routinely included.

Intrinsic Factor Secretion

IF secretion into the medium was determined by assessing the binding of ^{57}Co-labeled cyanocobalamin (^{57}Co-CBL) in the filtrate or supernatant, as follows: 500 ul of sample was allowed to react for 40 min with 0.5 ml of ^{57}Co-CBL. Bound label was then separated from free label by the addition of hemoglobin-coated charcoal suspension, which binds free CBL but not the IF-CBL complex. The suspension was centrifuged for 15 min at 3000 rpm in a Sorvall centrifuge, and samples of supernatant were counted in a Packard Instruments gamma counter (United Technologies, Downers Grove, IL). IF secretion was expressed as the amount of bound ^{57}Co-CBL radioactivity (counts per minute) of stimulated samples, $\pm$ SEM. Net secretion was defined as the difference between the means for stimulated and unstimulated samples. The significance of the differences between means of replicate runs was determined by Student's unpaired t-test, with $p < 0.05$.

^{14}C-aminopyrine Accumulation

Secretion of acid by parietal cells was measured by ^{14}C-aminopyrine (^{14}C-AP) accumulation under basal conditions and in response to stimulation by the appropriate agents. The procedure has been described in detail elsewhere [8]. Identical aliquots of gland suspension were preincubated in two flasks with dimethylamine-^{14}C-aminopyrine(Amersham or New England Nuclear), 0.1 uCi/ml, for 30 min with 100 percent O_2 at 37°C in ashaker bath. At zero time, 4 ml of incubation medium containing an appropriate concentration of stimulating agent was added to the labeled gland suspension in the experimental flask, and an equal volume of medium alone was added to that in the control flask. At 5, 15, 30, 45, and 60 min thereafter, 1.5 ml aliquots were withdrawn in quadruplicate and centrifuged at 10,000 x g for 1 min.

The supernatants were withdrawn, and two 0.5-ml aliquots were taken for determination of ^{14}C-AP. The surface of the pellet was washed in fresh medium to remove tracer in residual supernatant. The pellet was then dissolved in 250 ul concentrated nitric acid for 10 min at 50 degrees C and diluted to 550 ul with H_2O. 500-ul samples of dissolved pellet and corresponding supernatant were counted for 1 min in a Beckman LS7000 scintillation counter (Beckman Instruments, Fullerton, CA) with automatic quench correction. The ratio of ^{14}C-AP in the pellet to that in the supernatant was calculated. Results are expressed as percent increase, $\pm$ 1SEM, in the ^{14}C-AP ratio for stimulated glands above that for unstimulated glands. The significance of the difference between means of replicate runs was determined by Student's unpaired t-test, with $p < 0.05$.

Oxygen Consumption

The consumption of oxygen by unstimulated and stimulated glands was measured over time using a Clark-type polarographic electrode system (Model 53 Biological Oxygen Monitor, Yellow Springs Instrument Co., Yellow Springs, OH) precalibrated with air-saturated water. One ml of either incubation medium or incubation medium plus secretagogue was placed in the standard sample chamber at 37°C for 3 min. Following this, 3 ml of gland suspension which had been oxygenated with 100% O_2 for 2 min was added to each chamber and excess gas was expelled. The percent saturation of O_2 was recorded initially and at 5 min intervals for a period of 1 hr or until no measurable O_2 was present, whichever was less. The change in percent saturation in each sample was calculated over time, and the oxygen consumed in ul/min was calculated, based on 25 ul of O_2 in a 100% O_2-saturated incubation medium.

PPG in Glands and Medium

PPG in the medium was assayed by an acid protease method in

which a fixed aliquot of medium is incubated with hemoglobin solution at pH 2.0 for a fixed time interval; the enzyme reaction is terminated by adding trichloroacetic acid [16]. The hemoglobin reagent contained 20 mg/ml hemoglobin titrated with HCl to a final pH of 2.0. After a preliminary 10-min activation with 0.3 ml of 0.04 N HCl, 100 ul of medium was incubated with 0.8 ml of hemoglobin reagent for 20 min at 37°C. Undigested proteins were precipitated with 1.8 ml of 5% trichloroacetic acid and allowed to react for 10 min. Thecontents were filtered through Whatman No. 4 filter paper. The extent of hemoglobin digestion was assessed in 1.0 ml of filtrate by adding 5.0 ml of 0.2 N NaOH and, after 10 min, 0.5 ml pf 1 N phenol (Folin-Ciocalteu) reagent. Spectrophotometry was performed at 760 nm after 30 min. PPG activity was obtained in terms of peptic units (PU) released per 2-ml sample, 1 PU corresponding to the amount of enzyme yielding an absorbance change of 0.001 in one min under the assay conditions. The significance of differences between means of replicate runs was determined by Student's paired or unpaired t-test, as appropriate, with $p < 0.05$.

Graphical Representation

The data were depicted graphically by means of an IBM PC/XT equipped with Lotus Symphony software and a Hewlett-Packard 7475A plotter. For uniform representation all IF, ^{14}C-aminopyrine, and PPG data were expressed as percent of the maximum stimulation observed in the experiment, i.e., defining the (minimum) unstimulated value as 0 and the maximum stimulated value as 100. For clarity, individual standard errors < 5% were not displayed. For curves containing points with 1 SEM > 5%, the program displayed a complete envelope representing ± 1 SEM.

RESULTS

Characteristics of Isolated Gland Preparation

A single rabbit stomach typically yielded about 1 g wet weight of glands, enough for 200 or more incubations at a dilution of 70,000-90,000 glands per ml. This dilution was appropriate for the experiments described above, on the basis of the sensitivity of the assay methods used.

Examination of perfused fundic mucosa, mucosa digested for 15 min, and washed glands by scanning electron microscopy (Fig. 1) showed the glands initially imbedded in the fibrous connective-tissue matrix in a uniform parallel array (Fig. 1A). After collagenase incubation for 15 min (Fig. 1B), collagen digestion was far advanced at the serosal ends of the glands, where most of them had become separated. The mucosal ends of many glands were still attached to the mucosal surface, while other glands had broken loose at the neck. In the final suspension, the filtered and washed glands had lost their connective-tissue covering but retained their

essentially tubular morphology. A portion of a gland is shown at high magnification in Fig. 1C. Two morphologically different outer cell surfaces, one smooth and the other covered with microvilli, were distinguishable at this stage.

On the basis of their ability to exclude Trypan Blue at least 95% of the gland cells were viable. On stimulation of their cellular adenylate cyclase systems for 30 min with 10^{-5} M forskolin, the glands responded with a marked increase in cAMP content. The ratio of forskolin-stimulated to unstimulated cAMP levels ranged from 6.0 ± 0.3 to 36.9 ± 3.6 in different experiments. A useful index of functional cell integrity in gland experiments was the ratio of total stimulated release of IF or PPG to the unstimulated release of that protein. Loss of secretory response or significant cell damage would be expected to reduce this ratio toward unity. In representative experiments reported below, the maximum secretion in 30 min was approximately 26 times the unstimulated release in the case of IF, and 11 times the unstimulated release in the case of PPG.

Stimulation of IF Secretion

Histamine stimulated parietal cells of isolated gastric glands to secrete IF, as measured by comparing IF release into the medium, during a 30-min period, in the absence of histamine and in response to histamine at concentrations from 10^{-6} M to 10^{-3} M (Fig. 2). At 10^{-6} M, histamine caused a small but significant increase in IF release over unstimulated levels ($p < 0.05$). The mean IF secretory responses at 10^{-5} M and 10^{-4} M were $62 \pm 22\%$ and $84 \pm 7\%$, respectively, of the maximum response obtained, at 10^{-3} M.

The results in Fig. 2 are expressed as percentages of maximal stimulation, as are most of the results in this chapter section. To put such percentage figures in perspective, in the experiments with histamine cited above the ratio of maximal secretion to unstimulated secretion was 26.3 ± 2.5; that is, the magnitude of the unstimulated response (not shown) was only 3.8% of the total secretion in response to histamine, and histamine stimulated secretion by 2,500%. IF secretion in response to other secretagogues is described below.

Time Course of Histamine Effect on IF Secretion

When IF release by isolated gastric glands was sampled sequentially, at 5, 15, 30, 45, and 60 min, in the presence and absence of 10^{-5} M histamine (Fig. 3), during the course of the observations IF levels in unstimulated glands did not change significantly ($p > 0.05$). In the presence of histamine, on the other hand, IF secretion into the medium at 5 min was already $9 \pm 1\%$ of the maximum (i.e., 60-min) level. This value was significantly different ($p < 0.05$) from the unstimulated level of $0.0 \pm 0.5\%$. Thereafter,

histamine-stimulated IF secretion rose almost linearly by significant increments ($p < 0.05$) over each successive interval. The increase between 5 and 15 min was slightly greater than for other intervals. The 15-min value was 34 $\pm$ 1% of the 60-min period, yielding a calculated secretion rate 50% greater than the overall rate for 60 min.

Effects of Histamine on the Accumulation of ^{14}C-aminopyrine

In the same experiment (Fig. 3) histamine also stimulated ^{14}C-aminopyrine accumulation in glands, expressed as the ratio of ^{14}C in glands to ^{14}C in medium. In unstimulated glands, accumulation of ^{14}C-aminopyrine did not change significantly during the observation period from 5 min to 60 min ($p > 0.05$). By the time of the initial 5-min measurement, accumulation of the label by stimulated glands had attained a value equal to 47 $\pm$ 10% of the 60-min level, significantly greater ($p < 0.05$) than the value of 0.0 $\pm$ 0.6% for unstimulated glands. At this point the aminopyrine ratio for stimulated glands was 2.3 times the ratio for unstimulated glands. The difference continued to increase until 30 min, when the ^{14}C-aminopyrine level in stimulated glands became stable, reaching 97 $\pm$ 10% of the 60-min maximum. This value corresponds to an aminopyrine ratio for stimulated glands equal to 3.6 times the ratio for unstimulated glands.

Concurrent Effects of 8-Br cAMP on the Secretion of IF and the Accumulation of ^{14}C-aminopyrine

The cAMP-mimetic analog 8-Br cAMP also stimulated IF secretion, with a maximal response at 10^{-4} M. When glands stimulated with 8-Br cAMP at this concentration were sampled sequentially from 5 min to 60 min, IF secretion displayed a time course (Fig. 4) comparable to that for 10^{-5} M histamine stimulation (Fig. 3). At 5 min, IF secretion was 0.8 $\pm$ 0.1% of maximum for the stimulated glands and 0.0 $\pm$ 0.1% for the unstimulated glands, a small but significant difference ($p < 0.05$). At each later time period, IF secretion increased almost linearly in response to 8-Br cAMP stimulation, by significant increments ($p < 0.05$).

In the same experiment, 8-Br cAMP stimulated ^{14}C-aminopyrine accumulation significantly over the basal level ($p < 0.05$) at all times studied, from 5 to 60 min. After 5 min, for example, the ^{14}C ratio for stimulated glands was 9.7 $\pm$ 0.2% of the 60-min maximum, compared with 0.0 $\pm$ 0.3% for unstimulated glands ($p < 0.05$). After 45 min the stimulated ratio was 83 $\pm$ 5% of maximum, and there was no significant further increase in ^{14}C-AP accumulation ($p > 0.05$).

Stimulation of Oxygen Consumption by Histamine

The time course of the effect of histamine (10^{-5} M) on O_2 consumption by isolated gastric glands at 5-min intervals over60 min is shown in Fig. 5. Initially all chambers were

42% saturated with O_2. The O_2 consumption rate (slope of the curve) decreased slightly with time. The difference between stimulated and unstimulated O_2 consumption was significant at all time points from 5 min on ($p < 0.05$).

CCK-8 Stimulation of PPG and IF Secretion

The peptide CCK-8 stimulated the secretion of both IF and PPG. Parallel dose-response curves were obtained for the simultaneous secretion of IF and PPG by isolated glands in response to CCK-8 at concentrations from 10^{-11} to 10^{-7} M (Fig. 6). At 10^{-11} M and 10^{-10} M, CCK-8 did not significantly stimulate secretion of IF or PPG over the basal levels of 0 $\pm$ 5% and 0 $\pm$ 4%, respectively ($p > 0.05$). At 10^{-9} M and higher concentrations, CCK-8 significantly stimulated secretion of both macromolecules ($p < 0.05$). At 10^{-9} M CCK-8, for example, the release of IF was 55 $\pm$ 10% of maximal, while PPG release was 31 $\pm$ 1% of maximal. A further significant increase was observed between 10^{-9} M and 10^{-8} M CCK-8 ($p < 0.05$). The results of experiments with CCK-8 stimulation of aminopyrine accumulation are given below (Discussion).

Effects of Carbachol and Isoproterenol on PPG Secretion

Stimulation of PPG secretion by isoproterenol, 10^{-6} M, by carbachol at concentrations ranging from 3 x 10^{-7} M to 10^{-4} M, and by combinations of these is shown in Fig. 7, where the results are expressed as percentages of the maximum stimulation obtained with carbachol alone. PPG secretion was significantly increased at all concentrations of carbachol from 10^{-6} M to 10^{-4} M ($p < 0.05$). The maximum secretion obtained with carbachol alone was 6.3 $\pm$ 2.0 times the unstimulated secretion. At 10^{-6} M, carbachol increased secretion from the basal level of 0 $\pm$ 1% of maximal to 25 $\pm$ 2% ($p < 0.05$). Isoproterenol alone at the submaximal concentration of 10^{-6} M caused a net PPG release of 81 $\pm$ 3% of the maximum obtained with carbachol (100 $\pm$ 11%), significantly less than the latter ($p < 0.05$). The combination of isoproterenol, 10^{-6} M, with carbachol significantly increased PPG secretion at every concentration of carbachol ($p < 0.05$). The combined action of 10^{-4} M carbachol and 10^{-6} M isoproterenol stimulated PPG secretion equal to 169 $\pm$ 6% of the maximum obtained with 10^{-4} M carbachol alone. The combined response was 10.8 $\pm$ 1.2 times the unstimulated secretion. PPG secretion with other secretagogues is described below (Discussion).

DISCUSSION

The results of these studies demonstrate several features of the isolated gastric gland preparation that bear important implications for the study of gastric secretory function. The experiments show that isolated rabbit gastric glands display appropriate gastric secretory responses, delineated below, when stimulated by a variety of secretagogues. The parietal

cells in these glands respond appropriately, secreting IF into the medium. They appropriately accumulate ^{14}C-aminopyrine, an indirect indication that hydrogen ions are being secreted by these cells. Oxygen consumption by the glands increases appropriately in response to stimulation. In addition, thechief cells in these glands respond appropriately by secreting PPG into the medium.

It must further be emphasized that the use of isolated gastric glands allows different parietal cell and chief cell secretory functions to be activated or inhibited simultaneously, and to be measured in the same experiment. No less significant is the fact that the essential tubular structure of the glands is preserved, as scanning electron microscopy reveals. Thus the isolated gastric gland, as the multicellular structural unit of gastric secretory function, provides a unique model for *in vitro* study of the structural and functional interrelations of gastric secretory cells and their secretory products.

IF secretion by isolated rabbit gastric glands was markedly stimulated by histamine (Figs. 2 and 3). In the rabbit IF is secreted exclusively by the parietal cells [17]. Histamine stimulation of IF secretion by rabbit gastric mucosa has been documented for mucosal biopsies in organ culture [3] and dispersed mucosal cells [5]. Thus IF secretion by isolated rabbit gastric glands represents an appropriate response by the parietal cells in these glands to histamine stimulation.

Besides 8-Br cAMP, discussed below, four other secretagogues were tested, of which three stimulated IF release to varying degrees. The muscarinic agonist carbachol and the peptide hormones CCK-8 and G-5 also stimulated IF release, but less effectively than histamine and 8-Br cAMP. Carbachol at its maximally effective concentration, 3×10^{-5} M, elicited a response that was 7.0 ± 0.6 times the unstimulated level (in other words, 600% over unstimulated), or 27 ± 5% of the maximum obtained with 10^{-3} M histamine in the same experiments. The IF secretory responses to maximally effective concentrations of CCK-8 and pentagastrin (G-5) in separate experiments were smaller still. The maximal responses to CCK-8, at 10^{-8} M, and pentagastrin (G-5), at 10^{-7} M, were only 50 ± 19% and 24 ± 6%, respectively, over the unstimulated level. These increases, while small, were nevertheless significant ($p < 0.05$). Of secretagogues tested, only the beta-adrenergic agonist isoproterenol failed to stimulate IF release at any concentration from 10^{-8} to 10^{-4} M--although isoproterenol proved to be an effective stimulus for PPG release in this concentration range (below).

With sequential sampling, IF secretion in response to histamine after 5 min and 15 min was 9% and 34%, respectively, of the 60-min total. Thus the onset of IF secretion by isolated glands in response to histamine was virtually immediate, apparently within the first minute. The rate of

secretion diminished only slightly in subsequent intervals, since the maximum secretion rate, between 5 min and 15 min, was only 50% greater than the overall rate for 60 min. A qualitatively similar time course of IF secretion in response to histamine has been reported for dispersed mucosal cells [5].

Histamine, 10^{-5} M, also vigorously stimulated ^{14}C-aminopyrine accumulation by isolated gastric glands, confirming that the parietal cells of these glands secrete acid *in vitro* in response to this classic stimulus. The onset of aminopyrine accumulation occurred rapidly. After only 5 min inthe presence of histamine, the glands contained 2.3 times as much aminopyrine as unstimulated glands.

Aminopyrine accumulation in response to histamine reached a plateau at 30 min. This steady state does not necessarily imply that proton secretion had slowed. Aminopyrine builds up in the parietal cell vesicles and gland lumen as it is trapped by secreted protons. Since the glands are an open (secreting) system, the aminopyrine level in the glands increases only until the rate of aminopyrine entrapment by secreted protons is equalled by the flow of protonated aminopyrine from the glands. The rate of aminopyrine accumulation in glands is thus a qualitative or semiquantitative reflection of the rate of proton secretion, but is not proportional to it. We have previously noted this plateau effect with CCK-8 stimulation [19].

Histamine stimulated oxygen consumption by isolated gastric glands (Fig. 5). In parallel experiments (not shown) 8-Br cAMP caused a highly significant increase in the rate of O_2 consumption, from 51 $\pm$ 6 percent per hour to 131 $\pm$ 10 percent per hour ($p < 0.05$). Since histamine stimulated parietal cells but failed to stimulate PPG secretion by chief cells, stimulation of oxygen consumption by histamine suggests that stimulation of IF and acid secretion by isolated gastric glands is accompanied by an immediate increase in aerobic metabolic activity and thus in oxygen demand. This result is consistent with the behavior of isolated gastric glands in previous studies [8,18] and parietal cells in other preparations [4]. The parietal cells are rich in mitochondria, and the minimum energy cost of the osmotic work required to concentrate protons 10^6-fold is quite large [4]. Less is known about the metabolic requirements for the biosynthesis, storage, and secretion of PPG and IF. Since 8-Br cAMP stimulated secretion by both parietal and chief cells, it is likely that a portion of the stimulation of oxygen secretion by 8-Br cAMP reflects its action on chief cells, but further studies will be required to estimate the magnitude of this contribution precisely.

The cAMP-mimetic analog 8-Br cAMP stimulated IF secretion and ^{14}C-aminopyrine accumulation (Fig. 3) as well as oxygen consumption by the glands. These results are consistent with

the interpretation that cAMP is an intracellular mediator in the pathways by which histamine, as an H2 agonist, stimulates parietal cells in gastric glands to secrete both IF and acid [13,19]. It has previouly been observed that 8-Br cAMP stimulates aminopyrine accumulation and oxygen consumption in isolated rabbit gastric glands [8] and IF secretion in isolated rabbit mucosal cells [5].

While histamine was a very effective stimulus for IF secretion, aminopyrine accumulation, and oxygen consumption, it failed to stimulate PPG secretion at any concentration tested. By contrast, the peptide hormone CCK-8 stimulated secretion of both IF and PPG by isolated gastric glands at concentrations from 10^{-9} M to 10^{-7} M (Fig. 6). Moreover, in separate experiments CCK-8, 10^{-8} M, also caused a significant increase in ^{14}C-aminopyrine accumulation over time (not shown). The ^{14}C ratio for stimulated glands was 1.6 times that for unstimulated glands, a statistically significant difference ($p < 0.05$), and this proportion increased gradually to a maximum of2.0 at 60 min, a significant increase ($p < 0.05$). Further details concerning CCK-8 stimulation of IF secretion and aminopyrine accumulation in isolated glands have been discussed in a separate chapter [20].

In addition to CCK-8, three other secretagogues stimulated PPG secretion. Isoproterenol and carbachol, when tested alone and in combination, were found to have marked stimulatory effects which were also additive (Fig. 7). The most effective stimulus for PPG secretion, in terms of the maximum response obtainable at any concentration, was the cAMP analog 8-Br cAMP. At the maximally effective concentration, 10^{-3} M, 8-Br cAMP elicited 2.7 times as much PPG secretion in 30 min as carbachol at 10^{-5} M, its maximally effective concentration. In contrast to CCK-8, another peptide of the gastrin family, G-5, failed to stimulate PPG secretion.

In addition to histamine, 8-Br cAMP, and CCK-8, IF secretion was stimulated by the muscarinic agent carbachol and the peptide hormone fragment G-5. PPG secretion was stimulated by isoproterenol, 8-Br cAMP, CCK-8, and carbachol. Whereas histamine and isoproterenol probably work through cAMP-mediated mechanisms, CCK-8, carbachol, and G-5 are not known to stimulate adenylate cyclase in parietal or chief cells, nor is there any other evidence to suggest that they act on these cells through the mediation of cAMP. The prevalent view is that these three secretagogues probably act in most cases by a calcium-dependent mechanism; I have discussed this concept elsewhere as it pertains to chief cells (Chapter IV). This idea has yet to be confirmed, but if it proves correct, then the stimulation of IF secretion and aminopyrine accumulation in isolated gastric glands can occur by two different pathways, one cAMP-dependent and the other calcium-dependent. In the former class, 8-Br cAMP and forskolin [14] stimulate both chief cells and parietal cells. In the latter class, carbachol and CCK-8 are found to

stimulate both types of cells.

Differences between parietal cell and chief cell sensitivities to various stimuli have also been observed. Histamine was an effective stimulus for parietal cells but not for chief cells. Isoproterenol showed the reverse pattern of responses, stimulating PPG secretion but not IF secretion or ^{14}C-aminopyrine accumulation. These observations support the presence of specific intact and functional receptors in the membranes of each of the different cell types within the isolated gastric gland complex. It is thus likely that parietal cells, but not chief cells, carry H2 receptors linked to adenylate cyclase, and that chief cells have beta-adrenergic receptors coupled to adenylate cyclase while parietal cells do not.

The results described above demonstrate that isolated gastric glands respond to a variety of physiological stimuli in a physiologically relevant manner. The effects may be observed under well controlled and highly reproducible conditions, in the absence of various extrinsic neural, hormonal, paracrine, and circulatory influences. Agents may be administered at concentrations varying over several orders of magnitude, up to high levels which, while physiologically relevant at the cellular level, may be difficult or impossible to achieve bysystemic administration in the intact animal. Secretory responses can be measured without the complications that sometimes arise in vivo, such as contamination with extraneous gastrointestinal secretions, like bile or pancreatic juice, or mucosal ulceration. In addition, multiple secretory parameters may be investigated under identical conditions or even in the same experiment, permitting the study of secretory interactions between parietal and chief cells, or between different parietal cell secretions, in the physiologically relevant structural context of the gastric secretory unit. Of particular interest is the opportunity to study in vitro the mechanisms of secretion in a cell system which generates both macromolecules and protons.

Thus, the isolated gastric gland preparation has clear benefits over whole-animal preparations for the study of gastric secretion, and also has specific advantages over isolated cells. I conclude that for many gastric secretion studies, especially those concerned with secretory interactions at the level of the gastric secretory unit, the isolated gastric gland preparation offers a significant practical alternative to both intact animal and isolated cell preparation. The ability to isolate a specific functional unit of the stomach composed of a number of different cells which are interrelated structurally and functionally provides a major tool in the study of gastric physiology. The clear morphological and functional interrelationship has led me to propose the term gastron for this uniquely organized arrangement of cells.

SUMMARY

In order to investigate gastric secretory processes, their interrelations, and the factors which modulate them, I have modified and developed a preparation of isolated glands of rabbit gastric fundus. The isolated gland preparation permits the separate or simultaneous estimation of multiple gastric secretory functions under highly controlled conditions in the absence of interference by extrinsic neural, hormonal, and circulatory influences. Histamine, 10^{-5} M, stimulated a four-fold increase in intrinsic factor release by parietal cells into the medium, more than doubled the accumulation of ^{14}C-aminopyrine in the glands (a measure of acid secretion), and nearly tripled overall oxygen consumption by the glands, but had no effect on pepsinogen secretion. Conversely isoproterenol, 10^{-6} M, stimulated pepsinogen secretion, but failed to show any effect on intrinsic factor secretion. An active cAMP analog, 8-Br cAMP, at 10^{-3} M, strongly stimulated all four secretory parameters tested, a result supporting the view that cAMP mediates gastric secretory stimulation by both histamine (H2) and isoproterenol (beta-adrenergic). The muscarinic agonist carbachol, 10^{-4} M, was slightly more effective in stimulating pepsinogen release than isoproterenol, 10^{-6} M, and combinations of carbachol and isoproterenol produced additive effects on pepsinogen secretion. As the multicellular structural unit of gastric secretory function ("gastron"), the isolated gastric gland retains potentially important relationships between distinct parietal and chief cell secretory processes. Thus, the isolated gastric gland preparation offers unique advantages for efforts to elucidate the physiology and pathology of gastric secretory processes.

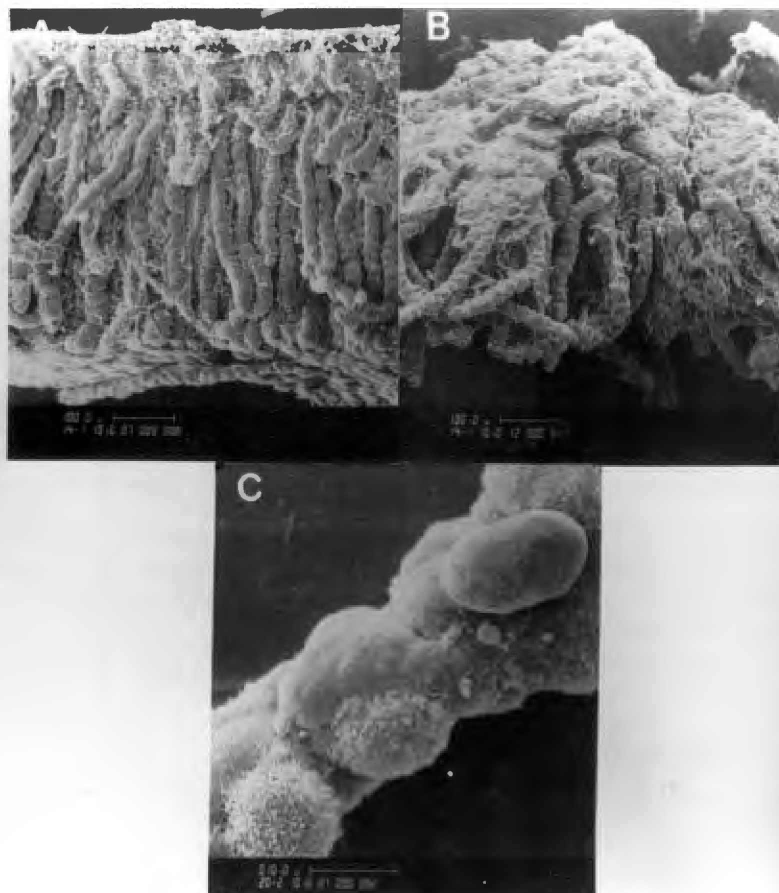

Figure 1. Scanning electron micrographs of rabbit gastric mucosa and isolated gastric glands. A--Perfused mucosa prior to collagenase digestion; view of cut edge, mucosal surface at top, showing parallel alignment of glands. B--mucosa after digestion with collagenase for 15 min; as in A, the mucosal surface is at the top, and most of the glands remain attached to the mucosal surface, but the orderly parallel arrangement of the glands is nearly lost, and in some regions separation of the glands is essentially complete. C--Portion of an isolated gland at higher magnification after digestion, filtration, and washing. Smooth and rough cell surfaces are visible. Scale markers: A and B, 100 um; C, 10 um.

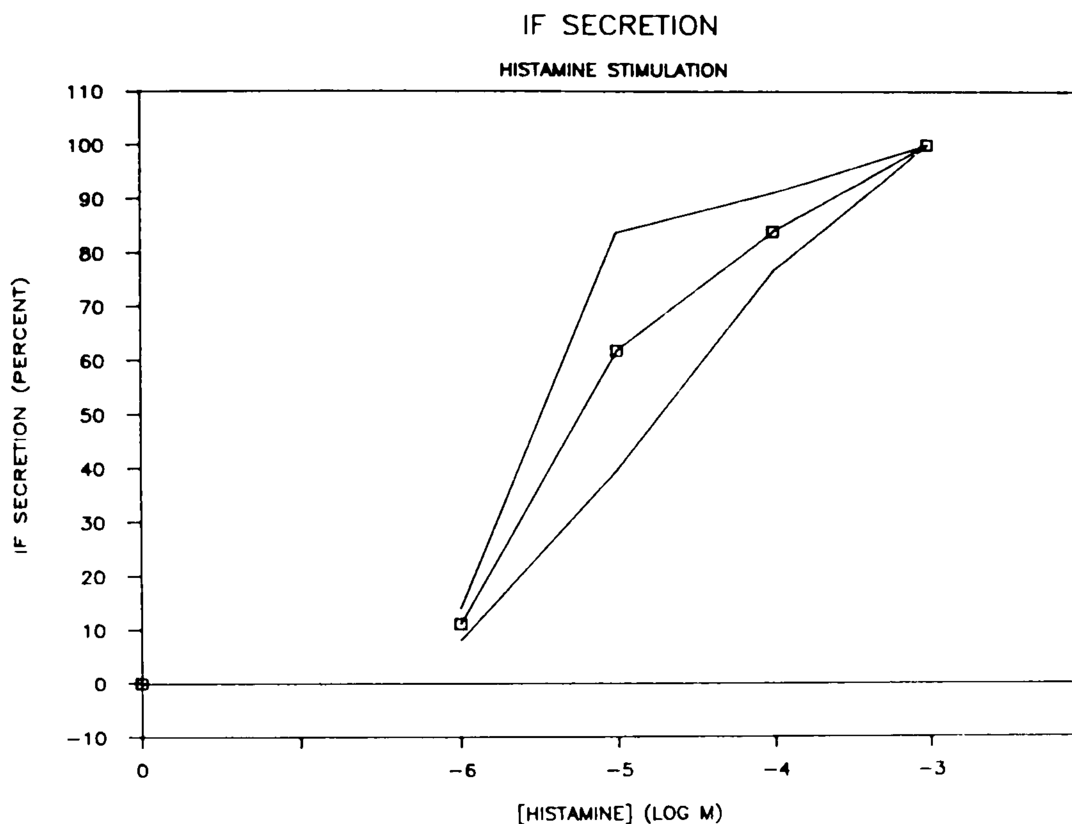

Figure 2. Concentration-response curve for histamine stimulation of IF secretion by parietal cells of isolated gastric glands. Release of IF into the medium over 30 min in the absence of histamine and in the presence of histamine at concentrations from 10^{-6}M to 10^{-3}M. Results are expressed as mean % of the maximum response obtained (response to 10^{-3}M = 100%), ± 1 SEM. Representation of standard errors in this and all subsequent graphs is explained in Graphical Representation (Methods).

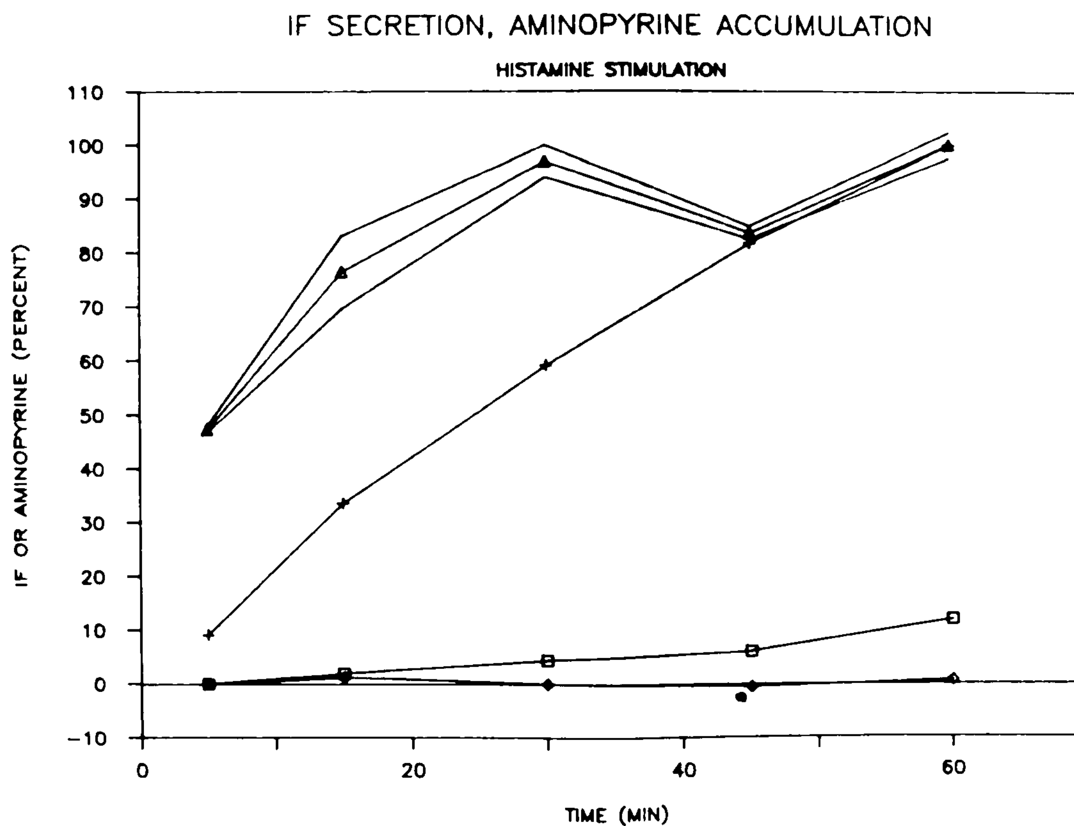

Figure 3. Time course of IF secretion and ^{14}C-aminopyrine accumulation by isolated gastric glands in the presence and absence of 10^{-5}M histamine. IF secretion, □ (unstimulated), + (stimulated); aminopyrine accumulation, ◇ (unstimulated), △ (stimulated). Results are expressed as % of the difference between the stimulated level at 60 min and the unstimulated level at 5 min.

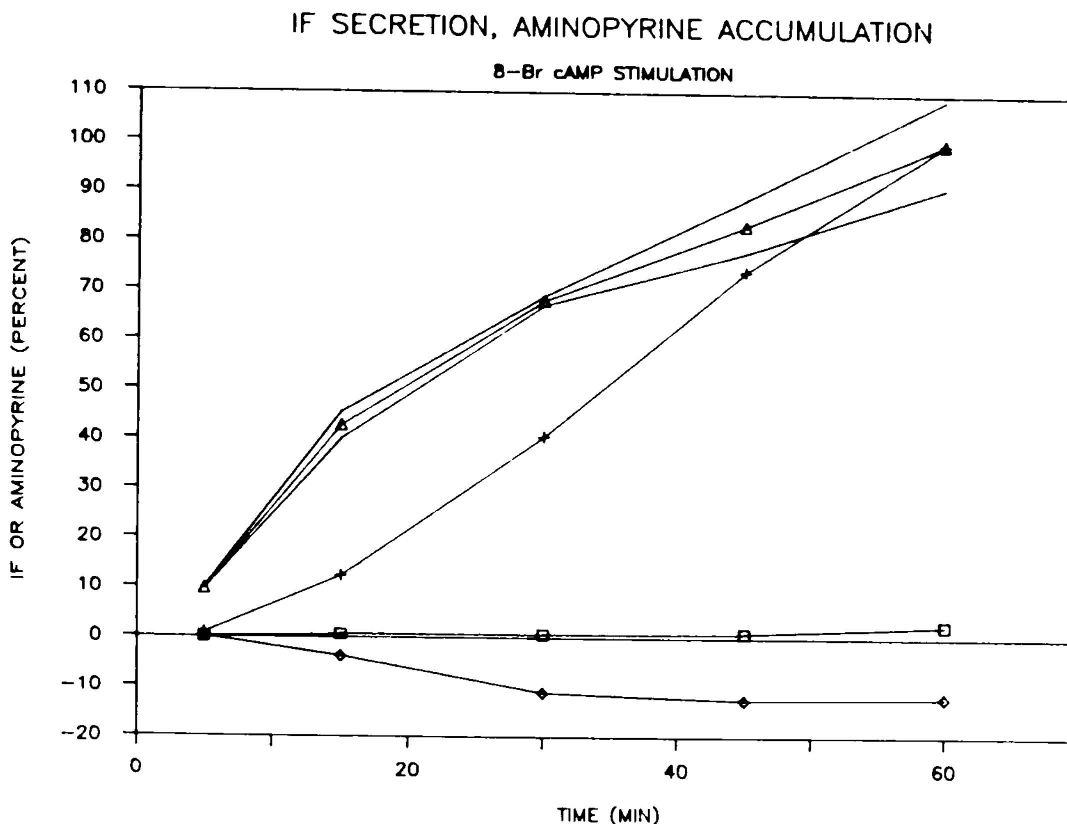

Figure 4. Time course of stimulation by 8-Br cAMP of IF secretion and ^{14}C-AP accumulation by parietal cells of isolated gastric glands, in the presence and absence of 10^{-4} M 8-Br cAMP. IF secretion, □ (unstimulated), + (stimulated); aminopyrine accumulation, ◇ (unstimulated), △ (stimulated). The results are expressed as in the preceding figure.

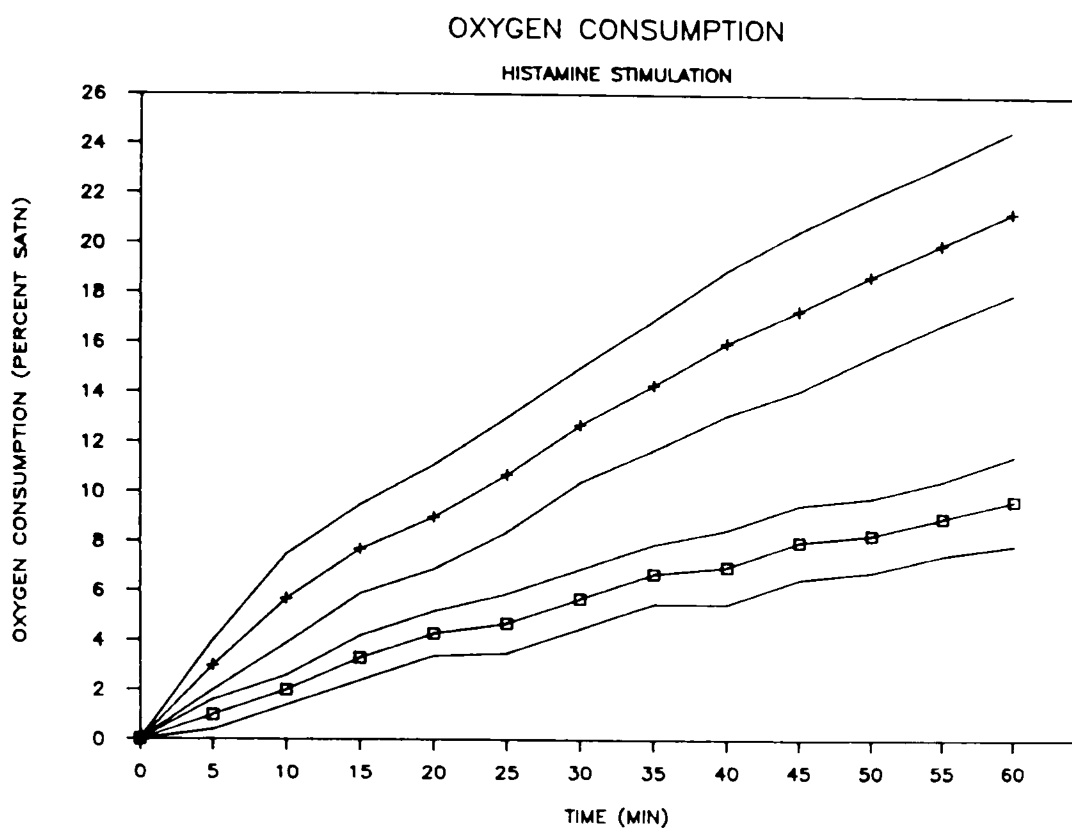

Figure 5. Time course of histamine stimulation of O_2 consumption by isolated gastric glands. Disappearance of O_2 from the metabolic chamber in the absence of histamine (+) and in the presence of histamine, 10^{-5} M (□). Results are expressed as the mean decrease in % O_2 saturation (from an initial level of 42% saturation). Each point represents the mean of three incubations ± 1 SEM.

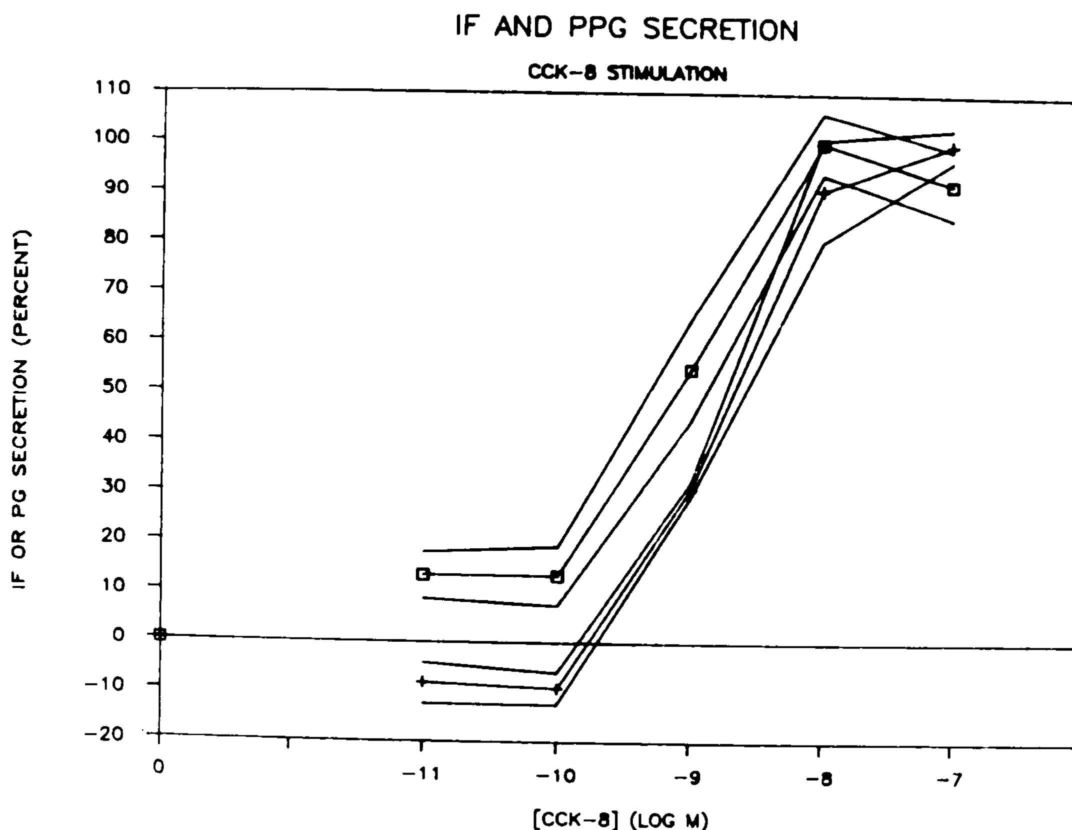

Figure 6. Concurrent stimulation of PPG and IF secretion by CCK-8. Concentration-response curves for release of PPG (+) and IF (□) in response to CCK-8 at concentrations from 10^{-11} M to 10^{-7} M. Values are expressed as % of maximum stimulated level over the unstimulated level.

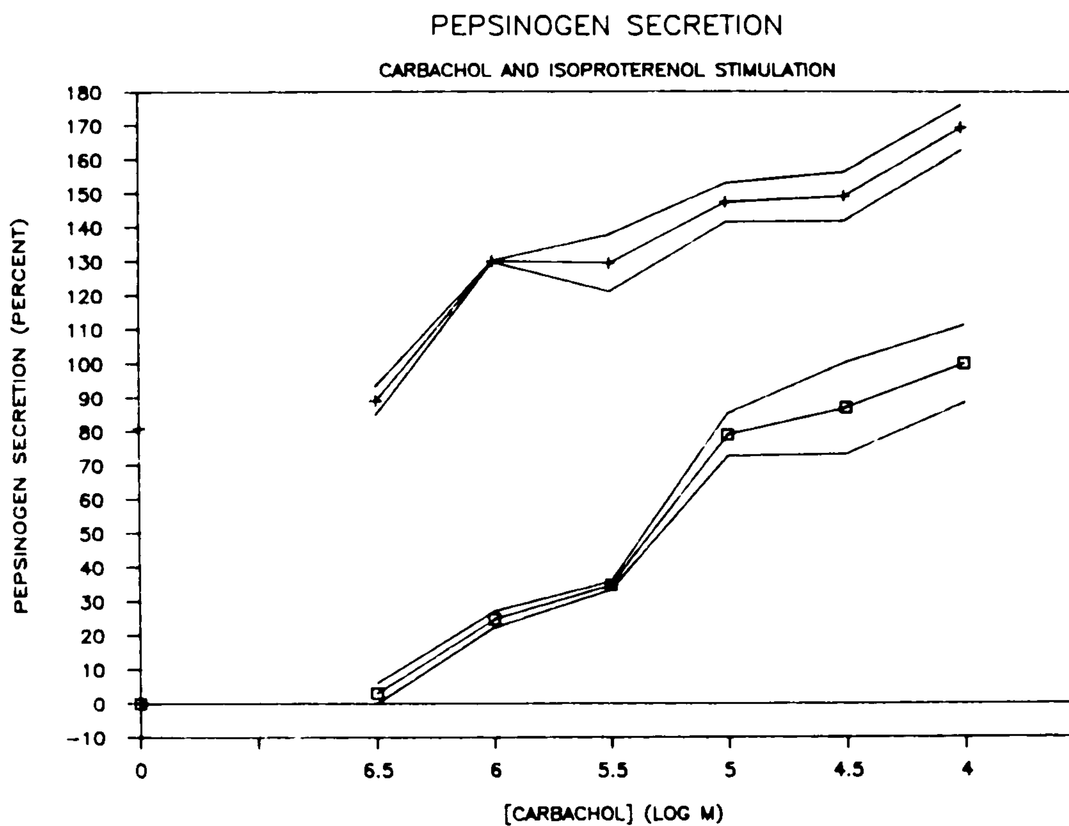

Figure 7. Stimulation of PPG secretion by carbachol and isoproterenol. Dose-response curves for carbachol-stimulated PPG release in the absence (□) and presence (+) of isoproterenol, 10^{-6} M, at carbachol concentrations from 3 x 10^{-7} M to 10^{-4} M. Results are expressed as % of the maximum carbachol-stimulated level over the unstimulated level, ± 1SEM.

REFERENCES

1. Forte JG, Machen TE: Transport and electrical phenomena in resting and secreting piglet gastric mucosa. J Physiol (Lond) 244: 35-51, 1975.

2. Sutton DR, Donaldson RM Jr: Synthesis and secretion of protein and pepsinogen by rabbit gastric mucosa in organ culture. Gastroenterology 69: 166-75, 1975.

3. Kapadia CR, Donaldson RM Jr: Macromolecular secretion by isolated gastric mucosa: Fundamental differences in pepsinogen and intrinsic factor secretion. Gastroenterology 74: 535-9, 1978.

4. Soll AH: Physiology of isolated canine parietal cells: Receptors and effectors regulating function. In Physiology of the Gastrointestinal Tract, ed LR Johnson, pp 673-91. New York, Raven Press, 1981.

5. Batzri S, Harmon JW, Walker MD, Thompson WF, and Toles RT: Secretion of intrinsic factor from dispersed mucosal cells isolated from guinea pig and rabbit stomach. Biochim Biophys Acta 720: 217-21, 1982.

6. Raufman J-P, Sutliff VE, Kasbekar DK, Jensen RT, Gardner JD: Pepsinogen secretion from dispersed chief cells from guinea pig stomach. Am J Physiol 247: G95-G104, 1984.

7. Berglindh T, Obrink KJ: A method for preparing isolated glands from rabbit gastric mucosa. Acta Physiol Scand 96: 150-9, 1976.

8. Berglindh TK, Helander HF, Obrink KJ: Effects of secretagogues on oxygen consumption, aminopyrine accumulation and morphology in isolated gastric glands. Acta Physiol Scand Suppl 97: 401-14, 1976.

9. Hersey SJ, Norris SH, Gibert AJ: Cellular control of pepsinogen secretion. Ann Rev Physiol 46: 393-402, 1984.

10. Hersey SJ, May D, Schyberg D: Stimulation of pepsinogen release from isolated gastric glands by cholecystokininlike peptides. Am J Physiol 244: G192-7, 1983.

11. Kasbekar DK, Jensen RT, Gardner JD: Pepsinogen secretion from dispersed glands from rabbit stomach. Am J Physiol 244: G392-6, 1983.

12. Koelz HR, Hersey SJ, Sachs G, Chew CS: Pepsinogen release from isolated gastric glands. Am J Physiol 243: 218-25, 1982.

13. Sewing K-F: Cyclic nucleotides as mediators of gastric secretion and drug action on gastric mucosa. In Konturek SJ, W Domschke, editors: Gastric secretion. Stuttgart/New York, 1981, Georg Thieme Verlag, pp 18-29.

14. Liddle GW, Hardman JG: Cyclic adenosine monophosphate as a mediator of hormone action. New Engl J Med 285: 560-6, 1971.

15. Modlin IM, Schafer DE, Tyshkov M, Ballantyne GH, Fratesi GR, Roberts JR, Zdon MJ. Forskolin-dependent (cyclic AMP-dependent) protein phosphorylation in isolated gastric glands. Arch Surg 121: 330-337, 1986.

16. Anson ML, Mirsky AE: The estimation of pepsin with hemoglobin. J Gen Physiol 16: 59-63, 1932.

17. Hoedemaeker PJ: Investigations on the site of production of Castle's gastric intrinsic factor. Doctoral dissertation. Groningen, The Netherlands, University of Groningen, 1965.

18. Zdon MJ, Ballantyne GH, Schafer DE, Fratesi GR, Roberts JR, Tyshkov M, Modlin IM: The effects of cholecystokinin on parietal cell secretion in isolated gastric glands. Surgery 98: 1038-1044, 1985.

19. Chew CS, Hersey SJ, Sachs G, Berglindh T: Histamine responsiveness of isolated gastric glands. Am J Physiol 238: G312-20, 1980.

20. Ballantyne GH, Zdon MJ, Schafer DE, Fratesi GR, Roberts JR, Tyshkov M, Modlin IM: Evidence for dual modulation of pepsinogen secretion using isoproterenol, carbachol, CCK-8, forskolin, 8-bromo cAMP, and A23187 probes. Ann Surg 204: 559-565, 1986.

Section III

EXPERIMENTAL

CHAPTER 10

Evidence for Dual Modulation of Pepsinogen Secretion Using Isoproterenol, Carbachol, CCK-8, Forskolin, 8-Bromo-cAMP and A23187

INTRODUCTION

A general model of pepsin secretion was postulated by J.N.Langley in 1881. Morphologic microscopic findings led him to propose that pepsin was stored in granules within the chief cell and released upon appropriate stimulation of the cell [1]. Subsequent research has supported this model with the stipulation that pepsin is stored in the form of an inactive precursor, pepsinogen [2].

The release of acid and pepsinogen following administration of certain gastric secretagogues (first messengers) such as gastrin has been frequently studied in intact animals [3]. Intervening neural, hormonal, and paracrine influences complicate the interpretation of in vivo results. An important effect in this category is the stimulation of pesinogen release by acid on the mucosal surface of the stomach through a local reflex [4]. Moreover, because of these complexities, in vivo studies cannot clarify the role of intracellular mechanisms (e.g., second messengers) in modulating pepsinogen secretion. Thus, there has been considerable interest in the development of in vitro models in which chief cell function may be studied in relative isolation with minimisation of extracellular influences.

In an early in vitro model - organ culture of gastric mucosal biopsies - acetylcholine, in the presence of the cholinesterase inhibitor physostigmine, stimulated pepsinogen secretion [5].

Similarly, isobutylmethylxanthine, which inhibits the degradation of cAMP to adenosine monophosphate (AMP) by phosphodiesterase, also stimulated pepsinogen secretion in both organ cultures of mucosal biopsies as well as in intact gastric mucosal strips in vitro [6]. These experimental systems suffered from several technical problems, including regional variations of secretory patterns within gastric mucosa.

Techniques that were initially developed for the isolation of pancreatic exocrine cells [7] could not be directly applied to isolating chief cells from rabbit mucosa. The extremely dense lamina propria of rabbit gastric mucosa does not permit efficient digestion by collagenase. Consequently, in initial studies I therefore examined pepsinogen secretion from isolated intact gastric fundic glands of the rabbit, adapting a method originally used for parietal cell studies [2]. In preliminary investigations by my colleague D. Shaefer, the glands remained rapidly responsive to acetylcholine [8]. Other laboratories have confirmed and extended these findings [2]. The present study utilizes isolated gastric glands (IGG)

to investigate intracellular mechanisms of pepsinogen (PPG) secretion in an environment largely free of neural, hormonal, and paracrine influences. PPG secretion from IGG was measured in response to a variety of test probes. The purpose of this study was to examine whether second-messenger mechanisms mediated by 3':5'-cyclic adenosine monophosphate (cAMP) or calcium-calmodulin (C-C), or both, govern PPG secretion from chief cells. First messenger probes were therefore used whose membrane receptors activate either cAMP mechanisms (histamine and isoproterenol) or C-C systems (carbachol and cholecystokinin-8, CCK-8). In addition, several probes that bypass membrane receptors were tested. In this group, I used forskolin (F), a direct stimulator of adenylate cyclase; 8-bromo cAMP (8B), a direct activator of cAMP-dependent protein kinases; and A23187 (A), a calcium ionophore, which specifically raises cytosolic calcium levels activating the C-C system.

METHODOLOGY

Study Design

Four test probes were chosen (Table 1) that act via membrane receptors and three that bypass receptor mechanisms. In each group, some act via cAMP-dependent mechanisms, and others are presumed to act by C-C-dependent mechanisms. Thus, four possible pathways for stimulation of PPG secretion were tested; [1] through membrane receptors acting via the cAMP system; [2] through direct activation of the cAMP system; [3] through membrane receptors acting through the C-C system; and [4] through direct activation of the C-C system.

Dose-response curves were predetermined for each probe (Figs. 1,2,3). Maximal or just submaximal doses were used in the experiments. IGG were incubated for 30 minutes in the presence and absence of the test agents and then separated from the medium by filtration. PPG was measured in the medium.

Statistical Analysis

In each experiment all incubations were performed in quadruplicate. Experiments were repeated in four or more rabbits for each agent. Thus, each cited value represents the mean of 16 or more PPG determinations. Mean values of stimulated PPG secretion (expressed as per cent) of total were compared to unstimulated values in the same rabbit using Student's t-test for unpaired data, with significance at $p<0.05$.

RESULTS

Receptor-activated Adenylate Cyclase Agents

(a) Isoproterenol increased PPG secretion significantly above baseline levels over the concentration range from 10^{-7} to 10^{-4}M ($p<0.05$). The results of a representative dose-response experiment giving PPG release as a function of isoproterenol concentration are shown in Figure 1. The stimulatory effect of 10^{-4}M isoproterenol was not significantly greater than the effect of 10^{-5}M ($p>0.05$). Isoproterenol 10^{-5}M significantly increased PPG release (Fig. 4), by 3.5 ± 0.9% of total gland PPG above the baseline value ($p<0.05$).
(b) Histamine, tested repeatedly in concentrations from 10^{-8} to 10^{-2}M, failed in every experiment to stimulate PPG secretions significantly above unstimulated levels ($p>0.05$). A net PPG release of -0.02 ± 0.30% of total gland PPG (not shown) was produced by 10^{-5}M histamine. The ability to dissociate the action of histamine on acid and pepsinogen secretion is dealt with in detail in a subsequent series of studies.

Direct Activation of Adenylate Cyclase

Forskolin caused a marked stimulation of PPG secretion over the concentration range from 10^{-7}M to 10^{-4}M. In a representative experiment (Fig. 3), net PPG release in response to forskolin at 10^{-7}M and higher concentrations was significantly greater than unstimulated release ($p<0.05$). There was no significant increase in PPG secretion between 10^{-6} and 10^{-4}M ($p>0.05$). In another series of experiments (Fig. 5), forskolin 10^{-5}M stimulated net PPG secretion significantly, by 10.6 ± 3.8% of total gland PPG ($p<0.05$).

Effects of a cAMP Analog

8-bromo cAMP was a highly effective stimulus for PPG release. At doses from 10^{-7} to 10^{-3}M a clear dose response curve was evident. Thus, 8-bromo cAMP (10^{-3}M) evoked a net increase of 13.8 ± 4.5% in PPG secretion ($p<0.05$) (Fig. 5).Receptor-activated Calcium-Calmodulin Systems

Carbachol (10^{-5}M) significantly stimulated PPG release (Fig. 4), by 5.1 ± 2.2% of total gland PPG over basal levels ($p<0.05$). In preliminary dose-response experiments, CCK-8 was tested at concentrations from 10^{-11}M to 10^{-7}M (Fig. 2). PPG secretion was significantly stimulated ($p<0.05$) over baseline levels by CCK-8 at concentration from 10^{-10}M to 10^{-7}M. There was no significant difference in CCK-8 stimulation between 10^{-8}M and 10^{-7}M ($p>0.05$). In a separate series of experiments (Fig. 4), CCK-8 (10^{-7}M) significantly raised PPG secretion with respect to baseline, by 5.3 ± 1.5% of total gland PPG

($p<0.05$).

Effects of a Calcium Ionophore

A23187 at a dose of 10^{-6}M (Fig. 5) significantly increased secretion over baseline levels by 2.1 $\pm$ 1.1% of total gland PPG ($p<0.05$).

Comparative Effects of the Probes

Forskolin and 8-bromo cAMP were overall the most effective of the seven agents tested. Stimulation of pesinogen secretion was significantly greater ($p<0.05$) for these two agents than for any of the others except carbachol ($p>0.05$). Carbachol, CCK-8, and isoproterenol stimulated about one third to one half as much pepsinogen release as forskolin and 8-bromo cAMP. There was no significant difference ($p>0.05$) between the levels of stimulation for carbachol, CCK-8, isoproterenol, and A23187.

Table 1. Agents Tested for Stimulation of Pepsinogen Secretion in Isolated Rabbit Gastric Glands

	Level of Action	
Probable Mediator	Receptor	Distal
cAMP	Histamine	Forskolin
	10^{-11} - 10^{-4}M	10^{-7}M - 10^{-4}M
	Isoproterenol	8-bromo cAMP
	10^{-7} - 10^{-4}M	10^{-7}M - 10^{-3}M
Ca-calmodulin	Carbachol	A23178
	10^{-9} - 10^{-4}M	10^{-9}M - 10^{-4}M
	CCK-8	
	10^{-11} - 10^{-7}M	

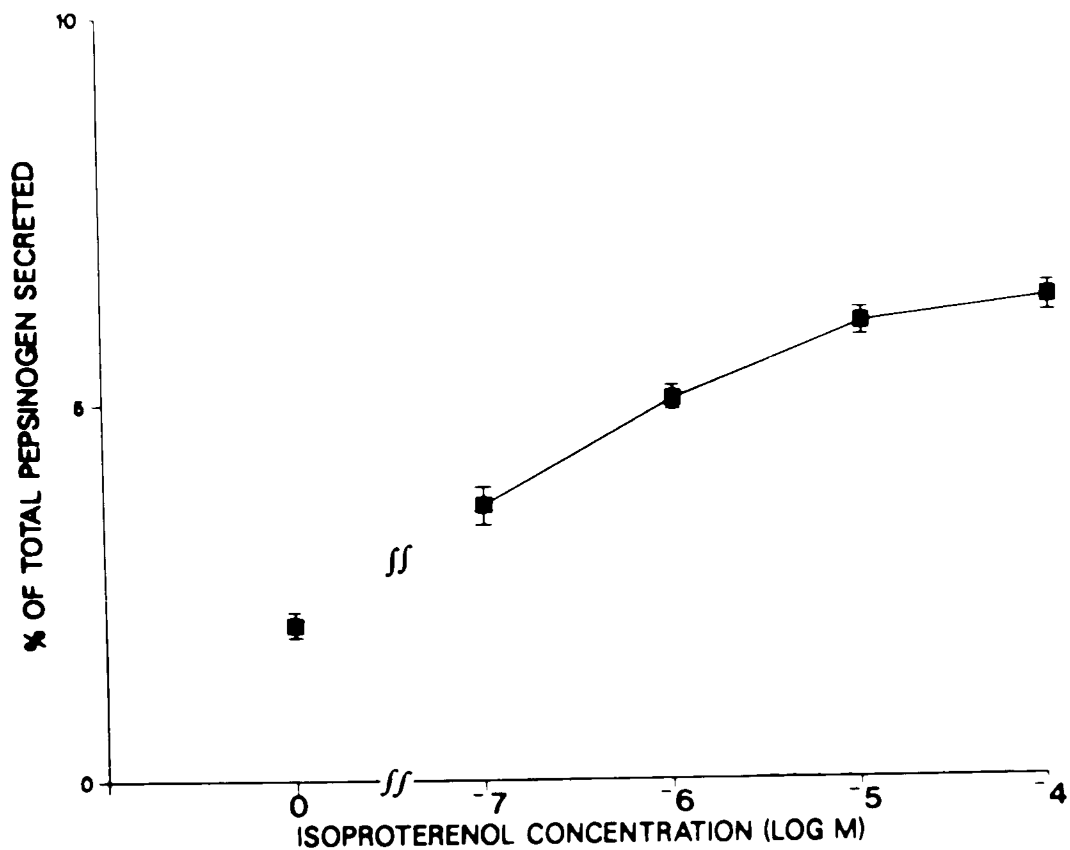

Figure 1. Dose-response relationship for isoproterenol stimulated pepsinogen secretion from isolated gastric glands (IGG). Pepsinogen secretion is expressed as per cent of the total pepsinogen secretion. A dose of 10^{-7}M isoproterenol (I) resulted in a significant secretion of pepsinogen over unstimulated levels ($p<0.05$). Maximal stimulation was obtained with doses of 10^{-5}M. PPG secretion stimulated by 10^{-4}M I was not significantly different from 10^{-5}M ($p>0.05$).

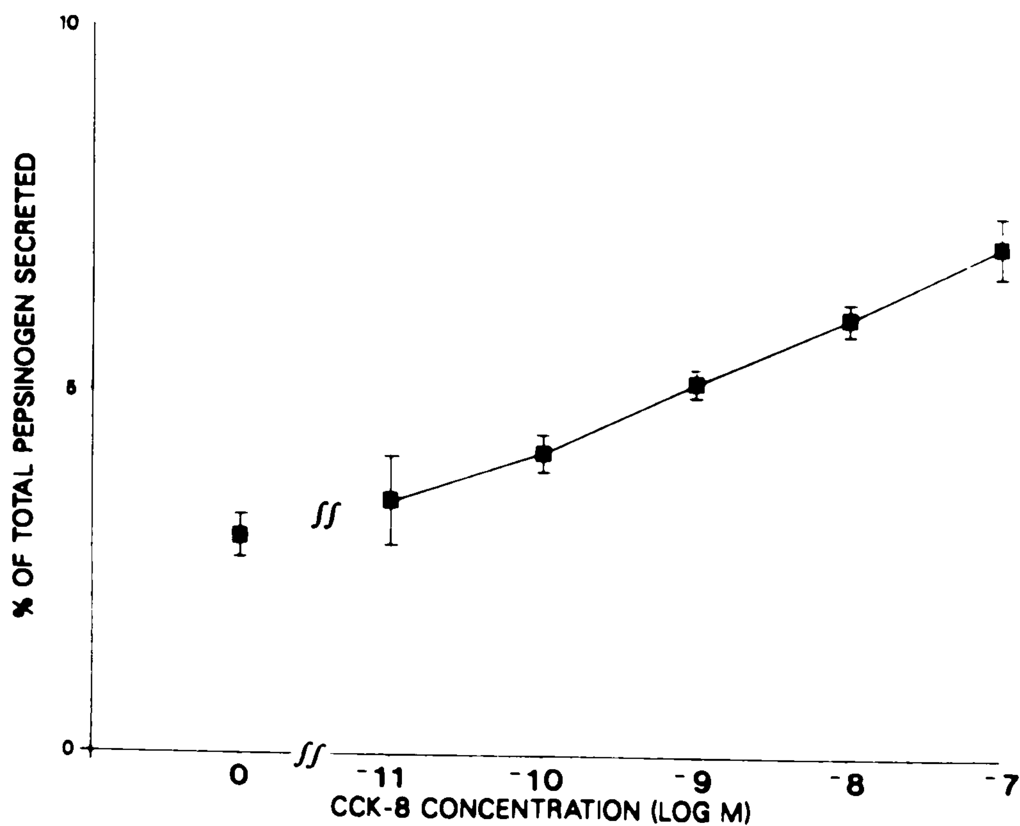

Figure 2. Dose-response relationship for CCK-8 stimulated pepsinogen secretion from IGG. Pepsinogen secretion is expressed as per cent of the total pepsinogen. CCK-8 at 10^{-11}M resulted in a significant stimulation of PPG secretion over unstimulated levels ($p<0.05$). Maximal stimulation was obtained at a dose of 10^{-7}M.

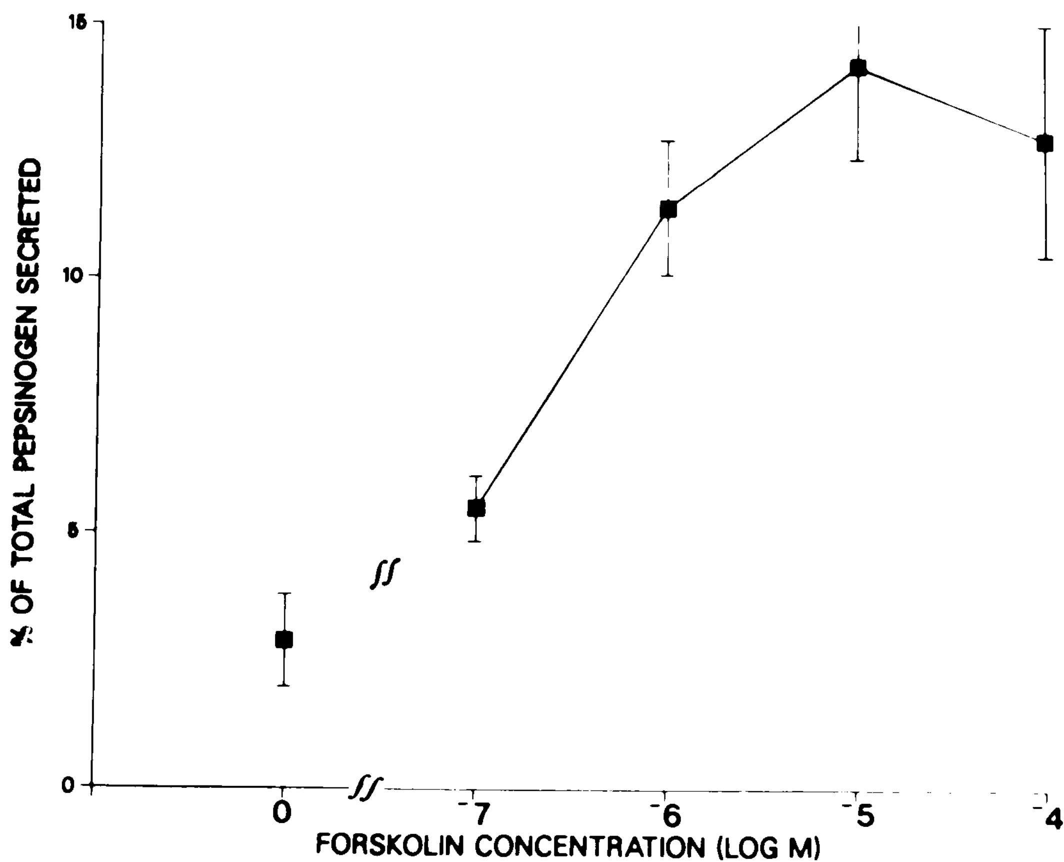

Figure 3. Dose-response relationship of forskolin stimulated pepsinogen secretion for IGG. Pepsinogen secretion is expressed as per cent of the total pepsinogen secreted. Forskolin 10^{-7}M resulted in a significant stimulation of PPG over unstimulated levels (p<0.05). Maximal stimulation was obtained at a dose of 10^{-5}M. There was significant difference in forskolin-stimulated PPG secretion at 10^{-5}M and 10^{-4}M (p>0.05).

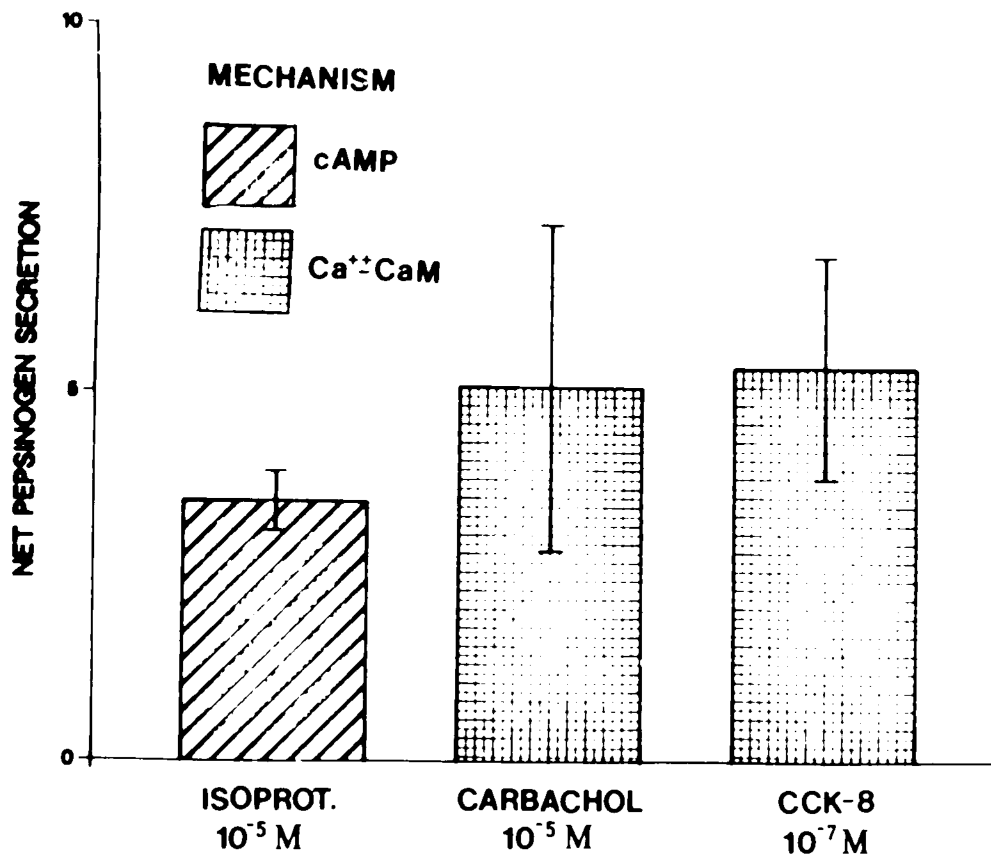

Figure 4. Stimulation of pepsinogen secretion from IGG by maximal doses of secretagogues that activate cAMP or calcium-calmodulin (Ca^{++}-CaM) second messenger system via membrane bound receptors. Results are shown as Mean $\pm$ SEM and are expressed as net pepsinogen secreted. i.e., per cent of the total pepsinogen secreted above baseline levels. Probes acting through either cAMP or C-C systems significantly ($p<0.05$) increased pepsinogen secretion above unstimulated levels.

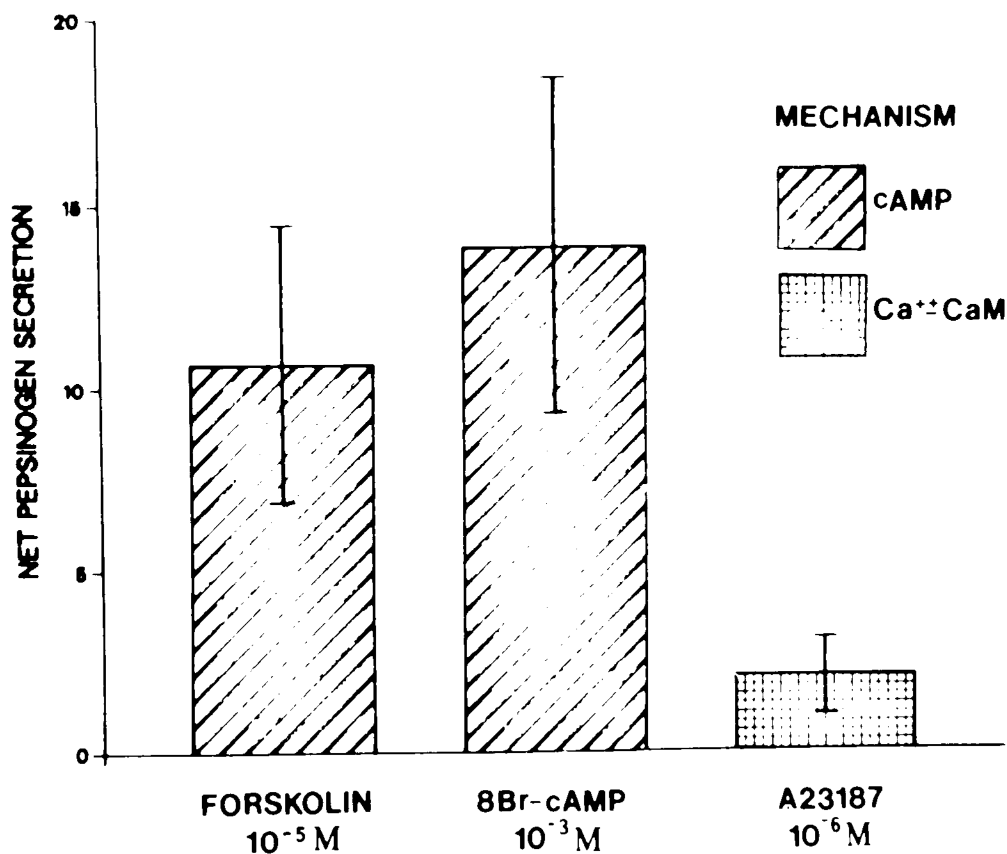

Figure 5. Stimulation of pepsinogen secretion from IGG by maximal doses of secretagogues that bypass membrane receptors directly activating cAMP or calcium-calmodulin (Ca^{++}-CaM) second messenger systems. Results are shown as Mean $\pm$ SEM and are expressed as net pepsinogen secreted i.e., per cent of the total pepsinogen secreted above baseline levels. Forskolin and 8-bromo cAMP, which directly activate cAMP mechanisms, produced the greatest increase of pepsinogen secretion above unstimulated levels.

DISCUSSION

The use of isolated gastric gland preparations permits the study of chief cell function in a simple, controlled environment. In the intact animal, chief cell function can be affected by alteration in blood pressure, shunting of blood past capillary beds, parasympathetic and sympathetic input, circulating hormones, and unrecognized regulatory systems. With IGG such extrinsic influences can be eliminated. The composition of the medium can be precisely controlled. Identical aliquots of cells from one animal can be simultaneously observed under different conditions. Thus, the direct effects of secretagogues on chief cells are more readily and unambiguously interpreted in this experimental system.

Unfortunately IGG are not a homogeneous population of chief cells. This functional gastric unit, the gastrone, consists of a mixture of parietal cells, chief cells, and small round cells. This last group consists mostly of endocrine cells. The exact functional relationships of these endocrine cells is not completely understood. Although IGG experimentation allows the study of chief cells in a simplified environment, these endocrine cells may be imparting an unrecognized paracrine influence on chief cell functions.

In some respects, isolated chief cell preparations represent an even simpler system than IGG [10]. However, at least atthe present time IGG have certain advantages over the isolated cell system. The calcium-free chelating media required for breaking tight junctions between cells may affect the function of calcium-dependent systems.

The density-gradient centrifugation required for separating enriched cell fractions may alter or injure exposed membrane receptors. With disruption of the fundic glands, chief cells lose their polarity and become leaky. Until these potential limitations of the isolated chief cell preparation can be evaluated, the IGG preparation may offer the best available means of studying chief cell function in a controlled physiological environment.

Of the seven agents tested as potential secretagogues in this study, only histamine failed to stimulate the secretion of PPG over unstimulated levels at any of the concentrations used ($p>0.05$). All the remaining agents caused significant stimulation of PPG release ($p<0.05$). The results of these experiments suggest that PPG secretion by IGG can be stimulated by at least five distinct classes of agents falling into two main categories: (1) agents that activate cAMP systems and (2) agents that "probably" activate C-C systems.

The observation that forskolin and 8-bromo cAMP were the two most effective stimulators of PPG secretion supports the proposal that cAMP may play a physiological role in the control of PPG secretion. It is probable that cAMP is an important intracellular regulator of this process.In this study, I have examined the effects of seven contrasting functional probes on PPG secretion. Each probe stimulates cellular function by a distinctive mechanism. Histamine binds to specific membrane H2 receptors, activating adenylate cyclase in rabbit gastric mucosa [9], resulting in an increase in intracellular cAMP [10]. In rabbit IGG, histamine stimulation results in acid secretion and intrinsic factor secretion from the parietal cells in a dose-dependent manner [13,14]. In the present study, however, histamine (10^{-5}M) failed to stimulate PPG secretion significantly above baseline values. One may thus conclude that histamine mechanisms are not primary modulators of PPG secretion in rabbit chief cells. A possible explanation of PPG release in response to histamine in some intact-animal experiments is that histamine stimulates acid secretion, which can then reflexly stimulate the release of PPG [4]. Alternatively, histamine may release other bioactive agents that are capable of stimulating PPG secretion. Although it is well documented that parietal cells in various species such as rabbits have H2 receptors, the present data suggests that chief cells in the rabbit have none.

Isoproterenol (10^{-5}M) significantly increased PPG secretion above baseline levels (Fig. 4). Whereas isoproterenol stimulates adenylate cyclase and increases cAMP content in rabbit IGG, it does not stimulate acid or intrinsic factor secretion [11,14]. Thus, beta-adrenergiccatecholamines acting through membrane adenylate cyclase and intracellular cAMP contribute to the regulation of PPG secretion in the rabbit.

Although isoproterenol was an effective stimulus for PPG secretion, its maximum effect was approximately half that of either forskolin or 8-bromno cAMP (Figs. 4 and 5). This observation suggests that it is not possible to stimulate chief cell adenylate cyclase fully by stimulating beta-adrenergic receptors. There are at least two possible explanations for this observation. Such a limitation might occur if other receptors are preferentially coupled to a portion of the chief cell adenylate cyclase. Alternatively, in a mobile receptor-cyclase system, there may be an excess of adenylate cyclase catalytic subunit over receptor molecules, perhaps to ensure optimum response with a limited number of receptors.

The diterpene forskolin is isolated from the roots of Coleus forskohlii and has been reported to activate adenylate cyclase by direct effect on its catalytic subunit [12]. In the

present study, forskolin significantly increased PPG secretion (Fig. 5). Forskolin has been reported to cause accumulation of cAMP in all tissues tested [15]. In particular, forskolin stimulates cAMP accumulation and activates cAMP-dependent protein kinases in isolated rabbit IGG [13]. It does not increase cAMP accumulation by inhibition of phosphodiesterase. Activationof adenylate cyclase by forskolin is reversible and independent of guanyl nucleotides, and it does not appear to depend on membrane receptors. Phentolamine (alpha-adrenergic blocker), propranolol (beta-adrenergic blocker), and cimetidine (H2 blocker) are ineffective in inhibiting forskolin-stimulated cAMP accumulation in brain slides [15]. It is believed that forskolin acts directly at the adenylate cyclase enzyme complex consisting of the guanyl nucleotide binding sub-unit and the catalytic subunit [15]. Thus, forskolin can stimulate pepsinogen secretion in IGG by bypassing membrane receptors and directly activating adenylate cyclase.

My observation with the cAMP analog 8 bromo-cAMP (8B) supports the hypothesis that cAMP is an important modulator of PPG secretion. 8B was the most effective stimulator of PPG secretion tested in this study (Fig. 5) although the dose used was somewhat excessive. The results are consistent with those found in rats [14] and guinea pigs, where cAMP analogs are potent stimulators of PPG secretion in vitro [10].

Carbachol, an acetylcholine analog that binds to muscarinic receptors, does not alter the cAMP content of gastric glands [2]. The mechanism of action of muscarinic agents on gastric glands is not known in detail; however, it appears that they function via the C-C system. In isolated pancreatic acini, carbachol elevates the cytoplasmic calciumconcentration [15] and increases phosphorylation of intracellular proteins in a pattern suggesting stimulation of a calmodulin-dependent protein kinase [16]. Studies with acetylcholine in rabbit IGG by Berglindh and colleagues [17] have shown that cholinergic stimulation of acid secretion, as measured by ^{14}C-aminopyrine accumulation, requires the presence of extracellular Ca^{++} exceeding at least 10^{-5}M. This suggests that some part of the cholinergic mechanism involves an influx of extracellular Ca^{++}. In contrast, histamine-stimulated acid secretion did not require the presence of such high calcium levels within the medium. In the present study, carbachol (10^{-5}M) significantly increased PPG secretion (Fig. 4). These results are consistent with the possibility that carbachol stimulates PPG secretion from chief cells by activating systems mediated by C-C.

The hormone (neurotransmitter) cholecystokinin (CCK) is a 33-amino acid peptide. CCK-8, the cholecystokinin octapeptide used in this experiment, is identical to the C-terminal

octapeptide of CCK, and the C-terminal pentapeptides of CCK, CCK-8, and gastrin are identical to each other. CCK-8 occurs naturally and has bioactivity similar to that of CCK in many systems. CCK-8 has been found in pig brain [18], and CCK-8 immunoreactivity has been identified in the small intestine [19]. In the present study, CCK-8 increased PPG secretion above unstimulated levels by 5.3 ± 1.5% of total gland PPG. CCK-8 reportedlystimulates PPG secretion sixfold in dispersed guinea-pig chief cells [10] and fourfold in dispersed rat gastric glands [17]. CCK-8 does not appear to act through cAMP mechanism in rabbit IGG, since adenylate cyclase activity in rabbit IGG does not increase following CCK-8 stimulation [20]. Absence of calcium from the medium inhibits CCK-8 stimulated chief cell function [23]. Furthermore, phosphorylation studies are consistent with the interpretation that CCK-8 acts through C-C dependent protein kinases [19]. Thus the evidence suggests that CCK-8 stimulates PPG secretion through C-C dependent mechanisms.

The cytosolic concentration of free calcium ions in most cells is normally in the submicromolar range. The ionophore A23187 selectively promotes the transport of calcium across biological membranes, thus transiently elevating the cytosolic concentration of free calcium ions [21]. In this study, A23187 modestly increased PPG secretions above basal levels. A moderate increase in PPG secretion has been observed with this agent in dispersed glands from rats [24]. The limited magnitude of this response may reflect the observation that A23187 may cofunction as a protonophore. It is possible that the resultant alteration of intracellular pH might alter the PPG secretary capacity. These observations further support a role for C-C systems in PPG secretion.

In summary, these experiments clearly support a dual modulation of pepsinogen secretion. Thus, isoproterenol and forskolin (adenylate cyclase activators), as well as the cAMP analogue 8-bromo-cAMP, significantly stimulate pepsinogen secretion in IGG. In addition, carbachol, CCK-8, and A23187, which all act via C-C systems, stimulate pepsinogen secretion. It is, therefore, possible that both cAMP and C-C systems participate in the modulation of pepsinogen secretion from rabbit chief cells.

REFERENCES

1. Langley, JN. On the histology and physiology of pepsin-forming glands. Philos Trans R Soc Lond 1181;172:664-711.

2. Hersey SJ, Norris SH, Gilbert AJ. Cellular control of pepsinogen secretion. Annu Rev Physiol 1984;46:393-402.

3. Walsh JH. Gastrointestinal hormones and peptides. In Johnson LR, ed. Physiology of the Gastrointestinal Tract. New York:Raven Press, 1981.

4. Johnson LR. Regulation of pepsin secretion by topical acid in the stomach. Am J Physiol 1972; 223:847-850.

5. Sutton DR, Donalsdon RM Jr. Synthesis and secretion of protein and pepsinogen by rabbit gastric mucosa in organ culture. Gastroenterology 1975;69:166-174.

6. Schafer DE, Ebersole ER, Donaldson RM Jr.Physostigmine and isobutylmethylxanthine stimulates pepsinogen secretion synergistically. Fed Proc 1979;39:885.

7. Amsterdam A, Jamieson JD. Studies on dispersed pancreatic exocrine cells. I. Dissociation techniques and morphologic characteristics of separated cells. J Cell Biol 1974;63:1037-1056.

8. Schafer DE. Stimulated secretion of pepsinogen by isolated fundic glands of rabbit mucosa. Physiologist 1980;23:66.

9. Sung CP, Jenkins BC, Burns LR, et al. Adenylate and guanyl cyclase in rabbit gastric mucosa. Am J Physiol 1973;225:1359-1363.

10. Chew CS, Hersey SJ, Sachs G, Berglindh TH. Histamine responsiveness of isolated gastric glands. Am J Physiol 1980;238(Gastroint Liver Physiol 1):G312-320.

11. Hersey SJ, Miller M, May D,Norris HS. Lack of interaction between acid and pepsinogen secretion in isolated gastric glands. Am J Physiol 1983;245(Gastrointest Liver Physiol 8):G775-779.

12. Seamon KB, Padgett W, Daly JW. Forskolin: unique diterpene activator of adenylate cyclase in membrane and in intact cells. Proc Natl Acas Sci USA 1981;78:3363-3367.

13. Chew CS. Forskolin stimulation of acid secretion and pepsinogen secretion in isolated gastric glands. Am J Physiol 1983;245(Gastrointest Liver Physiol 14):C371-C380.

14. Raufman JP, Kasbekar DK, Jensen RT, Gardner JD. Potentiation of pepsinogen secretion from dispersed glands from rat stomach. Am J Physiol 1983; 245(Gastrointest Liver Physiol 8):G525-G530.

15. Schultz I, Stolze HH. The exocrine pancreas: the role of secretogogues, cyclic nucleotides, and calcium in enzyme secretion. Ann Physiol 1980;42:127-156.

16. Burnham DB, Williams JA. Activation of protein kinase activity in pancreatic acini by calcium and cAMP. Am J Physiol 1984 (H4Gastrointest Liver Physiol 9):G500-G508.

17. Berglindh T, Sachs G, Takeguchi N. Ca2+ dependent secretogogue stimulation in isolated rabbit gastric glands. Am J Physiol 1980;239(Gastrointest Liver Physiol 2):G90-G94.

18. Muller JE, Strause E, Yallow RS. Cholecystokinin and its COOH-terminal octapeptide in the pig brain. Proc Natl Acad Sci USA 1977;74:3035-3037.

19. Dockray GJ. Immunoreactive component resembling cholecystokinin octapeptide in intestine. Nature 1977;270:351-361.

20. Hersey SJ, May D, Schyberg D. Stimulation of pepsinogen release from isolated gastric glands by cholecystokinin-like peptides. Am J Physiol 1983;244(Gastrointest Liver Physiol 7):G192-G1907.

21. Kasbekar DK, Jensen RT, Gardner JD. Pepsinogen secretion from dispersed glands from rabbit stomach. Am J Physiol 1983;244(Gastrointest Liver Physiol 7):G392-G396.

CHAPTER 11

Vasoactive Intestinal Peptide Augmentation of Cholecystokinin Stimulated Pepsinogen Secretion

INTRODUCTION

Vasoactive intestinal peptide (VIP) was isolated from the small intestines of hogs by Said and Mutt in 1970 [1]. The porcine octacosapeptide was sequenced and synthesized by Bodanzky et al. in 1973 [2]. VIP, secretin, glucagon, and gastric inhibitory peptide (GIP) are structurally related forming a family of gut hormones. Polak and colleagues, using immunohistochemical techniques, revealed a wide distribution of VIP containing cells throughout the mammalian and avian gut [3]. The highest concentration of VIP cells was in the colon. A dual role of VIP as a gastrointestinal hormone and as a neurotransmitter substance was proposed by Bryant et al [4] when they measured high concentrations of VIP within the central nervous system. Infusion studies have demonstrated that VIP is a more effective inhibitor of histamine-stimulated acid secretion and pentagastrin-stimulated pepsin secretion than secretin [5]. The clinical relevance of this hormone was confirmed when Said and Faloona [6] measured elevated levels of VIP in 28 patients with Watery-Diarrhea Hypokalaemia and Achlorhydria (WDHA) Syndrome.

VIP's mechanism of action has been well established in several in vitro systems. Acting through adenylate cyclase (AC), VIP causes accumulation of cyclic adenosine 3':5'-monophospate (cAMP) within jejunal mucosa cells [7,8]. Similarly, VIP has been reported to elevate levels of cAMP within isolated pancreatic acinar cells by activating AC [9,10,11]. The ability of VIP to activate AC in isolated gastric glands from the guinea pig fundus has recently been identified [12]. VIP affects cellular function of its end organs through the second messenger, cAMP.

It has been reported that the regulation of pepsinogen secretion from chief cells is dually mediated by two second messenger systems [13]. Isoproterenol, forskolin, and 8-bromo-cAMP stimulate PPG secretion by acting through cAMP-dependent systems. In contrast, carbamycholine and cholecystokinin-octapeptide (CCK-8) cause chief cell secretion by altering intracellular calcium levels and possibly activating the calcium-calmodulin second messenger system [13]. The stimulation of chief cells by compounds from these two groups produces at least an additive effect.

The purpose of this experiment was twofold: first, to establish the effect of VIP on chief cell secretion and second, to examine the interaction of cAMP (VIP) and calcium-mediated (CCK-8) secretagogues on pepsinogen secretion. Isolated rabbit gastric glands were prepared by the technique of Berglindh and Obrink [14]. This preparation provided an environment free of classical neural and hormonal

influences enabling a relatively direct interpretation of receptor-mediated cellular events. The effect of VIP on chief cells stimulated by a subthreshold dose of CCK-8 were examined. Since VIP acts through the cAMP second messenger system and CCK-8 through the calcium-dependent second messenger system, the augmentation of CCK-8-stimulated pepsinogen secretion by VIP would support the hypothesis that regulation of chief function is dually mediated by both the cAMP and calcium messenger systems.

METHODS

Gland preparation

Isolated gastric glands were prepared from 3-4kg New Zealand white rabbits using the techniques previously described in detail [13].

Peptides

Synthetic vasoactive intestinal peptide (Penninsula Laboratories) and cholecystokinin-octapeptide (Penninsula Laboratories) were used in all experiments.

Experimental design

Three series of experiments were performed. In the first set, a dose-response curve for VIP was generated. In these experiments concentrations of VIP from 10^{-7} to 10^{-11}M were added to the incubation medium. The level of pesinogen secretion at each concentration was determined in quadruplicate samples from four different rabbits (n=4). In the second set a subthreshold dose of CCK-8 was found by establishing a dose-response curve. Concentrations of CCK-8 from 10^{-11} to 10^{-7}M were used. In the third set of experiments, this subthreshold concentration of CCK-8 (10^{-9}M) was added to IGG with varying concentrations of VIP (10^{-7} to 10^{-11}M). Pepsinogen secretion at each combination was determined in quadruplicate samples [15,16] and the study repeated in four different animals (n=4).

Statistical analysis

At each test dose of VIP alone (10^{-7} to 10^{-11}M) or in combination with CCK-8 (10^{-9}M), 16 data points from 4 different animals were available for analysis. Comparison was done by Student's t test for unmatched samples. Statistical significance was defined as $P<0.05$.

RESULTS

VIP dose response

Unstimulated levels of pepsinogen secretion (Fig. 1) averaged 10 ± 2% of total pepsinogen. Total pepsinogen in these experiments averaged 4963 ± 622 ng/ml. VIP in concentrations from 10^{-11} to 10^{-7}M failed to stimulate the secretion of pepsinogen above unstimulated levels ($P>0.05$).

CCK-8 dose response

Unstimulated levels of pepsinogen secretion (Fig. 2) in these studies were 2.8 ± 0.2% of total pepsinogen. Total pepsinogen averaged 5756 ± 745 ng/ml. Maximal stimulation of pepsinogen secretion was achieved with a dose of 10^{-7}M. CCK-8 at a dose of 10^{-9}M did not significantly increase pepsinogen secretion above baseline levels ($P>0.05$). This dose of CCK-8 was used as a subthreshold dose in subsequent experiments.

CCK-8 in combination with VIP

Unstimulated levels of pepsinogen secretion (Fig. 3) in these experiments averaged 8 ± 3% total pepsinogen. Total pepsinogen in these experiments averaged 5156 ± 519 ng/ml. CCK-8 (10^{-9}M) alone did not significantly stimulate pepsinogen secretion above baseline levels ($P>0.05$). The combination of CCK-8 (10^{-9}M) with VIP (10^{-11} through 10^{-9}M) failed to significantly increase pepsinogen secretion above baseline values ($P>0.05$). The combination of CCK-8 (10^{-9}M) and VIP (10^{-8}M) significantly increased pepsinogen secretion above baseline levels ($P<0.05$). This combination (CCK-8 10^{-9} and VIP 10^{-8}M) did not significantly ($P=0.06$) stimulate more pepsinogen than the combination of CCK-8 (10^{-9}M) and VIP (10^{-11}M).

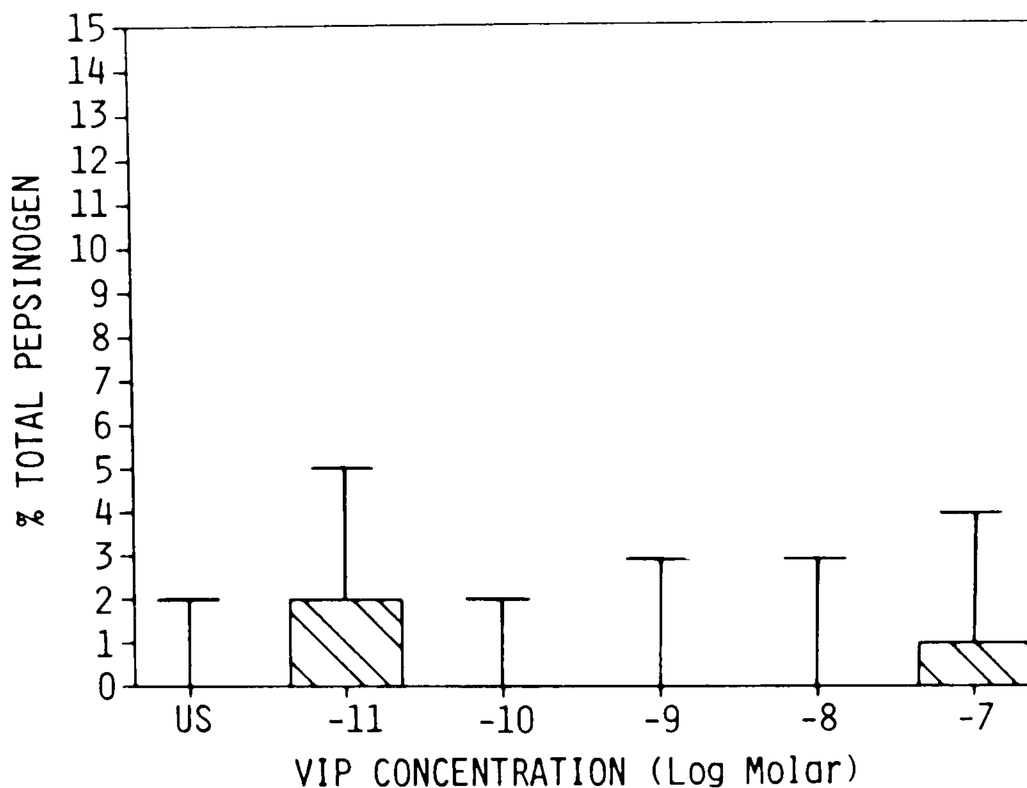

Figure 1. Vasoactive intestinal peptide (VIP) in concentration from 10^{-11} to 10^{-7}M when added to rabbit isolated gastric glands failed to significantly increase pepsinogen secretion above unstimulated (US) levels (P>0.05). Results are expressed as pecentage of total pepsinogen secreted above unstimulated levels.

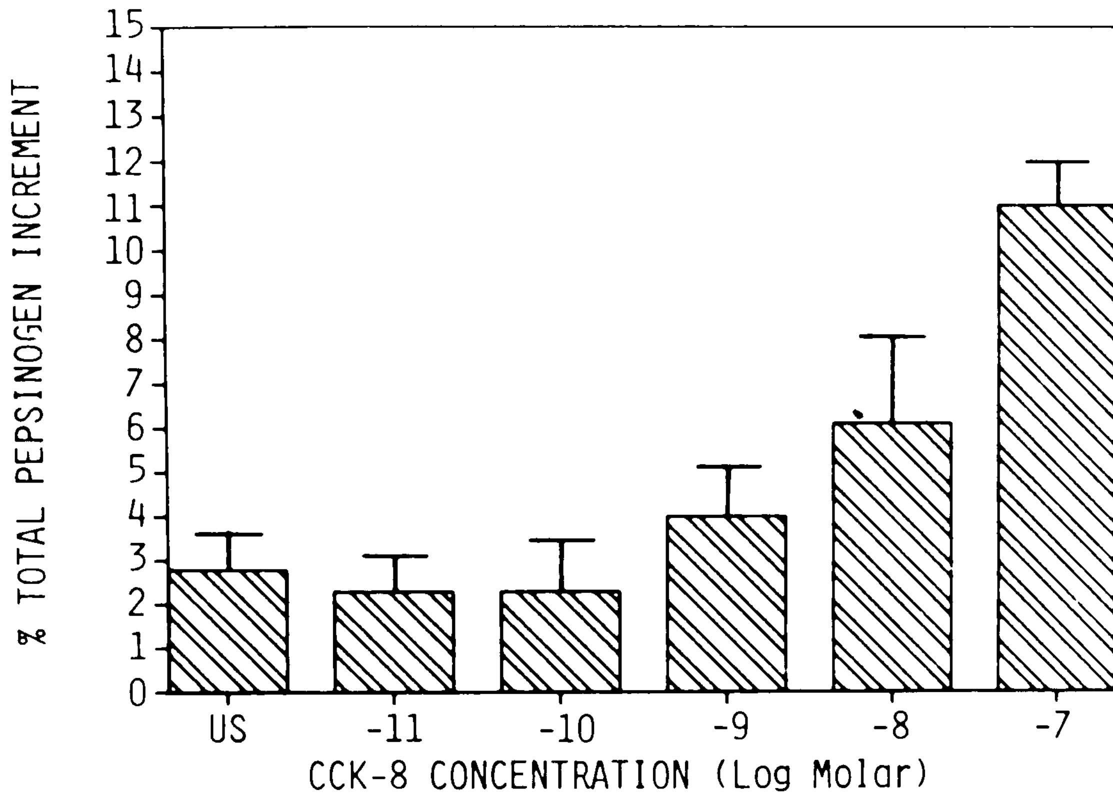

Figure 2. Pepsinogen secretion stimulated by cholecystokinin-8 (CCK-8) in rabbit isolated gastric glands. Maximal stimulation was achieved at a dose of 10^{-7}M. CCK-8 (10^{-9}M) did not significantly stimulate pepsinogen secretion (P>0.05). Results are expressed as percentage total pepsinogen secreted above baseline levels.

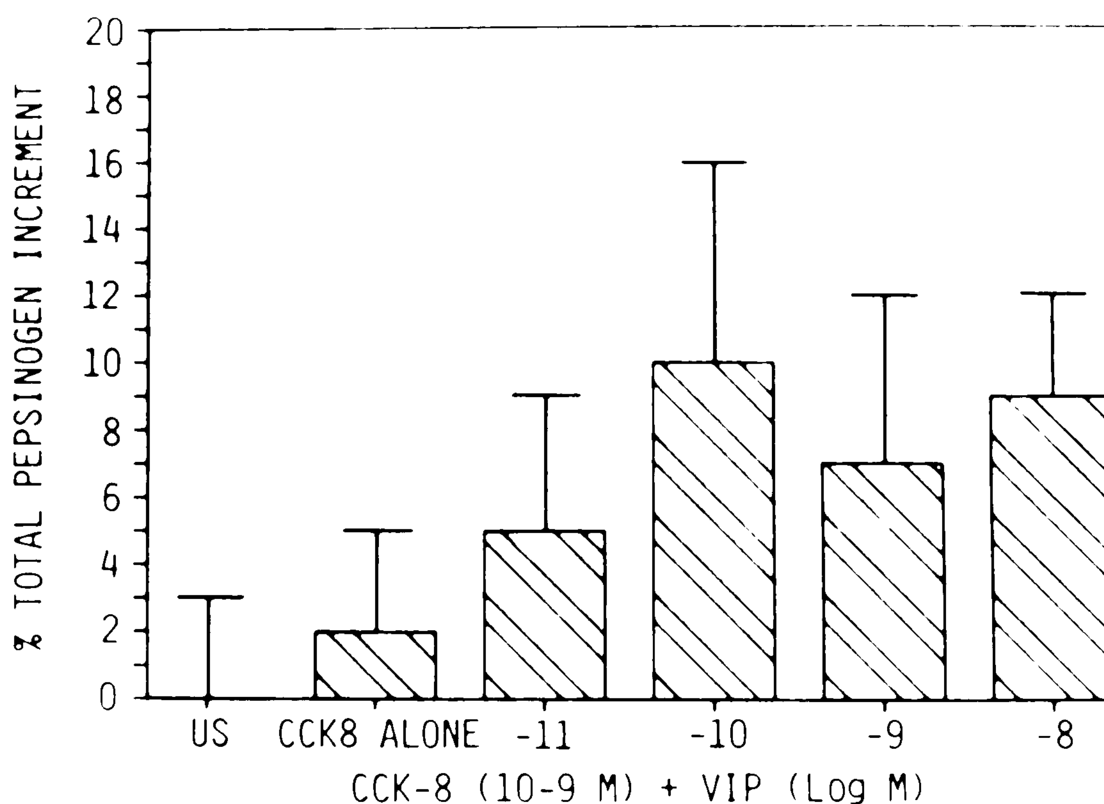

Figure 3. Pepsinogen secretion stimulated by CCK-8 (10^{-9}M) alone or in combination with varying doses of VIP (10^{-11} to 10^{-8}M). CCK-8 (10^{-9}M) alone failed to significantly stimulate pepsinogen secretion. The combination of CCK-8 (10^{-9}M) and VIP (10^{-8}M) significantly stimulated pepsinogen secretion above unstimulated (US) levels ($P<0.05$).

DISCUSSION

These studies demonstrated that vasoactive intestinal peptide alone in concentration from 10^{-11} to 10^{-7}M does not stimulate pepsinogen secretion from isolated gastric glands (IGG). Cholecystokinin-octapeptide at a concentration of 10^{-9}M also failed to significantly elevate levels of pepsinogen secretion above basal values. The combination of VIP (10^{-8}M) and CCK-8 (10^{-9}M), however, significantly increased pepsinogen secretion above basal values. Thus the combination of two peptides at concentrations which alone fail to stimulate secretion resulted in chief cell secretion.

VIP stimulation of pepsinogen secretion has not been previously demonstrated in rabbit IGG. In rat IGG, however, VIP (3 x 10^{-7}M) alone significantly increased pepsinogen secretion above unstimulated levels [17,18]. In similar studies with dispersed chief cells, Raufman and colleagues [19] doubled pepsinogen secretion with VIP. Maximum levels of secretion with VIP were achieved at a 10^{-9}M concentration. Chief cells in our rabbit preparation may be less sensitive to stimulation by VIP than rat or guinea pig chief cells.

CCK-K-8's ability to cause pepsinogen secretion by chief cells is well established. Ballantyne and co-workers [13] demonstrated that CCK-8 (10^{-7}M) increased levels of pepsinogen secretion by 5.3% (of total pepsinogen) $\pm$ 1.5% over basal levels. Studies in dispersed rat fundic glands found a fourfold elevation of pepsinogen secretioncaused by 10^{-8}M dose of CCK-8 [20]. Maximal stimulation of guinea pig dispersed chief cells was achieved at a 5 x 10^{-8} M CCK-8 [17]. CCK-8 at concentration greater than 10^{-8}M reliably produces pepsinogen secretion from rat, guinea pig, and rabbit chief cells.

Pepsinogen secretion in rabbit IGG can be evoked by two classes of agents; ones that activate adenylate cyclase and ones that alter cytosolic calcium. I have previously demonstrated that isoproterenol and forskolin (agents which increase AC activity) and 8-bromo-cAMP (a cAMP analog) significantly stimulate pepsinogen secretion from IGG [13]. Furthermore, I have shown that CCK-8, carbachol, and the calcium ionophore A23187 (drugs acting through calcium mechanisms) increase pepsinogen secretion. These observations have been supported by a number of different investigators [21,22,23]. It is therefore probable that regulation of chief cell pepsinogen secretion is dually modulated by both cAMP and calcium second messenger systems.

Binding of membrane receptors by secretagogues activates one of the second messenger systems (Fig. 4). Binding of receptors by agents which act through cAMP systems activates

membrane bound AC. This enzyme converts adenosine triphosphate (ATP) to cyclic adenosine 3':5'-monophosphate which acts as the intracellular (second messenger). cAMP is converted to adenosine monophosphate (AMP) when it phosphorylates a protein kinase. Phosphorylation activates the protein kinase allowing it to act on its protein substrate. Thus, binding of the membrane receptor by VIP leads to phosphorylation of intracellular protein kinases and protein substrates. In the gastricchief cell, this mechanism presumably results in secretion of pepsinogen.

Binding of membrane receptors by secretagogues acting through Ca^{2+} mechanisms opens calcium channels precipitating a rapid rise in cytosolic calcium concentration [20]. One result of this is the binding of Ca^{2+} by calmodulin. This calcium-calmodulin complex (C-C) can accomplish phosphorylation of protein substrates through several mechanisms including elevating cellular levels of cAMP of activating C-C dependent protein kinase [20]. These two second messenger systems probably communicate at several levels within the cell (Fig. 4). C-C can activate AC resulting in higher cellular levels of cAMP. It can also increase the activity of phosphodiesterase, an enzyme which degrades cAMP to AMP. This causes a decrease in cellular levels of cAMP. In addition, cAMP and C-C together may activate the same or similar protein kinases and thus trigger protein phosphorylation. It seems likely that these pathways allow for the two messenger systems to either amplify or mute each other [20].

VIP and CCK-8 promote pepsinogen secretion by different mechanisms. Thus, VIP is proposed to utilize the cAMP system as an intracellular second messenger while CCK-8 has been postulated to act via the C-C system. The effect of VIP on adenylate cyclase and intracellular cAMP has been studied in several cell types. VIP showed a dose-dependent increase in adenylate cyclase activity in human gastric and duodenal mucosa [24]. Maximal stimulation with a dose of 10 uM/ml produced a threefold increase in AC activity. Similarresults were found in dispersed guinea pig acinar cells [11,25] as well as human jejunal mucosa [7]. Other experiments have measured cAMP levels in cells stimulated by VIP.

Gardner and colleagues [10] examined the impact of VIP in the presence of theophylline, a phosphodiesterase inhibitor, on acinar cells isolated from guinea pig pancreas. VIP (10^{-6}M) caused a fourfold increase of cAMP as measured by radioimmunoassay. More recently Sutliff et al reported that vasoactive intestinal peptide and secretin increased cellular cAMP and pepsinogen secretion in dispersed chief cells from guinea pig gastric mucosa. With each peptide there was a

close correlation between the dose-response curve for changes in cellular cAMP and that for changes in pepsinogen secretion. Vasoactive intestinal peptide and secretin had no agonist activity and antagonized the actions of vasoactive intestinal peptide and secretin on cellular cAMP and pepsinogen secretion Studies of binding of ^{125}I-vasoactive intestinal peptide and of ^{125}I-secretin indicate that gastric chief cells possess four classes of binding sites for vasoactive intestinal peptide and secretin. Occupation of two of these classes of binding sites correlate with the abilities of vasoactive intestinal peptide and secretin to increase cellular cAMP and pepsinogen secretion [5]. VIP also increased cellular levels of cAMP in human jejunal mucosa [13]. Similarly, VIP (5 x 10^{-8}M) prompted a twofold increase in cAMP levels in rat colon sacs [3]. These studies indicate that VIP stimulates cells within the mammalian gastrointestinal tract by activating membrane bound AC - which then generates increased levels of intracellular cAMP. CCK-8 does not appear to stimulate cellular secretion via cAMP but rather by acting through calcium-mediated mechanisms. Hersey and colleagues [22] demonstrated that AC activity in rabbit IGG was not increased by CCK-8. This study also revealed that absence of calcium in the culture medium prevented CCK-8 stimulation of chief cell secretion. Additional evidence suggests that CCK-8 is dependent upon calcium-mediated mechanisms as a second messenger system. Recent phosphorylation studies [27] are consistent with CCK-8 acting through calcium-calmodulin-dependent protein kinases. It is , therefore, probable that CCK-8 stimulates chief cell function through calcium-dependent second messenger systems.

In the study reported here, peptides acting through two different second messenger systems were tested. This study does not address the level at which the summation of th two second messenger signals occurs. Previous studies with CCK-8 have failed to reveal alterations in cAMP levels. It is therefore unlikely that activated calcium-calmodulin functions by augmenting cAMP levels either by activation of AC or inhibition of phosphodiesterase. As mentioned above, studies with CCK-8 have not shown alterations in cAMP levels. Alternatively, both systems may cause phosphorylation of the same protein kinases. The role of peptide hormone interaction or of membrane bound phosphatidyl inositol remains to be elucidated.

I have shown in this study that VIP or CCK-8 alone(in the concentrations used)failed to stimulate pepsinogen secretion. However, the combination of VIP acting through AC and cAMP together with CCK-8 acting through calcium systems succeeded in significantly stimulating pepsinogen secretion from rabbit IGG. These findings are consistent with the hypothesis that

pepsinogen secretion is dually modulated via interactions of the cAMP and calcium-calmodulin second messenger systems.

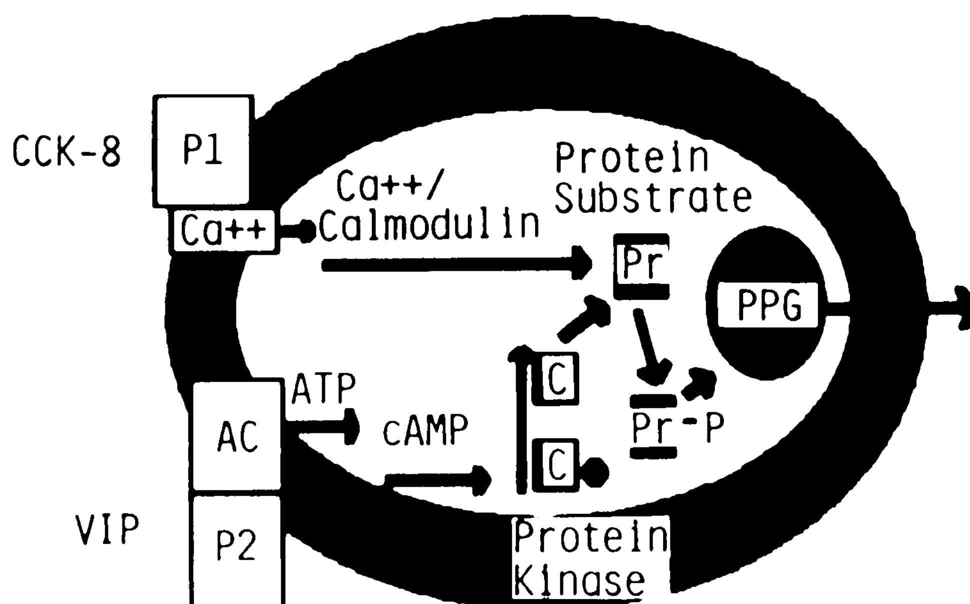

Figure 4. Stimulation of two classes of peptidergic membrane receptors (P1 and P2) can stimulate pepsinogen (PPG) secretion from gastric chief cells. Binding of P1 receptors by a secretagogue causes an influx of calcium (Ca^{2+}) into the cytosol. Calmodulin (C) binds this Ca^{2+} forming calcium-calmodulin (C-C). Binding of P2 by secretagogues activates adenylate cyclase (AC) which converts ATP to cAMP. The second messengers, cAMP and C-C, then cause secretion of PPG by the chief cell.

REFERENCES

1. Said, S.I., and Mutt, V. Polypeptide with broad biological activity: Isolated from small intestines. Science 169:1217,1970.

2. Bodansky, M., Klausner, Y., and Said, S. Biological activities of synthetic peptides corresponding to fragments of and to the entire sequence of the vasoactive intestinal peptide. Proc. Natl. Acad. Sci. USA 70:382,1973.

3. Polak, J.M., Pearse, A.G.E., Garaud, J.C., and Bloom, S.R. Cellular localization of a vasoactive intestinal peptide in the mammalian and avian gastrointestinal tract. Gut 15:720,1974.

4. Bryant, M.G., Polak, J.M., Modlin, I., Bloom, S.R., Albuquerque, R.H., and Pearse, A.G.E. Possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotransmitter substance. Lancet 1:991,1976.

5. Sutliff, Vincent E., Jean Pierre Raufman, Robert T. Jensen, and Jerry D. Gardner. Actions of vasoactive intestinal peptide and secretin on chief cells prepared from guinea pig stomach. Am. J. Physiol. 251 (Gastrointes. Liver Physiol. 14):G96-G102, 1986.

6. Said, S.I., and Faloona, G.R. Elevated plasma and tissue levels of vasoactive intestinal polypeptide in the watery-diarrhea syndrome due to pancreatic, bronchogenic and other tumors. N.Engl. J. Med. 293:155,1975.

7. Klaeveman, H.L., Conlon, T.P., Levy, A.G., and Gardner, J.D. Effects of gastrointestinal hormones on adenylate cyclase activity in human jejunal mucosa. Gastroenterology 68:667,1975.

8. Schwartz, C.J., Kimberg, D.V., Sheerin, H.E., Field, M., and Said, S.I. Vasoactive intestinal peptide stimulation of adenylate cyclase and active electrolyte secretion in intestinal mucosa. J. Clin. Invest. 54:536,1974.

9. Deschodt-Lanckman, M., Robberecht, P., De Neef, P., Labrie, F., and Christophie, J. In vitro interactions of gastrointestinal hormones on cyclic adenosine 3':5'-monophosphate levels and amylase output in the rat pancreas. Gastroenterology 68:318,1975.

10. Gardner, J.D., Conlon, T.P., and Adams, T.D. Cyclic AMP

in pancreatic acinar cells: Effects of gastrointestinal hormones. Gastroenterology 70:29,1976.

11. Long, B.W., and Gardner, J.D. Effects of cholecystokinin on adenylate cyclase activity in dispersed pancreatic acinar cells. Gastoenterology 73:10087,1977.

12. Gespach, C., Hoa, D.H.B., and Rosselin, G. Regulation by vasoactive intestinal peptide, histamine, somatostatin-14 and -28 of cyclic adenosine monophosphate levels in gastric glands isolated from the guinea pig fundus or antrum. Endocrinology 112:1597,1983.

13. Ballantyne, G.H., Zdon, M.J., Schafer, D.E., Fratesi, G.R., Roberts, J.R., Tishkoff, M., and Modlin, I.M. Evidence for dual modulation of pepsinogen secretion using isoproterenol, carbachol, CCK-8, forskolin, 8 bromo-cAMP, and A23187 probes. Ann. Surg., 204(5) 559-65, 1986.

14. Berglindh, T., and Obrink, K.J. A method for preparing isolated glands from rabbit gastric mucosa. Acta Physiol. Scand. 96:150,1976.

15. Anson,M.L., and Mirsky. A.E. The estimation of pepsin with hemoglobin. J.Gen.Physiol. 16:59,1932.

16. Schlamowitz, M. and Petersen, L.U. Studies on the optimum pH for the action of pepsin on "native" and denatured bovine serum albumin and bovine hemoglobin. J. Biol. Chem. 234:3137,1959.

17. Raufman, J.P., Kasbekar, D.K., Jensen, R.T., and Gardner, J.D. Potentiation of pepsinogen secretion from dispersed glands from rat stomach. Amer. J. Physiol. 245:G525, 1983.

18. Carter, R., Bitar, K.N., Zfass, A.M., and Makhlouf, G.M. Inhibition of VIP-stimulated intestinal secretion and cyclic AMP production by somatostatin in the rat. Gastroenterology 74:726,1978.

19. Raufman, J.P., Sutliff, V.E., Kasbekar, D.K., Jensen, R.T., and Gardner, J.D. Pepsinogen secretion from dispersed chief cells from Guinea pig stomach. Amer. J. Physiol. 247:G96,1984.

20. Rasmussen, H., and Barret, P.Q. Calcium messenger system: An integrated view. Physiol. Rev. 64:938, 1984.

21. Chew, C.S. Forskolin stimulation of acid and pepsinogen

secretion from isolated gastric glands. Amer.J.Physiol. 245:C371,1983.

22. Hersey, S.J., Miller, M., May, D., and Norris, S.H. Lack of interaction between acid and pepsinogen secretion in isolated glands. Amer. J.Physiol. 245:G775, 1983.

23. Koelz, H.R., Hersey, S.J., Sachs, G., and Chew, C.S. Pepsinogen release from isolated gastric glands. Amer. J.Physiol. 243:G218,1982.

24. Simon, B., and Kather, H. Activation of human adenylate cyclase in the upper gastrointestinal tract by vasoactive intestinal polypeptide. Gastroenterology 74:722,1978.

25. Olinger, E.J., and Gardner, J.D. Action of VIP and sectin on adenylate cyclase activity in acinar cells from guinea pig pancreas. Gastroenterology 77:704,1979.

26. Walsh, J. Gastrointestinal hormone and peptides. In L.R.Johnson (Ed.), Physiology of the Gastrointestinal Tract. New York:Raven Press, 1981. Pp.59-144.

27. Burnham, D.B., and Williams, J.A. Activation of protein kinase activity in pancreatic acini by calcium and cAMP. Amer.J.Physiol. 246:G500, 1984.

CHAPTER 12

Effect of Trifluoperazine, A Calmodulin Antagonist, on Pepsinogen Secretion

INTRODUCTION

The therapy of peptic ulcer disease (PUD) has focused mainly on acid secretion. The role of the powerful gastric proteolytic enzyme pepsin in the pathogenesis of PUD is, however, much less well understood. This role may be deserving of investigation, since inhibition of either the proteolytic activity of pepsin or the release of its precursor, pepsinogen, may be among practical therapeutic options.

As the principal gastric protease, pepsin attracted the attention of some of the earliest physiologists and biochemists. Over a century ago Langley suggested, on the basis of microscopic investigations, that pepsin is stored in granules in the gastric chief cell and released on stimulation [1]. Pepsin and pepsinogen were among the first enzymes to be crystallized and extensively characterized [2]. It is only within the past decade, however, that a consistent picture of the physiological stimulation of pepsinogen release by chief cells has began to unfold [3].

I have previously reported that pepsinogen secretion is modulated at the intracellular level by both cAMP and calcium [4,5]. For example, pepsinogen secretion is stimulated by cholera toxin [6] and by isobutyl methylxanthine [7], both of which increase intracellular cAMP levels. It is also stimulated by acetylcholine [7-9], which is believed to act on the pancreatic acinar cell by raising levels of cytosolic-free calcium [10,11]. Cholecystokinin octapeptide (CCK-8), a potent stimulus for pepsinogen secretion, is also believed to raise cytosolic-free calcium concentrations comparable to those known to activate pancreatic acinar secretion. Evidence for dual modulation of pepsinogen secretion has also been obtained by other investigators [3,12].

While calcium ions may potentially influence biological processes in many different ways, only two general mechanisms have well-documented roles in the calcium messenger system that mediates physiological regulatory stimuli [13]. One of these mechanisms, the stimulation by calcium of phospholipid-dependent protein kinases (C kinases), is still in the early stages of investigation, and has not yet been unambiguously implicated in gastrointestinal secretory processes [14]. The second mechanism, which involves the binding of calcium ions to calmodulins, forming active calcium/calmodulin complexes, has been extensively studied since its discovery in 1970 [15]. Calmodulin may play a role in some calcium-mediated responses of the pancreatic exocrine cell to secretagogues [16].

In an effort to identify inhibitors of pepsinogen release with potential therapeutic application in PUD, and to obtain information about the possibility that calcium may stimulate pepsinogen secretion through the calmodulin mechanism, I have examined the effect of calmodulin antagonists on pepsinogen secretion. the phenothiazine antipsychotic drugs have long been known to block the stimulation of phosphodiesterase by calmodulin [17]. Over the past decade they have come to be among the most extensively investigated of the calmodulin blockers [17]. One of the most potent of the phenothiazines is trifluoperazine (TFP, Stelazine), with an IC_{50} of 10^{-5}M [17].

It is probable that there are calmodulins in rabbit chief cells, as in all other eukaryotic cells. Moreover, it is highly probable, on the basis of available data, that there exists a pepsinogen secretory pathway responsive to one or more chief cell calmodulins, which are sensitive to phenothiazines, including TFP; and that TFP can gain access to these calmodulins and thus inhibit the calmodulin-sensitive pepsinogen secretory pathway. We have therefore studied the effect of TFP on pepsinogen secretion in vitro, using the isolated gastric gland (gastron) system. The advantage of this preparation is that modulatory processes may be scrutinized apart from the interference of extrinsic neural, paracrine, or endocrine influences [5]. There is, however, a likelihood that several cell types present in the isolated gastric gland preparation may interact in a paracrine fashion. CCK-8 stimulates pepsinogen secretion by isolated rabbit and rat gastric glands [3,4,12], The purpose of this study was therefore to evaluate the effect of a calcium-calmodulin antagonist (TFP) on pepsinogen secretion stimulated by CCK-8.

METHODS

Isolated gastric glands were prepared from rabbit stomachs perfused in situ at high pressure with colloid-free buffered saline, as described by Berglindh and Obrink [8]. Stripped fundic mucosa was finely chopped and incubated in collagenase-containing medium for 45 minutes at 37°C. The digest was washed repeatedly with serial filtration through successively finer nylon mesh, and the resulting washed glands were resuspended and incubated in buffered medium containing glucose and albumin, as previously described [4,5]. In the present study, glands were incubated for 30 minutes in the presence and absence of CCK-8 and TFP.

The effect of CCK-8 and TFP, separately and combined, on pepsinogen secretion were evaluated quantitatively from dose-response curves obtained under three experimental conditions. In the first set of experiments, pepsinogen secretion was determined as a function of concentration of CCK-8 alone (10^{-11}M-10^{-5}M. These dosages reflect concentrations recognized to be within the physiological

range. The second set of experiments determined the effects of TFP (10^{-6}M-10^{-3}M) on pepsinogen secretion in the presence of a maximally effective concentration of CCK-8 (10^{-7}M). In the third set, the effects of TFP alone (2 x 10^{-5}M - 5 x 10^{-4}M) on pepsinogen secretion were measured.
Pepsinogen secretion was assayed by measuring net pepsinogen release into the medium. We used a modification of the proteolytic method of Anson and Mirsky [19], as previously described [4,5]. [^{14}C] Methemoglobin (New England Nuclear, Boston, Mass.), 10^5 cpm, was added per millimiter of assay buffer before proteolysis, and ^{14}C remaining in the digest supernatant after trichloroacetic acid precipitation was measured in a beta counter (Beckman Instruments, Fullerton,Calif). Total gland pepsinogen content was measured by freezing gland suspensions in liquid nitrogen, thawing, and assaying the supernatant for pepsinogen.

In another series of experiments, the release into the medium of lactate dehydrogenase (LDH) was measured to assess the possibility of nonspecific effects of TFP. LDH was measured by the method of Wacker et al [20], in which L-lactate is oxidized to pyruvate with simultaneous reduction of NAD to NADH, and NADH production is measured at 340nm. Assay reagents were obtained from Gilford Diagnostic, Cleveland, Ohio.

The effects of TFP on cellular cAMP levels were measured in parallel 30-min incubations terminated by boiling for 3 min and centrifugation. Supernatant cAMP concentrations were measured by ^{125}I-radioimmunoassay (Becton-Dickinson Immunodiagnostics, Orangeburg, N.Y.).

At each concentration of TFP or CCK-8, mean values of each of the measured variables were compared with each other and with unstimulated values of Student's t-test, and judged to be statistically significant at $P<0.05$.

RESULTS

CCK-8 stimulated pepsinogen secretion in a dose-related fashion (Fig. 1). At 10^{-11}M and 10^{-10}M, no effect of CCK-8 on pepsinogen release was seen. At 10^{-9}M, CCK-8 significantly stimulated the secretion of pepsinogen over basal levels ($P<0.05$). A further significant increase was seen at 10^{-8}M ($P<0.05$). There was no significant difference between 10^{-8}M and 10^{-7}M ($P>.0.05$).

When isolated gastric glands were stimulated with CCK-8, 10^{-7}M, in the presence and absence of TFP, 10^{-6}M to 10^{-3}M (Fig.2), CCK-8 alone gave a three-fold stimulation over basal levels (Fig. 2), and this response was unaffected by 10^{-6}M and 10^{-5}M

TFP ($P>0.05$). At 10^{-4}M and 10^{-3}M TFP, pepsinogen secretion was 5.6 and 6.5 times the basal level, respectively. This response was significantly greater ($P<0.05$) than that obtained with CCK-8 alone (Fig. 2). Thus CCK-8 stimulation of pepsinogen secretion was not inhibited at any TFP concentration (Fig. 2).

When TFP was tested alone, it had no significant effect on release of pepsinogen at concentrations up to 2 x 10^{-5}M (Fig. 3). At 5 x 10^{-5}M and above, TFP significantly stimulated pepsinogen secretion ($P<0.05$). The mean response increased in dose-related fashion. At 2 x 10^{-4}M and 5 x 10^{-4}M, the response was significantly greater than 5 x 10^{-5}M ($P<0.05$).

In experiments designed to assess the possibility that TFP might nonspecifically promote the release of cell protein, LDH release into the medium was not significantly greater in the presence of TFP, up to 2 x 10^{-4}M, than in the absence of stimulation (Fig. 4).

Likewise, in experiments to determine whether TFP might affect pepsinogen secretion by altering cAMP levels (Fig. 5), TFP at 10^{-8}M - 10^{-3}M had no significant effects on glandular cAMPlevels ($P>0.05$). In the same experiments the adenylate cyclase activator forskolin, 10^{-5}M, stimulated an increase of approximately 20-fold (Fig. 6).

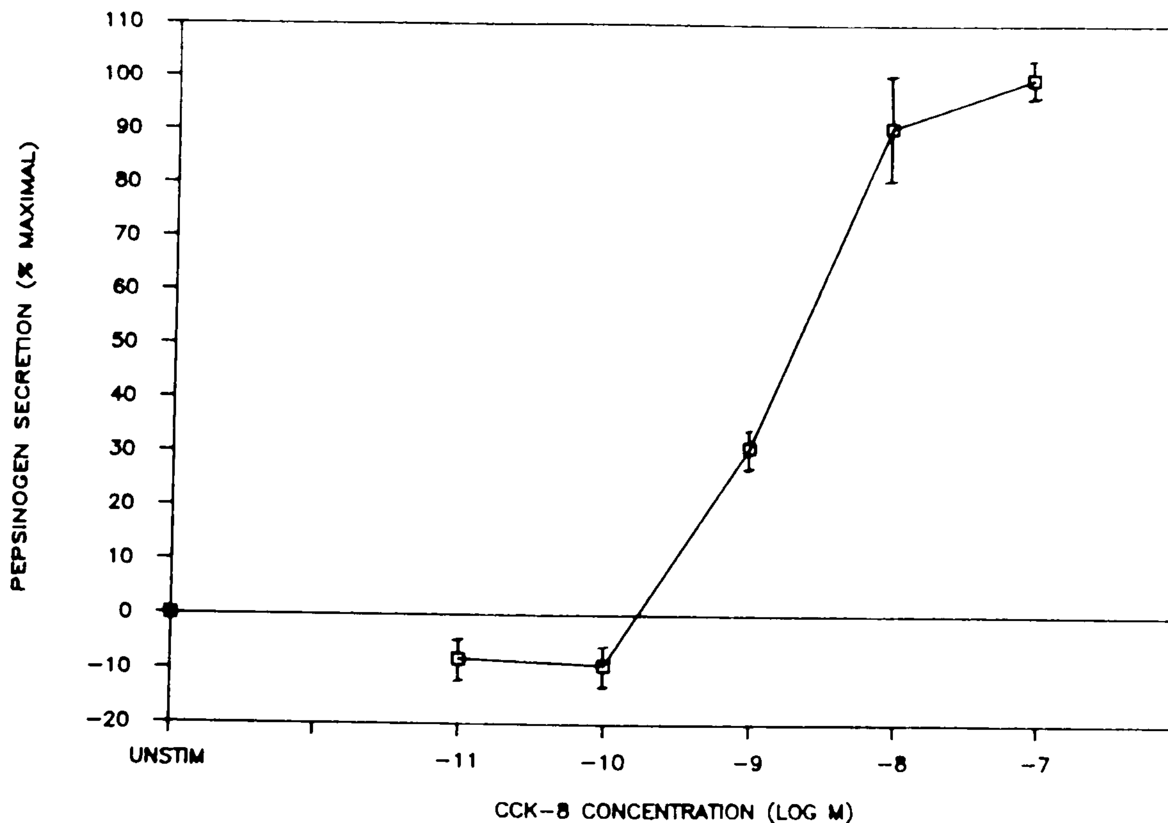

Figure 1. Dose-response curve for pepsinogen secretion as a function of CCK-8 concentration (10^{-11}M-10^{-7}M). Pepsinogen release is expressed as the mean percentage of the maximum observed in excess of basal (unstimulated) levels ± 1 SEM.

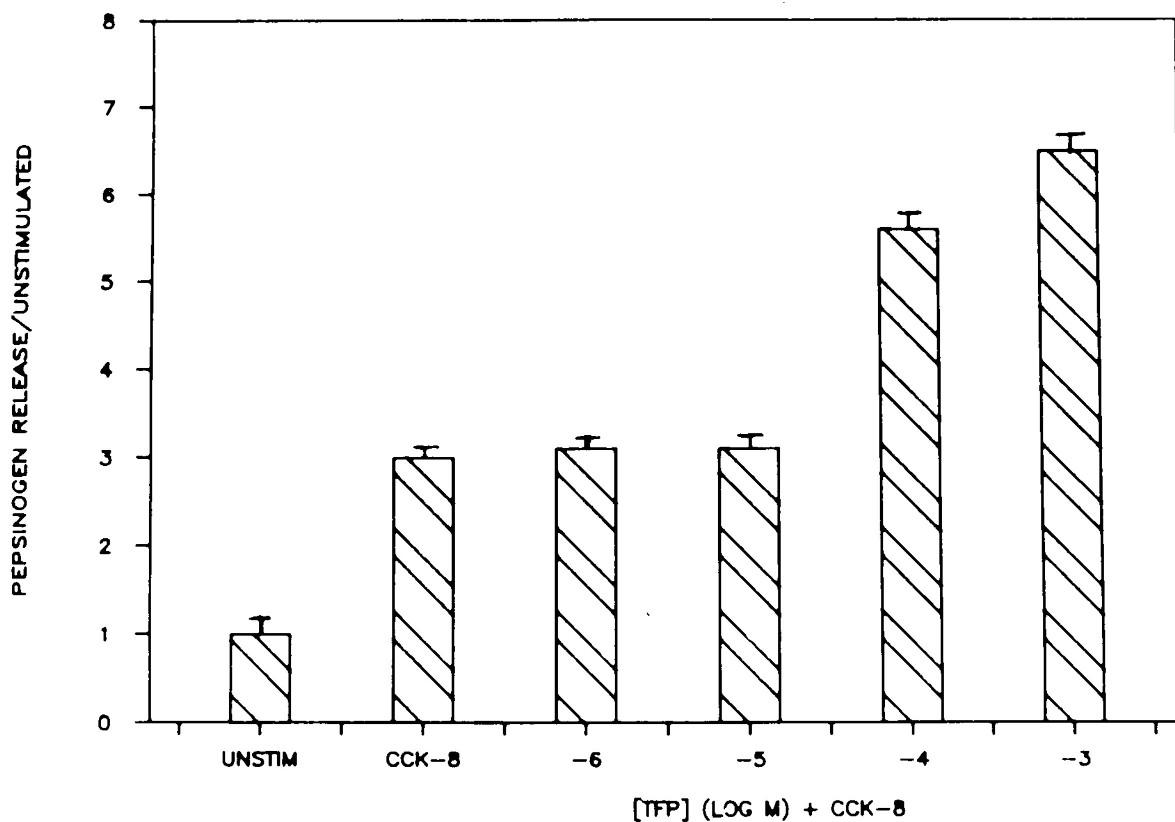

Figure 2. Effects of TFP (10^{-6}M-10^{-3}M) on pepsinogen secretion in the presence of 10^{-7}M CCK-8, a maximally effective concentration. The results are expressed relative to pepsinogen release by glands incubated in the absence of both TFP and CCK-8 (UNSTIM). Release by glands stimulated with CCK-8 alone is also shown (0). The values are means ± 1 SEM.

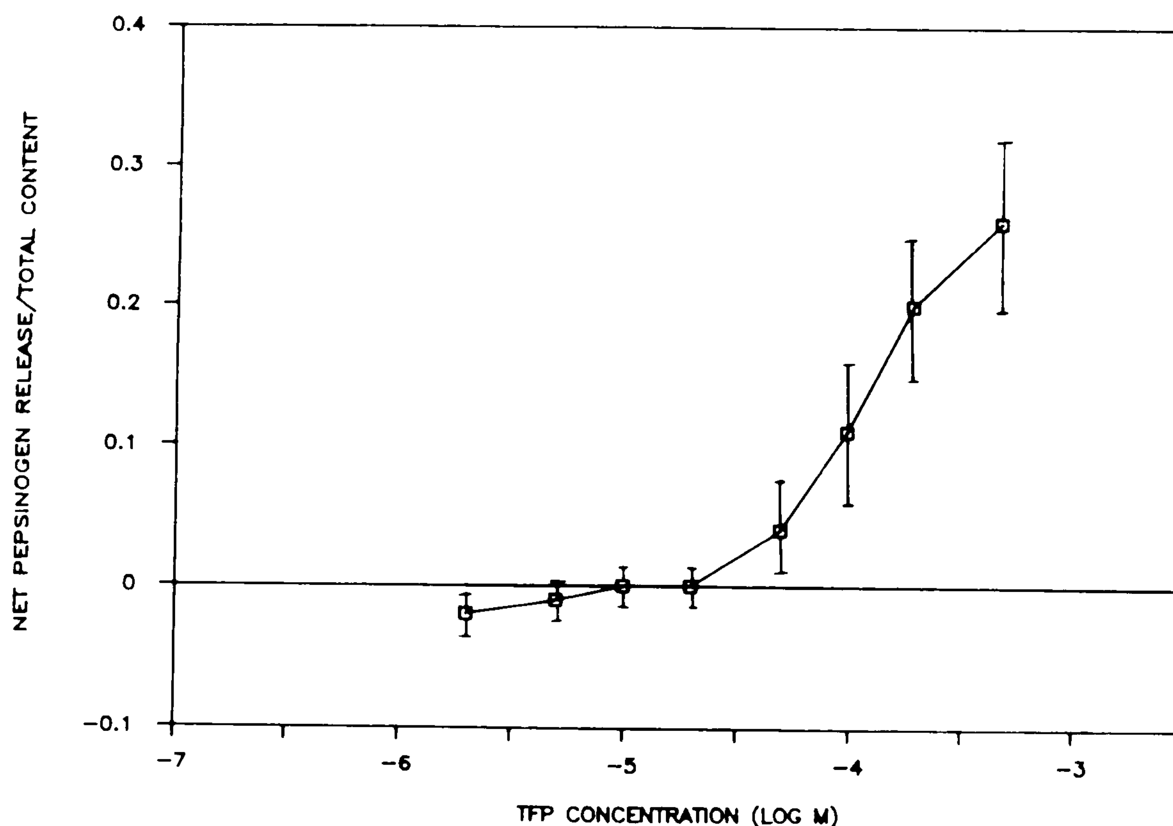

Figure 3. Dose-response curve for net pepsinogen secretion, in excess of basal (unstimulated0 levels, as a function of TFP concentration (2 x 10^{-6}M - 5 x 10^{-4}M), in the absence of CCK-8. Pepsinogen release is expressed as a fraction of total gland content of pepsinogen. Values are means ± 1 SEM.

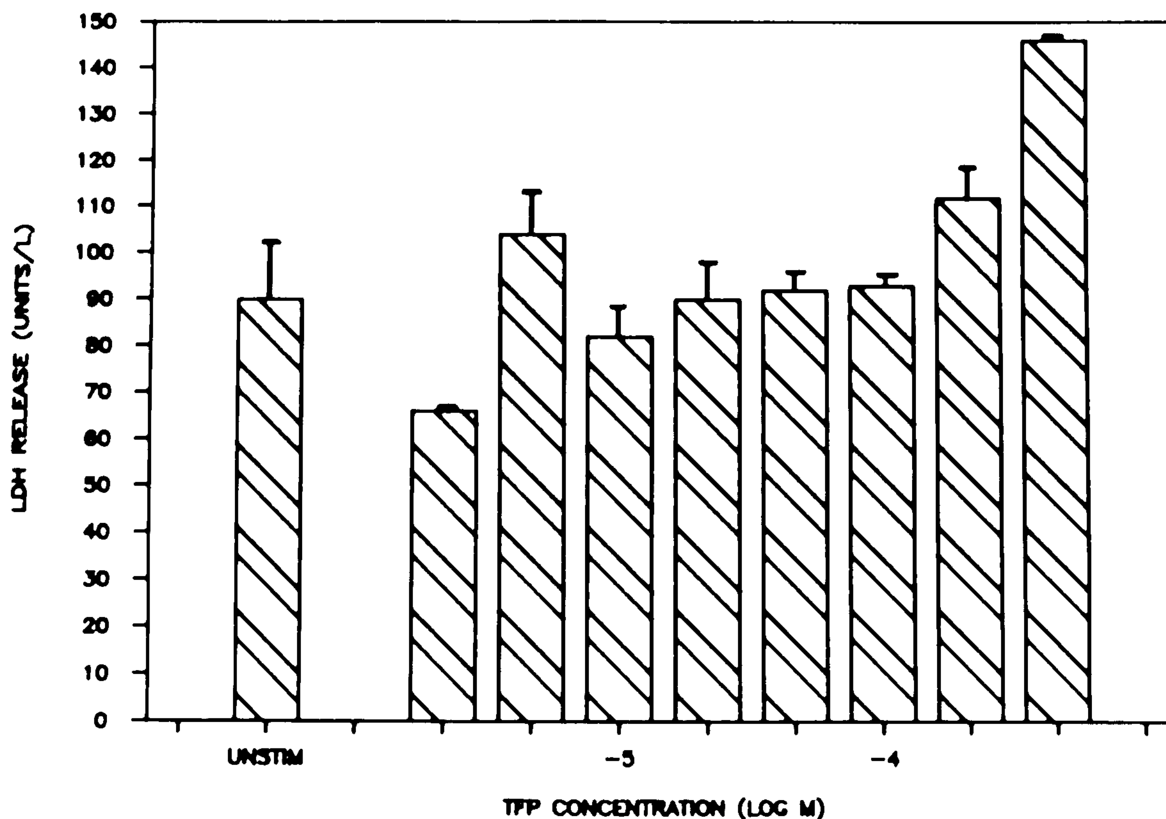

Figure 4. Effect of TFP (2×10^{-6}M - 5×10^{-4}M) on LDH release from isolated gastric glands in 30 min. LDH release from glands incubated for the same interval in the absence of TFP is also shown (UNSTIM). The values are means $\pm$ 1 SEM.

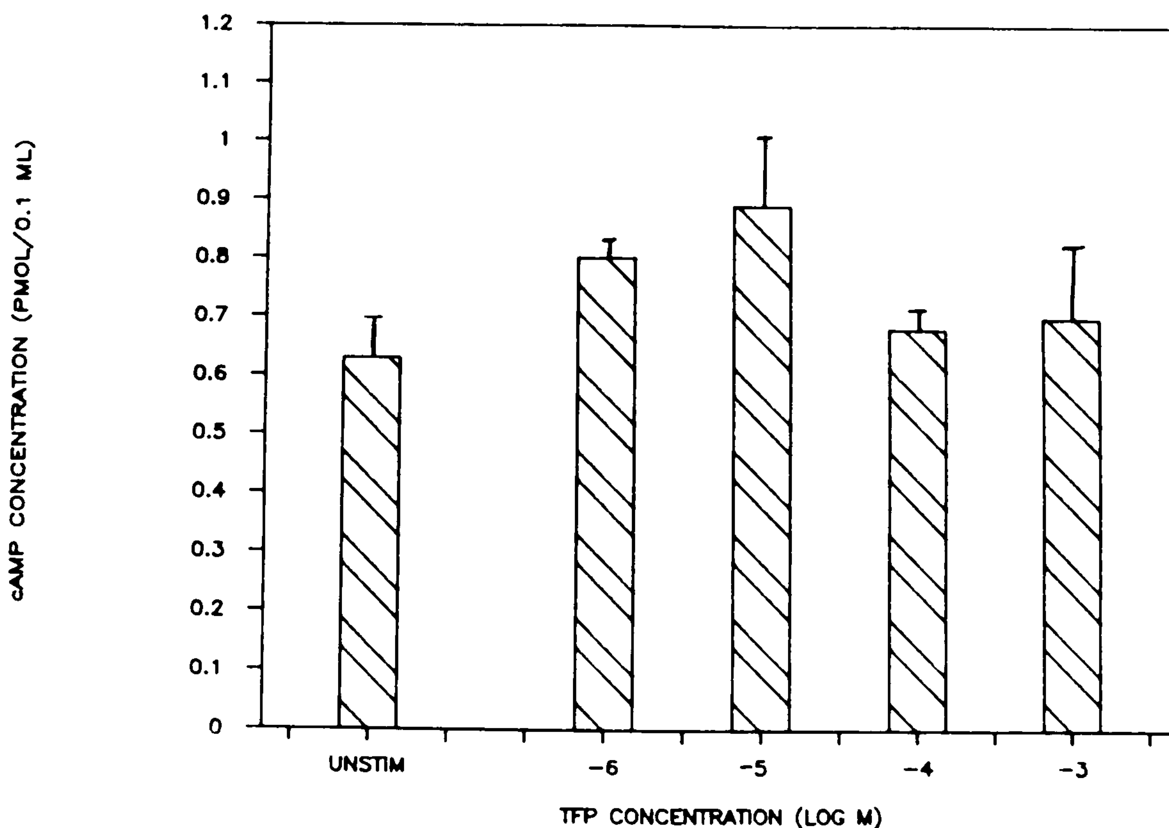

Figure 5. Dose-response curve for the effect of TFP (10^{-6}M-10^{-3}M) on cAMP levels in isolated gastric glands. cAMP levels in glands incubated without TFP are also shown (UNSTIM). The values are means $\pm$1 SEM.

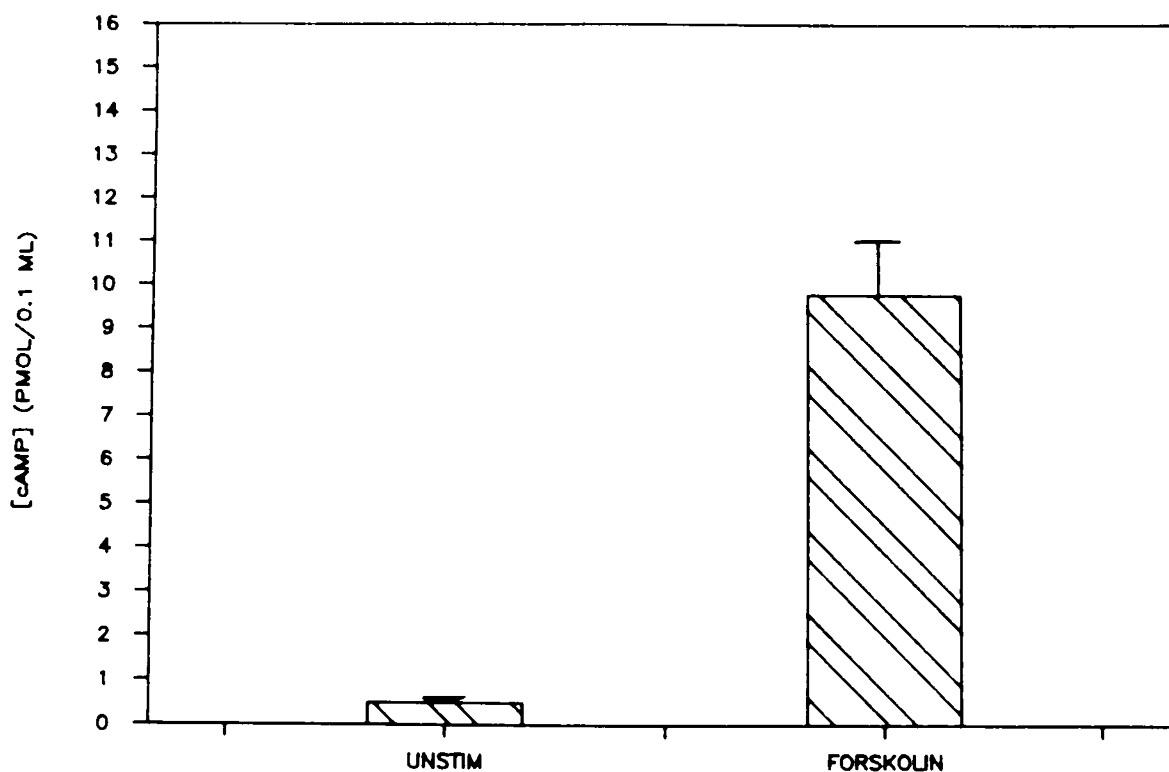

Figure 6. Effect of 30-min incubation with forskolin, 10^{-5}M, on cAMP levels in isolated gastric glands. The cAMP content of glands incubated in the absence of forskolin is also shown (UNSTIM). The values are means ± 1 SEM.

DISCUSSION

The present results show not only that TFP does not inhibit the stimulatory effects of CCK-8 on pepsinogen secretion at the low concentrations where it might be predicted to do so, but also that at somewhat higher concentrations TFP alone actually stimulates pepsinogen secretion. In an attempt to explain this unexpected and paradoxical result I investigated two possibilities. The first of these was that TFP might cause the release by a nonspecific mechanism that would liberate other cell proteins such as LDH [12]. The second was that TFP might stimulate by the other known mechanism for the modulation of pepsinogen secretion, namely, the cAMP-dependent mechanism.

Our results with LDH show that up to 10^{-4}M, TFP has no effect on the release of this marker protein. This concentration enhanced pepsinogen secretion in both the presence and absence of CCK-8. Thus up to this concentration, at least, the stimulatory effect of TFP is specific for pepsinogen secretion. Presumably in these experiments LDH was released by other gland cell types in addition to chief cells.

The results of the measurements of cellular cAMP show that cellular adenylate cyclase is exquisitely responsive to forskolin, 10^{-5}M, under the experimental conditions employed. Thus adenylate cyclase systems in these glands are functional and the assay is capable of detecting much smaller changes in cAMP levels than those produced by forskolin. Yet TFP produced no discernible effect on cell cAMP levels. Thus it is unlikely that TFP alters pepsinogen secretion by increasing cAMP levels.

The foregoing observations raise three very different questions, which must be considered separately: First, why does TFP not inhibit the stimulatory action of CCK-8 on pepsinogen secretion? Secondly, how does TFP itself stimulate the release of pepsinogen? And third, what implications do these observations have for our understanding of the physiological modulation of pepsinogen secretion and for its control?

TFP permeates cell membranes readily, and the IC_{50} for inhibition of calmodulin by TFP in a great variety of in vitro test systems is 10^{-5}M [17]. Thus, where TFP is observed to block calmodulin, it usually does so at concentrations below 5 x 10^{-5}M [17]. The present experiments encompassed the range 10^{-8}M to 10^{-3}M. Thus the absence of an inhibitory effect of TFP in these experiments probably cannot be attributed to the use of too high or too low a concentration of TFP.

Sanders et al. have reported that in monolayers of canine chief cells carbachol-stimulated PS is inhibited by the calmodulin antagonist chlorpromazine, a phenothiazine derivative analogous to trifluperazine [21]. Similarly, N,N-(diethylamin0)octyl-3,4,5-trimethoxybenzoate (TMB-8), which is believed to inhibit the mobilization of intracellular calcium, inhibits carbachol-stimulated PS [21]. The explanation of the apparent discrepancy between these results and mine is not yet clear, though several possibilities must be considered. While chlorpromazine and trifluoperazine commonly have qualitatively similar effects on the same tissues, the former istypically the less active of the two as a calmodulin antagonist. Thus, it is not probable that the difference is due to the agents used. Likewise it is possible that species differences in calcium-mediated pepsinogen secretion account for the difference. No major qualitative differences in this regard between the dog and the rabbit have received attention, however.

The calmodulin-sensitive pathway may not be sufficiently active under the experimental conditions used here, including CCK-8 stimulation, for its inhibition to have been detected. Possibly these discrepancies may even reflect fundamental differences between the mechanism of carbachol and CCK-8 stimulation. Neither mechanism has been unambiguously established for any cell system, and no direct evidence is available for the chief cell. A priori it would be simplistic to assume that a cholinomimetic neurotransmitter and a peptide hormone act in completely identical ways. In chief cells, CCK-8 may work primarily by a pathway that is not calmodulin-dependent. This concept is supported by the fact that N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), a calmodulin inhibitor belonging to a different class [22], also failed to inhibit CCK-8 stimulation of pepsinogen secretion in my system.

The explanation may also rest on pharmacokinetic differences between isolated glands and chief cell monolayers, or in the actual experimental design. The failure of TFP to inhibit pepsinogen secretion in these experiments may mean that TFP does not reach the calmodulin-sensitive pepsinogen secretory pathway at the proper time in sufficient amounts. Such failure could suggest that transport of TFP to the calmodulin site regulating the pathway is impeded. This interpretation leads to the second question. How does TFP at high concentrations stimulate pepsinogen secretion? Clearly TFP reaches some region of the chief cells. Thus it is unlikely that TFP failed at all concentrations in our experiments to reach chief cell calmodulin sensitive to TFP. It must not be assumed, however, that the inhibitory and stimulatory sites of TFP action are the same.

The precise nature of the stimulatory process cannot be deduced from the present studies. The phenothiazines have been reported to interact with numerous other cellular constituents, including several types of receptors for neurotransmitters, phospholipids and other calcium-binding proteins, such as trophin C [17]. The possibilities are especially numerous at the high TFP concentrations where the stimulation of pepsinogen secretion occurs. In principle it is impossible to absolutely rule out nonspecific effects. Thus, although LDH has been used as a marker for clear-cut nonspecific effects of other agents that stimulate pepsinogen secretion [23], it may not be sufficiently sensitive to reveal more subtle nonspecific actions. Even the demonstration that TFP does not appreciably alter cAMP levels in the isolated glands does not entirely exclude the possibility of significant cAMP accumulations at sensitive local sites within the chief cells.

In spite of these reservations, however, the stimulation of pepsinogen secretion by TFP cannot be ignored, especially in the light of the third question: What implications do these observations have for the understanding of the physiological modulation of pepsinogen secretion and for its control? Thefact is that the intracellular mechanisms controlling the release of pepsinogen are still largely obscure. The detailed events by which cAMP and calmodulin ultimately bring about the release of the pepsinogen molecule are mainly subjects of speculation. Thus the intriguing possibility remains that TFP stimulates pepsinogen secretion through a hitherto undescribed mechanism, which may point to a final pathway common to all regulatory mechanisms for pepsinogen secretion. The reported interactions of TFP with neurotransmitters and phospholipids, mentioned above [17], underscore the need for further investigation of this possibility.

These results indicate that TFP is unlikely to be of use as a secretory blocker for pepsinogen, at least to the extent that pepsinogen secretion is under the control of CCK-8. Its potential usefulness as an antagonist to pepsinogen secretion in response to other secretagogues such as acetylcholine is still to be determined. The identification of an intracellular inhibitor of pepsinogen secretion akin to a parietal cell proton pump blocker may be the next logical step in management of PUD.

REFERENCES

1. Langley,J.N. On the histology and physiology of pepsin-forming glands. Phil. Trans. R. Soc. London Ser. B172:664.

2. Herriott,R.M. Pepsinogen and pepsin. J. Gen. Physiol. 45:57,1962.

3. Hersey,S.J., Norris,S.H., and Gibert,A.J. Cellular control of pepsinogen secretion. Annu. Rev. Physiol. 46:393,1984.

4. Ballantyne,G.H., Zdon,M.J., Schafer,D.E., Fratesi,G.R., Roberts,J.R., Tyshkov,M., and Modlin,I.M. Evidence for dual modulation of pepsinogen secretion using isoproterenol, carbachol, CCK-8, forskolin, 8-bromo-cAMP, and A23187 probes. Arch. Surg. 204(5):559-565,1986.

5. Modlin,I.M., Schafer,D.E., Ballantyne,G.H., Fratesi,G.R., Tyshkov,M., and Zdon,M.J. Isolated gastric glands in the study of gastric secretory relationships. Surg. Res. Comm. 1(2):81-96,1987.

6. Schafer,D.E., and Garshfield,G.N. Cholera enterotoxin stimulates marked pepsinogen secretion by isolated gastric fundic glands. Fed. Proc. 41:1432,1982.

7. Schafer,D.E. Acetylcholine, calcium, and cyclic nucleotides in the cellular control of pepsinogen secretion. Biophys. J. 17:276a,1977.

8. Schafer,D.E., Ebersole,E.R., and Donaldson,R.M.,Jr. Physostigmine and isobutylmethylxanthine stimulate pepsinogen secretion synergistically. Fed. Proc. 38:885,1979.

9. Sutton,D.R., and Donaldson,R.M.,Jr. Synthesis and secretion of protein and pepsinogen by rabbit gastric mucosa in organ culture. Gastroenterology 69:166,1975.

10. Gardner,J.D., Conlon,T.P., Klaeveman,H.L., Adams,T.D., and Ondetti,M.A. Action of cholecystokinin and cholinergic agents on calcium transport in isolated pancreatic acinar cells. J.Clin. Invest. 56:366,1975.

11. Williams,J.A. Regulation of pancreatic acinar cell function by intracellular calcium. Amer. J. Physiol. 238:G269,1980.

12. Raufman,J.-P., Kasbekar,D.K., Jensen,R.T., and Gardner,J.D. Potentiation of pepsinogen secretion from dispersed glands from rat stomach. Amer. J. Physiol. 245:G525,1983.

13. Rasmussen,H., and Barrett,P.Q. Calcium messenger system: An integrated view. Physiol. Rev. 64:938,1984.

14. Kishimoto,A., Takai,Y., Mori,T., Kikkawa,U., and Nishizuka,Y. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphoinositol turnover. J. Biol. Chem. 255:2273,1980.

15. Cheung,W.Y. Calmodulin plays a pivotal role in cellular regulation. Science 207:19,1980.

16. Gorelick,F.S., Cohn,J.A., Freedman,S.A., Delahunt,W.G., Gershoni,J.M., and Jamieson,J.D. Calmodulin-stimulated protein kinase activity from rat pancreas. J. Cell Biol. 97:1294,1983.

17. Weiss,B. Techniques for measuring the interaction of drugs and calmodulin. In A.R. Means and B.W. O'Malley Eds.), Methods in Enzymology Vol. 102, Hormone Action, Calmodulin and Calcium-Binding Proteins, Part G. New York: Academic Press, 1983. pp. 171-184.

18. Berglindh,T., and Obrink,K.J. A method for preparing isolated glands from the rabbit gastric mucosa. Acta Physiol. Scand. 96:150,1976.

19. Anson,M.L., and Mirsky,A.E. The estimation of pepsin and hemoglobin. J. Gen. Physiol. 16:59,1932.

20. Wacker,W.E.C., Ulmer,D.D., and Vallee,B.L. Metalloenzymes and myocardial infarction II. Malic and lactic dehydrogenase and zinc concentrations in serum. N. Engl. J. Med. 255:449,1956.

21. Sanders,M.J., Amirian,D.A., and Soll,A.H. Stimulus-secretion coupling of pepsinogen release in canine chief cell monolayers. Fed. Proc. 42:591,1983.

22. Hidaka,H., and Tanaka,T. Naphthalenesulfonamides as calmodulin antagonists. In A.R.Means and B.W.O'Malley (Eds.), Methods in Enzymology Vol.102, Hormone Action, Calmodulin and Calcium-Binding Proteins, Part G. New York: Academic Press, 1983. pp. 185-194.

23. Raufman,J.P., Sutliff,V.E.,Kasbekar,D.K., Jensen,R.T., and Gardner,J.P. Pepsinogen secretion from dispersed chief cells from guinea pig stomach. Amer. J. Physiol. 247:G95,1984.

CHAPTER 13

Pepsin Release by a Prostaglandin E_1 Analogue

INTRODUCTION

Prostaglandins are potent inhibitors of gastric acid secretion [1-3]. Furthermore, they appear to be capable of protecting the gastric mucosa against a wide variety of noxious agents. This "cytoprotective" property appears to be independent of acid inhibition since it is apparent at much lower concentrations and is observed to occur even with prostaglandins that do not inhibit acid secretion [4,5].

The effects of prostaglandins on gastric pepsinogen secretion are less clear. Since pepsin is a potent mucosal barrier breaker strongly implicated in ulcerogenesis, [6] changes in pepsinogen secretion due to prostaglandin therapy may be of consequence in determining the therapeutic potential of prostaglandins in the treatment of peptic ulcer disease. The effects of prostaglandins on gastric secretion in human and intact animal studies have been difficult to interpret because of complex endocrine, paracrine, and neural influences. In addition, changes in acid and fluid secretion create artifacts in the measurement of pepsinogen secretion.

Therefore, the purpose of this study was to delineate the effect of a prostaglandin E_1 (PGE_1) analogue (misoprostol) [7] on acid and pepsinogen secretion in an experimental model exempt from endocrine and neural influence. The actions of misoprostol on the secretion of pepsinogen and acid were accordingly studied in isolated gastric glands and parietal cells of the rabbit.

METHODS

Isolated gastric glands and parietal cells for pepsinogen and acid studies were prepared from gastric mucosa of 3 - 4 kg male New Zealand white rabbits.

Isolated gastric glands were prepared by methodology previously described in detail. Isolated parietal cells were prepared as per methodology detailed earlier in this thesis. Acid secretion of the parietal cells was assessed by the uptake of diphenylamine carbon 14-labelled aminopyrine. This is a weak base that is nonionized at neutral pH and freely diffuses across the cell membrane. Within the acidic secretory canaliculi of a stimulated parietal cell, aminopyrine becomes ionized and is trapped within the cell. Accumulation of labeled aminopyrine in the parietal cell is therefore an indirect measure of acid secretion. Parietal cell suspensions were incubated with ^{14}C-aminopyrine for one hour at 37° C in oxygenated respiratory medium containing the agents under study. After incubation, the cells were filtered and the percentage of aminopyrine taken up by the cells was counted using a beta counter.

EXPERIMENTAL DESIGN

The gastric glands were incubated for 40 minutes with misoprostol in concentrations ranging from 10^{-11} to 10^{-6}mol/L at 37°C with continuous oxygenation. Misoprostolwas initially dissolved in ethyl alcohol and then serially diluted using a buffered incubation medium. Pepsinogen secretion of the treated glands was expressed as a multiple of the rate of secretion by untreated glands. No histamine was used.

Using these techniques, studies of acid secretion were performed in isolated parietal cells and studies of pepsinogen secretion in isolated gastric glands. Enriched parietal cell suspensions were initially incubated with identical concentrations of misoprostol and vehicle for one hour at 37°C during oxygenation.

In a second series of experiments, enriched parietal cell suspensions were incubated with the same concentrations of the misoprostol and the vehicle in the presence of 10^{-6}mol/L of histamine. After each experiment, the aminopyrine uptake of the misoprostol-treated cells was measured and compared with control cells (unstimulated for the first series of experiments and histamine-stimulated for the second.)

Data presented represent the mean of six experiments. Data were analyzed by Student's paired t-test; $P<.05$ was considered statistically significant.

RESULTS

Misoprostol stimulated pepsinogen secretion in isolated gastric glands in a dose-dependent manner. This effect was statistically significant at concentrations as low as 10^{-10}mol/L ($P<.05$). Half-maximal stimulation occurred at 10^{-8}mol/L whereas treatment of the glands with misoprostol at 10^{-7}mol/L concentrations resulted in a doubling of pepsinogen secretion over the unstimulated state (Fig. 1). The alcohol vehicle had no effect on pepsinogen secretion at the concentrations required to deliver 10^{-6}mol/L misoprostol.

Misoprostol alone had no effect on acid secretion from unstimulated isolated parietal cells, even at 10^{-6}mol/L (1.1 ± 0.2 times unstimulated). However, misoprostol significantly blocked histamine stimulation of acid secretion at doses as low as 10^{-10}mol/L ($P<.05$; Fig. 2). Half-maximal blockade was seen at 10^{-9}mol/L and 78% inhibition of acid secretion occurred with 10^{-7}mol/L misoprostol. The alcohol vehicle used to dissolve the misoprostol had no effect on basal or histamine stimulated acid secretion at similar concentrations. Histamine at 10^{-6}mol/L alone stimulated acid secretion in isolated parietal cells to 2.1 ± 0.3 times the unstimulated rate.

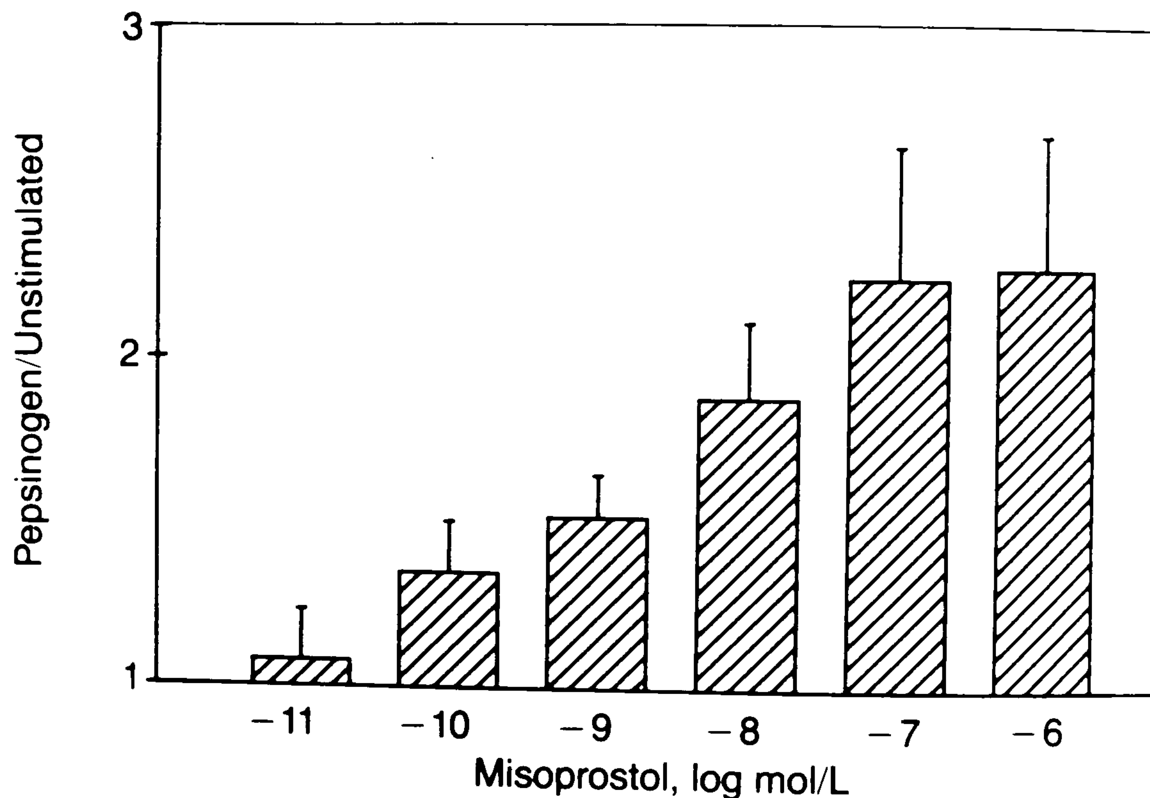

Figure 1. Misoprostol stimulates pepsinogen secretion (given as multiple of unstimulated values) in isolated rabbit gastric glands. Data are normalized to unstimulated state and represent mean of six experiments. Values differ significantly from unstimulated state ($P<.05$) except for 10^{-11}mol/L. Unstimulated pepsinogen secretion (not shown) is 1.0.

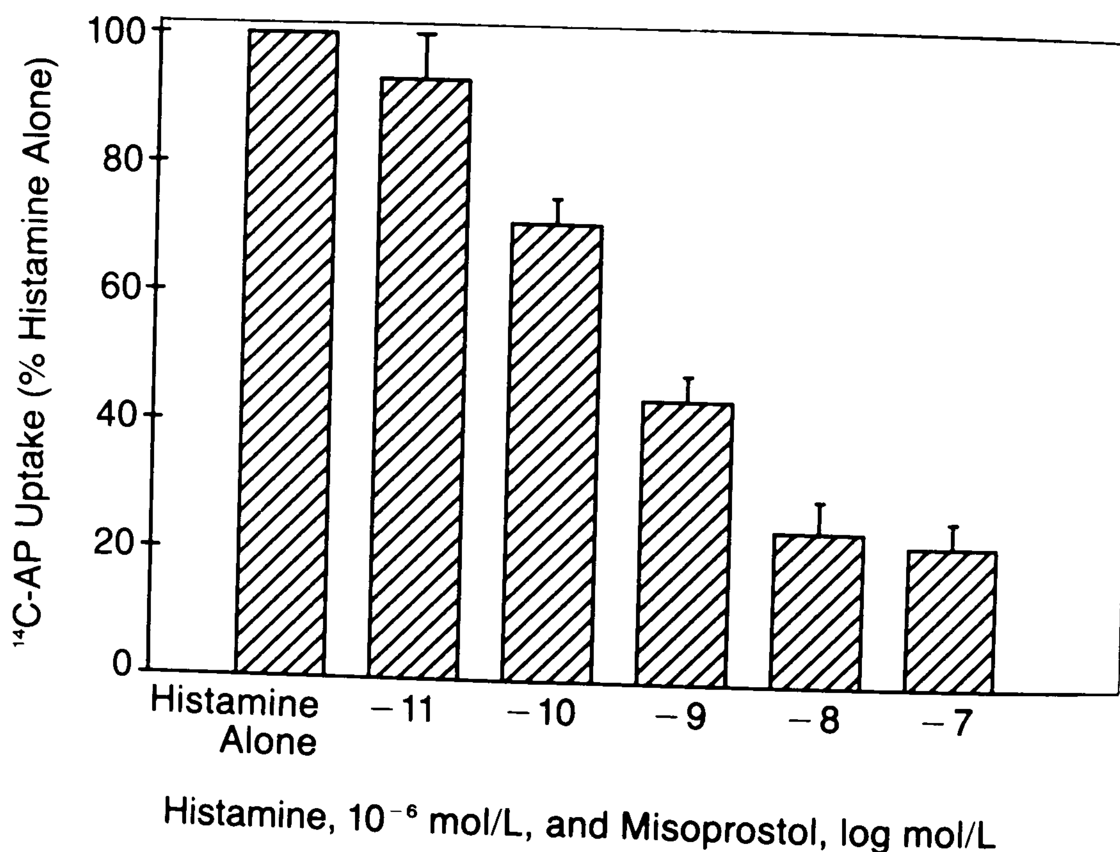

Figure 2. Misoprostol inhibits acid secretion in histamine-stimulated isolated rabbit parietal cells. Data are normalized to rate of acid secretion in parietal cells exposed to 10^{-6}mol/L of histamine without misoprostol and represent mean of six experiments. All values differ significantly from unstimulated state ($P<.05$) except 10^{-11} and 10^{-10}mol/L. ^{14}C-AP indicates carbon 14-labeled aminopyrine.

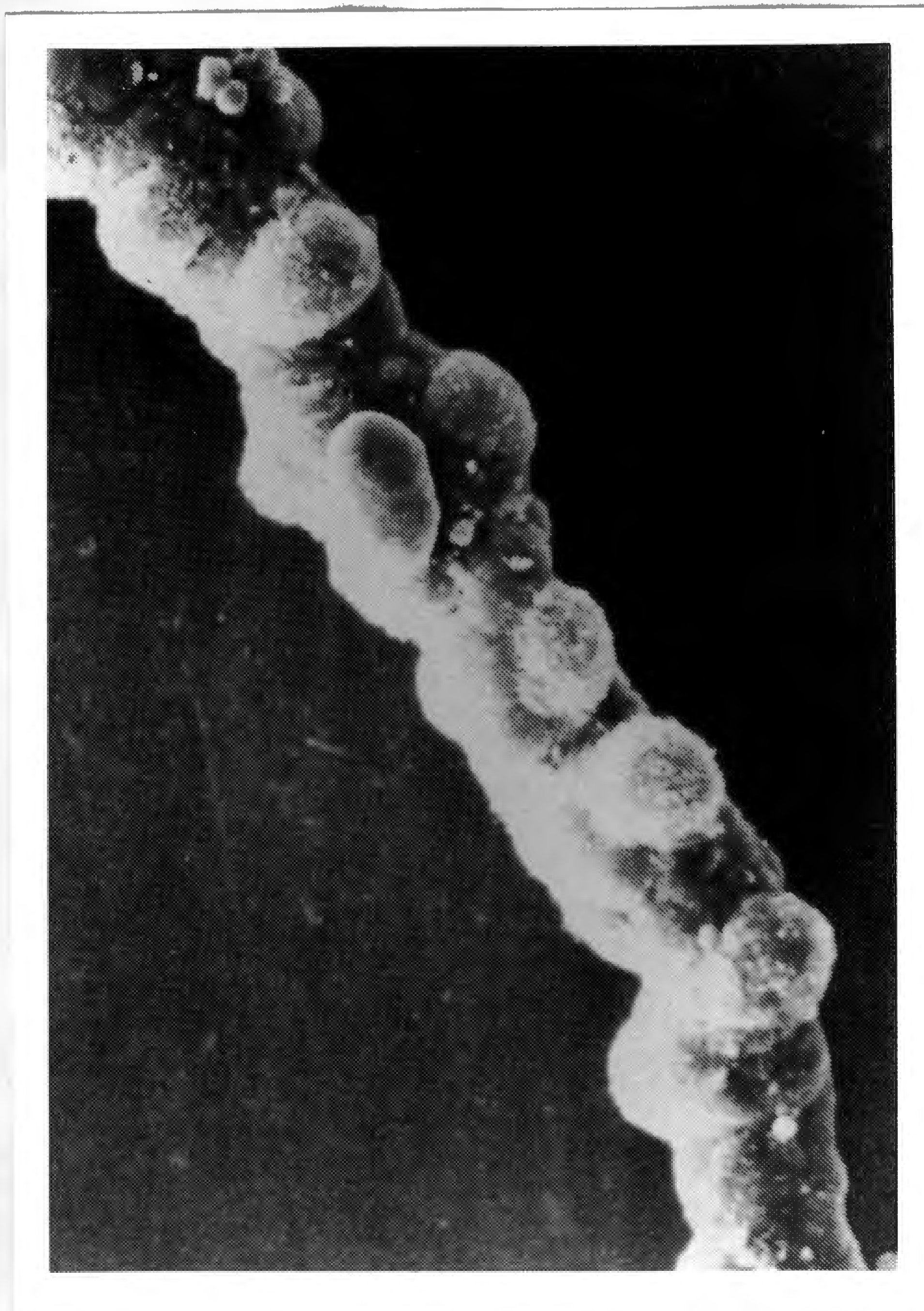

Figure 3. Scanning electron micrograph of rabbit gastric glands isolated from rabbit fundic mucosa by collagenase digestion (x 1000).

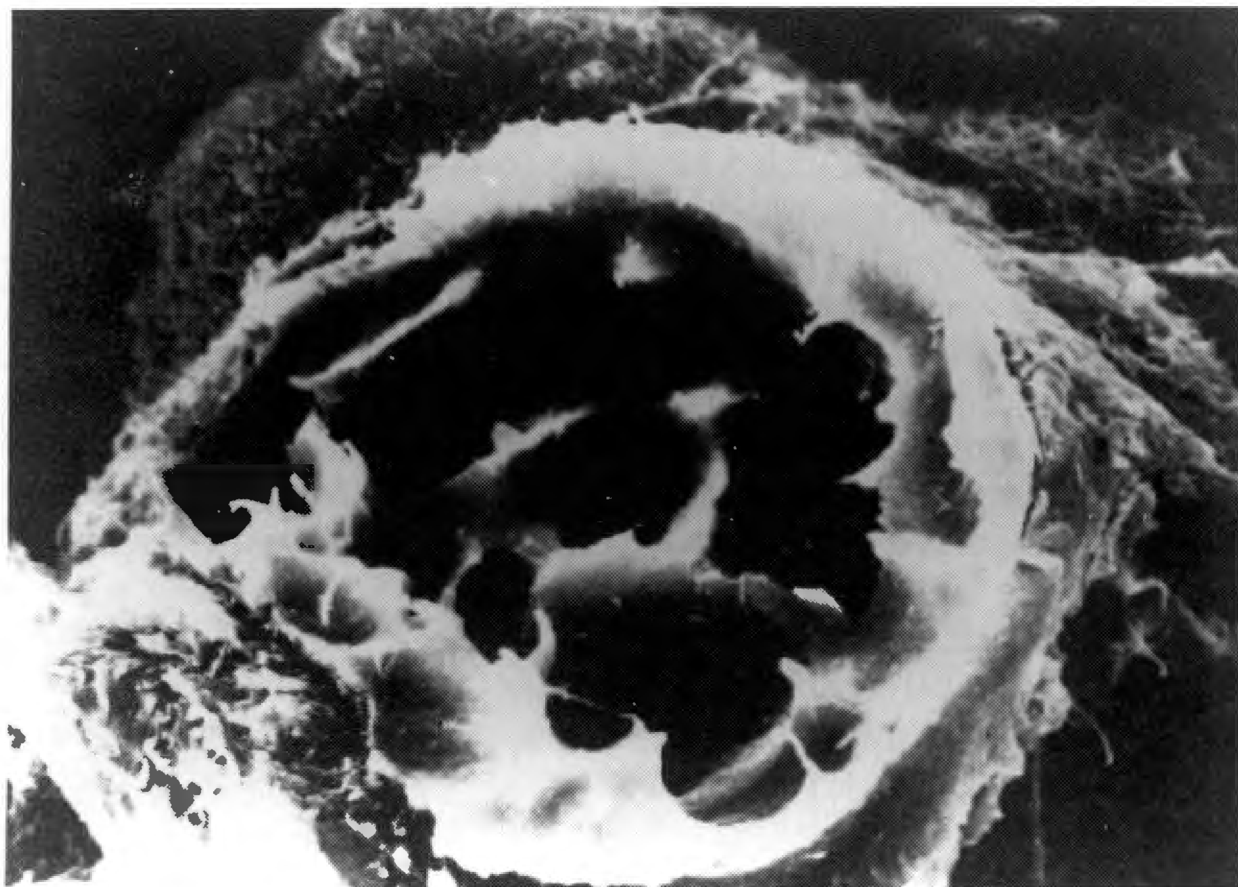

Figure 4. Scanning electron micrograph of fractured parietal cell isolated by collagenase digestion, chelation, and elutriation (x 2000).

DISCUSSION

Misoprostol proved a potent inhibitor of histamine-stimulated acid secretion by isolated rabbit parietal cells. This is consistent with results of other in vitro [1,3,11] and in vivo [13] studies.

Although misoprostol inhibited acid secretion, this PGE_1 analogue significantly increased pepsinogen secretion in isolated rabbit gastric glands. Previous studies by Berger and Raufman [13] have demonstrated a similar stimulatory effect in isolated guinea pig gastric glands. In vivo studies with the PGE_3 analogue enprostil and with prostaglandin E_3 in dogs with gastric fistulas [14,15] suggest that prostaglandins inhibit pepsinogen secretion. These studies, however, need to be interpreted with caution since the measurement of pepsin under these experimental conditions may considerably underestimate actual secretory rates. Pepsin may be inactivated by the less acidic pH of gastric juice samples taken from patients whose acid secretion is potently inhibited [16]. Furthermore, acid itself may stimulate pepsinogen secretion, [17] and the loss of this stimulation in acid-suppressed patients might also conceal prostaglandin stimulation of pepsin secretion. Reported findings of decreased peptic activity in the gastric lumen of acid-suppressed patients may therefore be clinically misleading.

It is unclear whether the stimulation of pepsinogen secretion reported herein represents a physiologic or only a pharmacologic response, since the actual levels of prostaglandins in the target tissue are unknown. However, local concentrations of prostaglandins in the stomach are known to be high [18]. Serum levels would be meaningful only if the prostaglandins were exclusively circulating hormones. In fact, prostaglandins probably act as autocrine or paracrine messengers, and their effective concentration at the cellular membrane is undoubtedly orders of magnitude higher than in the peripheral circulation.

Prostaglandin stimulation of pepsinogen secretion is probably mediated by cyclic adenosine monophosphate (cAMP) [19]. The calcium-mediated pepsigogue carbachol potentiates PGE_2 stimulation of pepsinogen secretion in isolated rabbit gastric glands [13]. This suggests that prostaglandins act through a pathway independent of calcium. The other major intracellular pathway resolves around cAMP. In fact, prostaglandins also increase the cAMP content of enriched canine chief cell suspensions [19]. Furthermore, protaglandins have previously been shown to inhibit acid secretion by decreasing cAMP in parietal cells [1]. Thus, both the stimulation of acid secretion and the inhibition of

pepsinogen secretion are likely to occur through differential effects of the prostaglandins on cAMP content in adjacent gastric fundic cell types.

Methylated prostaglandin analogues such as misoprotol were expected to be valuable in the treatment of peptic ulcer disease. However, clinical trials with several prostaglandin compounds have generally failed to demonstrate a significant advantage over conventional antacid therapy or histamine blockade [20,21].

The explanation may partially lie in the effects of the prostaglandins on pepsin secretion. It has been postulated since the first half of this century that luminal pepsin may be a significant ulcerogenic agent [22]. More recent studies continue to support the concept of pepsin as a potent mucosal barrier breaker that permits luminal acid to injure the mucosa [16,23].

Prostaglandin analogues with diminished or absent pepsigogic potency might be more efficacious against peptic ulcer disease. Alternatively, pepsinogen-blocking agents might be useful adjuncts to prostaglandin therapy. Pepstatin is a direct pepsin inhibitor derived from the polypeptides in inactive pepsinogen that block the active site of the enzyme [24]. Colloidal bismuth is also believed to inhibit pepsin whereas carbenoxolone and several other drugs inhibit pepsinogen secretion [25]. It is possible that a therapeutic trial combining prostaglandins with such agents might yield better results than prostaglandin therapy alone. It may also be useful in future clinical trials of the prostaglandins to monitor pepsinogen secretion via serum pepsinogen levels or mucosal biopsies.

Changes in pepsinogen secretion should be considered in the choice and development of new regimens for the treatment of peptic ulcer disease. As these results show, it cannot be assumed that because an agent inhibits acid secretion, it has the same effect on the secretion of pepsinogen.

REFERENCES

1. Soll AH: Specific inhibition by prostaglandin E_2 and I_2 of histamine-stimulated [^{14}C]aminopyrine accumul- ation and cyclic adenosine monophosphate generation by isolated parietal cells. J Clin Invest 1980,65:1222-1229.

2. Mahachai V, Walker K, Sevelius H, et al: Antisecretory and serum lowering effect of enprostil in patients with duodenal ulcer disease. Gastroenterology 1985;89: 555-561.

3. Zucker KA, Zdon MJ, Tyshkov M,et al: Prostaglandin E_2 analogue inhibition of intrinsic factor and acid secretion in the isolated rabbit parietal cell. Gastroenterology 1986;90:1705.

4. Robert A, Lancaster C, Davis JP, et al: Cytoprotection by prostaglandin occurs in spite of penetration of absolute ethanol into the gastric mucosa. Gastroenterology 1985;88:328-333.

5. Whittle BJR, Steel G: Evaluation of the protection of rat gastric mucosa by a prostaglandin analogue using cellular enzyme marker and histologic techniques. Gastroenterology 1985;88:315-327.

6. Basson MD, Modlin IM: Pepsinogen: Prolate ellipsoid or unrecognized pathogen. J Clin Gastroenterol 1987;9:475-479.

7. Bauer RF: Misoprostol:Preclinical pharmacology. Dig Dis Sci 1985;30:118S-125S.

8. Modlin IM, Schafer DE, Ballantyne GH,et al: Isolated gastric glands in the study of gastric secretory relationships. Surg Res Commun 1987;1:81-96.

9. Zdon MJ, Ballantyne GH, Schafer DE, et al: Evidence for non-gastrin mediated somatostatin inhibition of parietal cell function. Surgery 1986;100:954-961.

10. Basson MD, Adrian TE, Modlin IM: A new model for the study of mammalian pepsinogen secretion. Gastroenterology 1987;95:1310.

11. Levine RA, Kohen KR, Schwartzei EH, et al: Prostaglandin E2-histamine interaction on cAMP, cGMP, and acid production in isolated fundic glands. Am J Physiol 1982;242:G21-G26.

12. Steiner JA: Misoprostol clinical pharmacology: Establishment of activity in man. Dig Dis Sci 1985;30:136S-141S.

13. Berger S, Raufman J-P: Prostaglandin-induced pepsinogen secretion from dispersed gastric glands from guinea pig stomach. Am J Physiol 1985;249:G592-G598.

14. Deakin M, Ramja J, Paul A, et al: Effects of enprostil, a synthetic prostaglandin E_2, on gastric secretion, gastrin release and mucosal blood flow in dogs. Dig Dis Sci 1980;25:673-679.

15. Konturek SJ, Robert A, Hanchar AJ, et al: Comparison of prostacyclins and prostaglandin E_2 on gastric secretion, gastrin release and mucosal blood flow in dogs. Dig Dis Sci 1980;25:673-679.

16. Pearson JP, Ward R, Allen A, et al: Mucus degradation by pepsin: Comparison of mucolytic activity of human pepsin 1 and pepsin 3: Implications in peptic ulceration. Gut 1986;27:243-248.

17. Bynum TE, Johnson LR: Stimulation of human pepsin output by topical hydrochloric acid. Dig Dis Sci 1975;20:607

18. Wright JP, Young GO, Klaff LJ, et al; Gastric mucosal prostaglandin E levels in patient with gastric ulcer disease and carcinoma. Gastroenterology 1982;82:263-267.

19. Wollin A, Soll AH, Samloff IM: Actions of histamine, secretin and PGE_2 on cyclic AMP production by isolated canine fundic mucosal cells. Am J Physiol 1979;237:437-443.

20. Nicholson PA: A multicenter international controlled comparison of two dosage regimens of misoprostol and cimetidine in the treatment of duodenal ulcer in outpatients. Dig Dis Sci 1985;30:171S-177S.

21. Lauristen K, Rask-Madsen J: Prostaglandins and clinical experiences in peptic ulcer disease. Scand J Gastroenterol 1986;21(suppl 125):174-180.

22. Howes EL, Flood CA, Mullins CR: The influence of pepsin and hydrochloric acid on the healing of gastric defects. Surg Gynaecol Obstet 1936;62:149-157.

23. Kivilaakso E, Barzilai A, Schiessel R, et al: Ulceration of isolated amphibian gastric mucosa. Gastroenterology 1979;77:31-37.

24. Ford TF, Grant DA, Austen BM, et al: Intramucosal activation of pepsinogen in the pathogenesis of acute gastric erosions and their prevention by the potent semisynthetic amphipathic inhibitor pepstatinyl-glysyl-lysyl-lysine. Clin Chim Acta 1985;145:37-47.

25. Colin-Jones DG: There is more to healing ulcers than suppressing acid. Gut 1986;27:475-480.

CHAPTER 14

Dissociation of Pepsinogen and Acid Secretion in the Guinea Pig

INTRODUCTION

Pepsin is a potent proteolytic enzyme strongly implicated as a cofactor in the genesis of experimental gastric ulcers [1-3] and by epidemiologic data in human ulcers [4,5]. Yet, prophylaxis and therapy for ulcers and gastritis have emphasized acid almost to the exclusion of the contribution of pepsin to breaking the mucosal barrier. This disregard has been partly rooted in the clinical intuition (based on older in vivo experiments) that pepsin release always parallels the secretion of acid. Indeed, pepsinogen secretion generally increases in vivo and in isolated stomach preparations in response to agents which stimulate acid secretion [6,7] and decreases in vivo with H_2 blockers [8,9,10] and substituted benzimidazoles [11,12]. It has, therefore, been hypothesized that chief cell pepsinogen secretion may require a flow of water and acid from adjacent parietal cells [12]. Although pepsin and acid secretion have been reported to vary independently in dispersed gland suspensions [13,14], pepsin secretion may be different in isolated gastric glands than from within intact mucosa. Furthermore, acid secretion continued at a basal (although not increased) rate in these studies with gastric glands. Such observations do not therefore preclude a requirement for a moderate level of parietal cell secretion to activate pepsinogen secretion or to wash pepsinogen from the gastric pits into the gastric lumen. Alternatively, stimulated parietal cells might exert local influences on adjacent chief cells not evident in isolated gastric gland preparations because of dilution by the suspending medium.

To investigate these possibilities, I studied the effects of cholecystokinin, histamine and carbamylcholine on the secretion of pepsinogen and acid. I utilized isolated intact sheets of guinea pig fundic mucosa, a new model which I have developed for the study of mammalian pepsinogen secretion [15]. In a second phase of this study, I investigated the effects of proton pump inhibition by the potent H^+/K^+ ATPase inhibitor omeprazole on the basal, carbamylcholine-stimulated and cholecystokinin-stimulated secretion of acid and pepsinogen in this model.

METHODS

Fundic mucosae from 200 gram female Hartley guinea pigs were stripped of their serosal layers and mounted in Ussing chambers as previously described [15,16]. Pepsinogen secretion was measured using ^{125}I-albumin as a substrate as previously described, using 15 hour incubation times [14]. All samples were assayed in triplicate and each assay was controlled by simultaneous assay of a series of known standard pepsinogen concentrations (Sigma).

EXPERIMENTAL DESIGN

In the first phase of the study, preparations were treated with 10^{-8}M cholecystokinin-octapeptide (Kinevac, Squibb Diagnostics, New

Brunswick, NJ), 10^{-8}M cholecystokinin, 10^{-4}M histamine (Sigma, St. Louis, MO), or $3x10^{-7}$M carbamylcholine (Sigma). All secretagogues were dissolved inmammalian Ringer's solution. Dithiothreitol (DTT 0.001%) was added to submucosal solutions in all cholecystokinin experiments to prevent inactivating oxidation of the cholecystokinin and was also studied separately as a control. Eight experiments were done for each agent.

In the second part of this study, 16 preparations were treated with 10^{-4}M omeprazole (Astra, Hasle) after 40 minutes of basal secretion. Twenty minutes later, half of these were stimulated with $3x10^{-7}$M carbachol while the other eight received 10^{-9}M cholecystokinin. The omeprazole was initially dissolved in methanol, but was then diluted in Ringer's solution so that the final methanol concentration in the submucosal solution was only 0.05%. Measured pepsinogen and acid secretory rates were normalized to the basal rates of each preparation as recorded during the initial forty o sixty minutes after equilibration. Since data were not normally distributed, statistical analysis was performed by Wilcoxon signed rank and Wilcoxon rank sum tests. Ninety-five percent confidence intervals were also determined nonparametrically.

RESULTS

The mean basal rate of pepsinogen secretion of these preparations was 0.07 ± 0.01 units $hr^{-1}cm^{-2}$ while the unstimulated acid secretory rate was 0.43 ± 0.18 ueq H^{+} $hr^{-1}cm^{-2}$. The equilibrium transmucosal potential difference ranged from -20 to -40 mv with tissue resistances of 40 to 100 Ohm x cm^{2}. In none of the studies conducted was there any significant difference between the electrical characteristics of control and experimental tissues. Ninety-five percent confidence intervals for acid and pepsin secretion in these experiments are shown in Table 1.

Histamine at 10^{-4}M concentrations increased acid secretion by a factor of 5.1 ($p<0.01$) but had no significant effect on pepsinogen secretion ($p>0.4$). Dithiothreitol increased neither the secretion of pepsinogen nor the secretion of acid ($p>0.4$ for both). Cholecystokinin at 10^{-9}M stimulated pepsinogen secretion by a factor of 9.1 ($p<0.01$) without altering acid secretion. Similarly, cholecystokinin at 10^{-8}M had no effect on acid secretion in this system but increased pepsinogen release to 23.1 times the basal rate ($p<0.01$). Carbamylcholine at 3 x 10^{-7}M raised acid secretion by a factor of 4.0 ($p<0.01$) and pepsinogen secretion by a factor of 10.9 ($p<0.01$) (Fig. 1). None of these agents significantly altered transmucosal potential difference or tissue resistance.

Omeprazole decreased acid secretion to below measurable levels within ten minutes of administration in all preparations. Pepsinogen secretion in omeprazole-treated tissues was 1.8 times control levels but this was not statistically significant ($p>0.05$) (Table 1). Omeprazole-treated tissues secreted no measurable acid

even after exposure to 3x10^{-7}M carbachol. However, these tissues did secrete pepsinogen in response to carbachol, increasing pepsinogen secretion by a factor of 10.0, which was unchanged from stimulation in mucosal sheets treated with carbachol alone (Fig. 2). The omeprazole-treated tissues exposed to 10^{-8}M cholecystokinin also secreted no acid but increased pepsinogen secretion by a factor of 9.1 afterstimulation with cholecystokinin (Fig. 3). Thus, omeprazole had no statistically significant effect on the stimulation of pepsinogen secretion by these doses of carbachol and cholecystokinin. There was also no significant alteration in potential difference or electrical resistance in these tissues during omeprazole pretreatment and subsequent carbachol and cholecystokinin treatment.

Table 1. 95% Confidence Intervals for Observed Acid and Pepsinogen Secretion

	Pepsinogen		Acid	
Dithiothreitol	0.4 - 2.7	0.9 - 1.1		
10^{-9}M CCK	5.8-22.0	0.9 - 1.1		
10^{-8}M CCK	12.8 - 34.0	0.9 - 1.1		
10^{-4}M Histamine			0.6 - 1.3	4.3 - 7.9
10^{-4}M Histamine + 10^{-9}M CCK			3.7 - 12.4	2.8 - 5.1
$3x10^{-7}$M Carbachol			3.0 - 25.1	2.5 - 8.2
10^{-4}M Omeprazole	0.9 - 2.1	0		
10^{-4}M Omeprazole + 3 x 10^{-7}M Carbachol			3.5 - 7.2	0
10^{-4}M Omeprazole + 10^{-9}M CCK			6.0 - 11.9	0

(Data normalized to basal secretory rates)

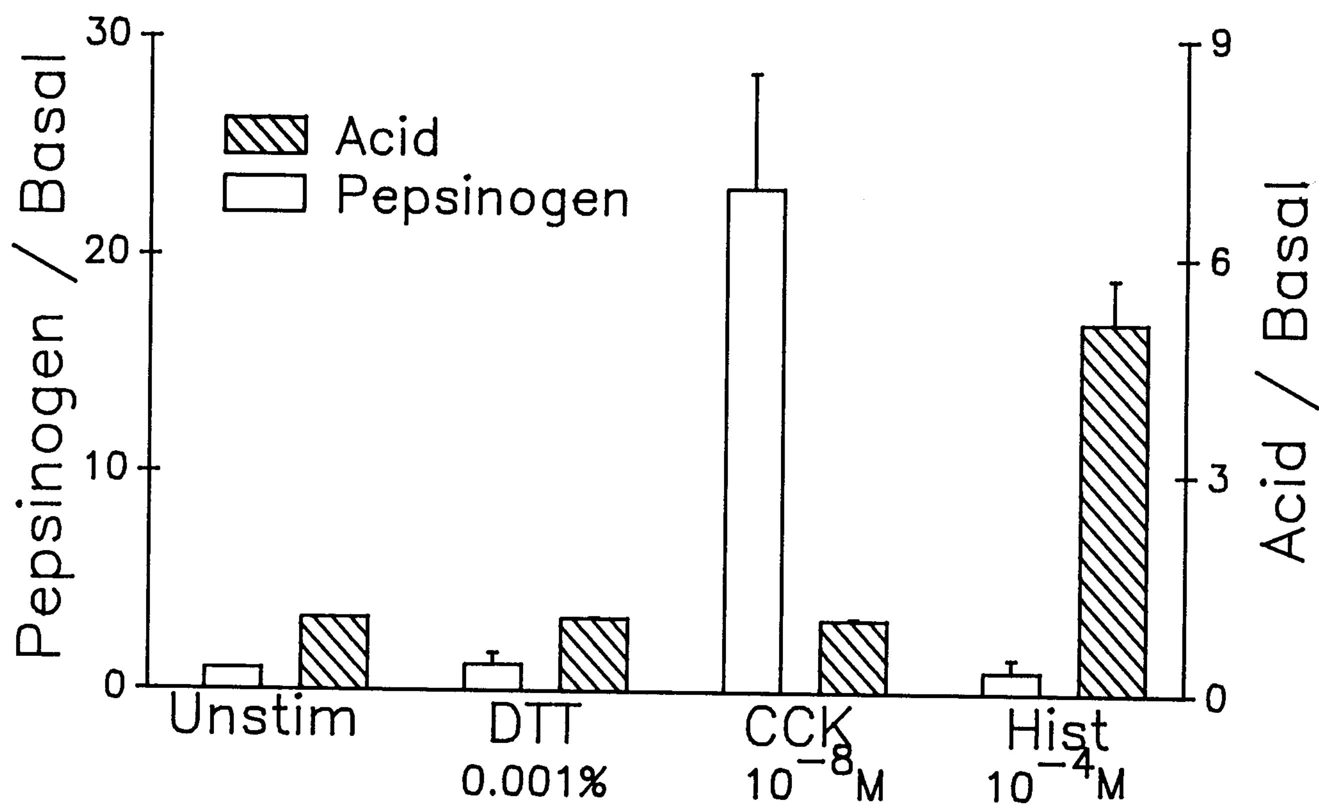

Figure 1. Pepsinogen secretion (mean over 20 min normalized to basal) is stimulated by 10^{-8}M cholecystokinin (CCK) but not by the 0.001% dithiothreitol (DTT) used to prevent oxidation of the CCK or by 10^{-4}M histamine (HIST). Acid secretion (peak secretory rate normalized to basal) is stimulated by histamine but not by cholecystokinin (CCK). Values shown represent means and standard errors from eight preparations each.

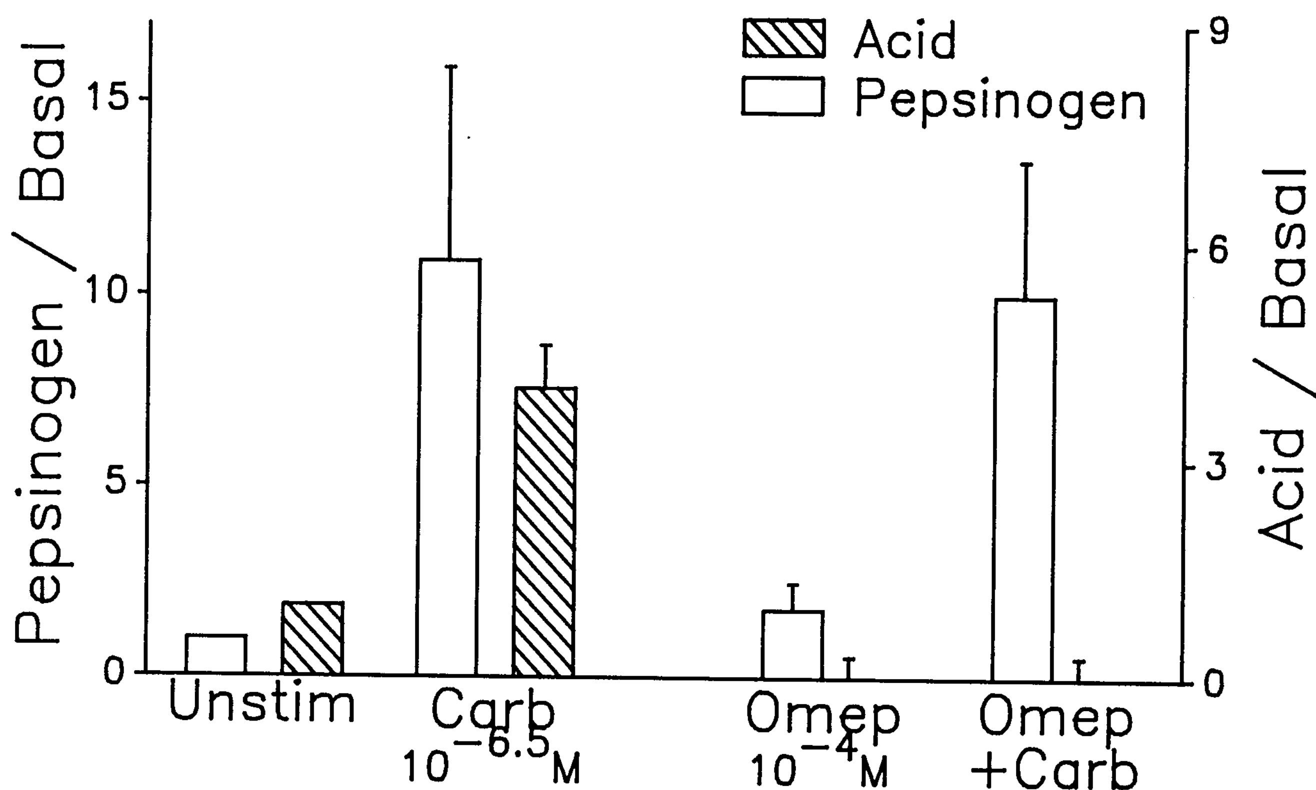

Figure 2. Carbachol (CARB) $3x10^{-7}$M stimulates both pepsinogen (mean over 20 min normalized to basal) and acid (peak secretory rates normalized to basal) secretion. Omeprazole 10^{-4}M (OM) abolishes measurable acid secretion without altering basal pepsinogen secretion. Tissues treated with omeprazole and then exposed to carbachol (Omep + CARB) still secrete no measurable acid but increase pepsinogen secretion indistinguishably from control tissues treated with carbachol. Values represent means and standard errors from eight preparations each.

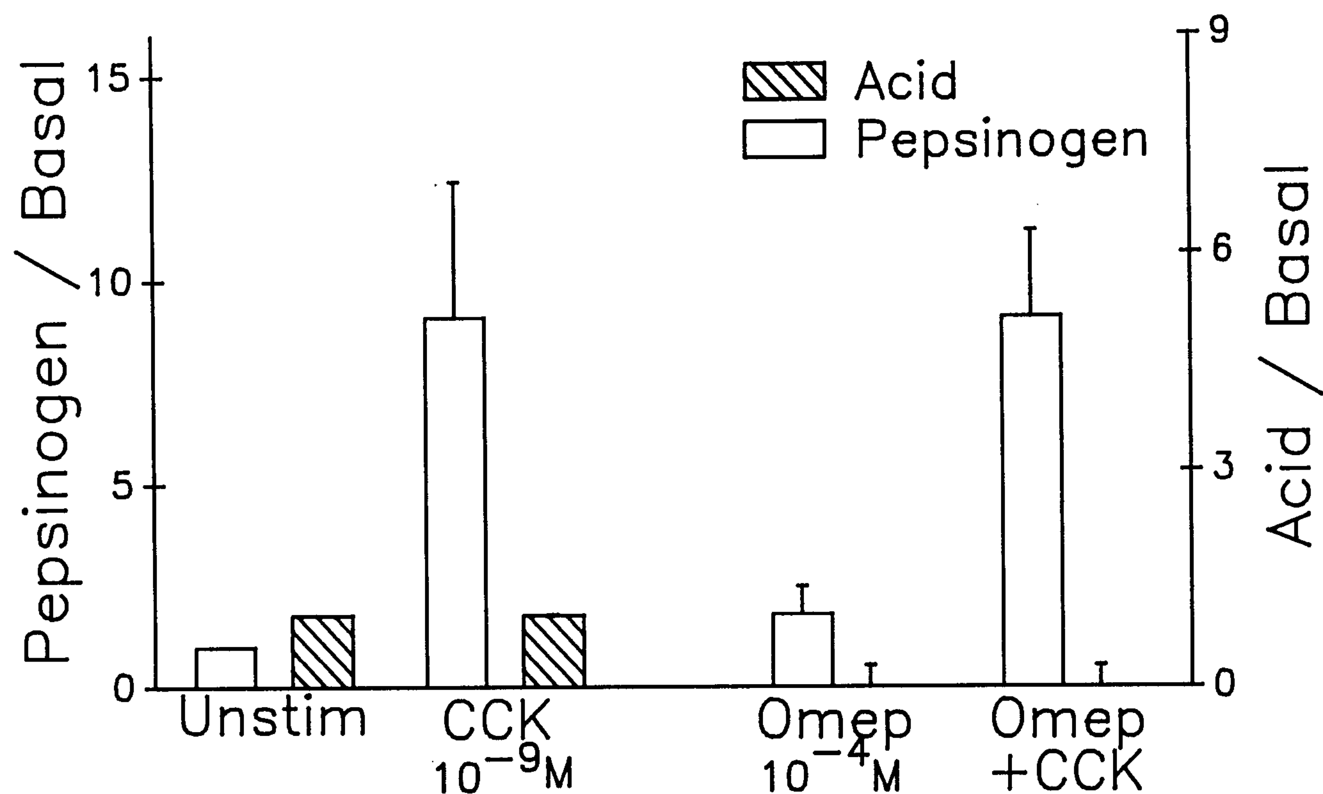

Figure 3. Cholecystokinin-8 (CCK) 10^{-9}M releases pepsinogen (mean over 20 min normalized to basal) without affecting acid secretion (peak secretory rates normalized to basal). Omeprazole (Omep; 10^{-4}M) had no effect on basal pepsinogen secretion despite complete abolition of acid secretion. Tissues treated with omeprazole and then with 10^{-9}M cholecystokinin (Omep + CCK) responded to cholecystokinin as did control preparations. Values represent means and standard errors from eight preparations each.

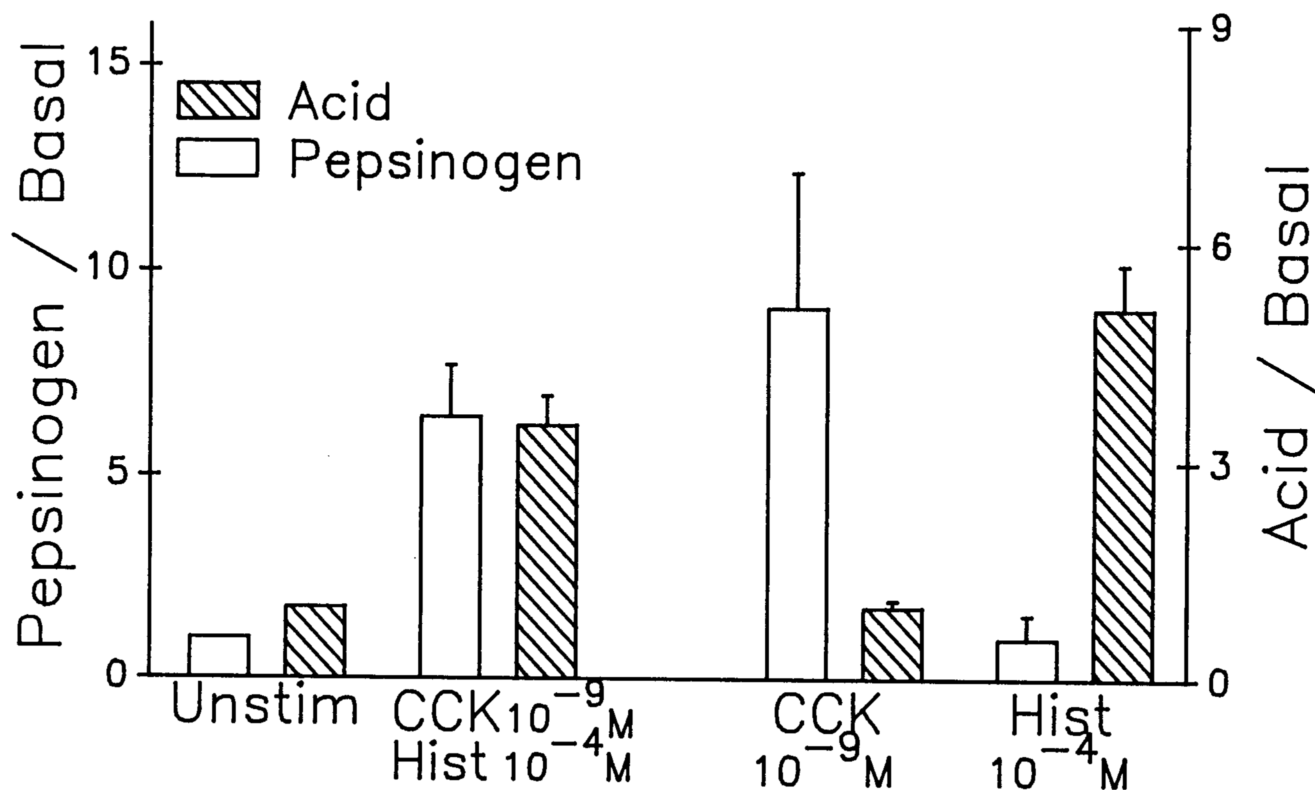

Figure 4. Tissues treated with cholecystokinin (CCK; 10^{-9}M) together with 10^{-4}M histamine demonstrate an acid secretory response equivalent to tissues treated with 10^{-4}M histamine alone and a pepsinogen secretory response equivalent to tissues treated with 10^{-9}M cholecystokinin-octapeptide alone. Values represent means and standard errors for eight preparations each.

DISCUSSION

The guinea pig intact mucosal sheet is an in vitro model which is more isolated and better controlled than intact animal preparations and yet preserves normal tissue morphology and polarity [15]. It is thus ideally suited to illuminate reported contrasts between *in vitro* and *in vivo* results. The transmucosal potential differences

and electrical resistances of the preparations used for these experiments were comparable to previous work with this model [15,16].

The observation of stable potential differences and tissue resistances demonstrates the continued viability of the mucosal preparations. In addition, the fact that electrical characteristics were unchanged during acid stimulation by carbachol and histamine suggests that acid secretion contributes little to the transmucosal potential difference exhibited by mammalian gastric mucosa. This is consistent with Rehm's hypothesis that this electrical gradient is primarily dependent on an active chloride pump distinct from the cation exchange which drives acid secretion [17]. Recent studies of chloride fluxes in omeprazole-treated bullfrog gastric mucosa have further substantiated Rehm's model [18].

The doses of 10^{-4}M histamine and 10^{-8}M cholecystokinin chosen for this study are maximal for this system while 3 x 10^{-7}M carbachol and 10^{-9}M cholecystokinin represent ED_{50} doses [16]. Histamine and cholecystokinin concentrations were initially maximized to optimize the likelihood of demonstrating pepsinogen or acid secretory effects with these agents. ED_{50} doses of carbachol and CCK were subsequently chosen to facilitate inhibition by omeprazole. The omeprazole dose utilized here is comparable to and consistent with that used in other in vitro systems [18,19].

Carbamylcholine stimulated the secretion of both acid and pepsinogen. However, histamine stimulated acid secretion in this system without any observable effect on pepsinogen secretion. Cholecystokinin, in contrast, was a potent pepsigogue for the intact fundic mucosal sheet despite its inability to alter acid secretion. Omeprazole is a powerful proton pump inhibitor which decreases acid secretion in vitro even below unstimulated levels [20].

Omeprazole decreased acid secretion by the isolated fundic mucosal sheet to undetectable levels. Despite total inhibition of acid secretion, basal pepsinogen secretion continued unabated in the presence of omeprazole and omeprazole-treated mucosae responded normally to 10^{-9}M cholecystokinin. Furthermore, although omeprazole also abolished any measurable acid secretory response to carbamylcholine, the pepsigogic potency of this cholinergic agonist was identical to that seen in preparations not treated with omeprazole.

The results of the carbachol, cholecystokinin and histamine experiments are consistent with previous studies in the isolated mucosal sheet [15] and in isolated gastric gland suspensions [21-23]. These data suggest that although pepsigogic stimulation may sometimes coincide with the stimulation of acidsecretion, increased acid secretion is neither necessary nor sufficient to

increase pepsinogen secretion, even in intact mucosa. Furthermore, the absence of any effect of omeprazole on basal pepsinogen secretion or on its stimulation by carbachol or cholecystokinin suggests that pepsinogen secretion is independent of even a minimal tonic level of acid secretion.

It therefore seems unlikely that parietal cell acid secretion is related to chief cell pepsinogen secretion or to the release of pepsinogen from the fundic mucosa in any direct or causal fashion. Previous observations in vivo and in isolated stomach preparations of close parallels between acid and pepsin secretion [6,7] and inhibition of pepsin secretion by H_2 blockers [8-10] and proton pump inhibitors [11,12] cannot be denied. However, the results of this study would seem to render the "acid washout effect" an untenable hypothesis.

Alternative explanations for reports of simultaneous increases in acid and pepsin secretion must therefore be postulated. Some agents (e.g. carbamylcholine) may have parallel effects on parietal cells and chief cells by coincidence or by virtue of sharing similar receptor and/or intracellular second messenger systems. Alternatively, agents which do not act on the chief cell directly may cause in vivo release of pepsinogen by stimulating submucosal neurons to release such primary pepsigogues as acetylcholine or cholecystokinin. Such effects would be absent in isolated glands or mucosal sheets from which the submucosa and serosa have been removed.

Furthermore, luminal acid may itself be a potent pepsigogue in vivo [24]. While it might be argued that this observation reflects release rather than secretion, the report that this response is blocked by atropine suggests that it is unlikely to represent toxic chief cell lysis [25]. Similar activity has been demonstrated in isolated gastric glands [26] and in rabbit mucosal biopsies [27]. An increase in gastric acid secretion by an acid-agonist or a decrease by an acid-blocking agent might therefore alter pepsinogen secretion not because the experimental agent acts on the chief cell but through changes in local hydrogen ion concentration. Such effects are eliminated in the Ussing chamber by autotitration to a constant "luminal" pH.

Acid in the duodenum has also been reported to release such pepsigogues as CCK [28] and vasoactive intestinal peptide [29]. Acid-pepsinogen parallels in vivo might also be mediated by these processes.

These results show that, while agents such as carbamylcholine may stimulate acid and pepsinogen secretion together, acid secretion is, in fact, neither necessary nor sufficient for basal or stimulated pepsinogen secretion in isolated guinea pig fundic mucosae. Thus, if acid and pepsin secretion change

simultaneously *in vivo*, explanations other than "acid washout" must be sought [30].

In addition to illuminating the processes of mammalian secretion, the demonstration of the independence of pepsin secretion from parietal cell function is of potential clinical significance for the treatment of gastric ulcer disease, towhich abnormalities in pepsin production have been linked [31-33]. Although gastric ulcers are clearly not a disease of overproduction of acid, most treatment modalities for patients with gastric ulcers still aim at preventing or neutralizing this acid. These results suggest that attempts to block acid secretion may not prevent the release of pepsinogen, a likely cofactor for at least some mucosal lesions.

It is impossible to ignore the likelihood that the secretion of a major proteolytic enzyme in such large quantities may play a role in the pathogenesis of gastrointestinal mucosal disease. Further delineation of the exact secretory mechanism for pepsin is required to allow formulation of pharmacotherapeutic probes to ablate intragastric peptic activity.

SUMMARY

In vivo observations have suggested that acid secretion may potentiate pepsinogen release. We measured pepsinogen and acid secretion by guinea pig fundic mucosal sheets stimulated by 10^{-4}M histamine, 10^{-8}M and 10^{-9}M cholecystokinin, and $3x10^{-7}$M carbamylcholine and then investigated the effects of 10^{-4}M omeprazole on basal, carbachol-stimulated, and cholecystokinin- stimulated secretion. Histamine increased basal acid secretion five-fold ($p<0.01$) without altering pepsinogen secretion. Cholecystokinin did not stimulate acid secretion but increased pepsinogen secretion by factors of 23.1 at 10^{-8}M and 9.1 at 10^{-9}M (both $p<0.01$). Carbamylcholine increased acid secretion and pepsinogen secretion by factors of 4.0 and 10.9, respectively (both $p<0.01$). Pretreatment with 10^{-4}M omeprazole abolished basal and carbachol-stimulated acid secretion. However, pepsinogen secretion was unaffected ($p>0.05$). Furthermore, omeprazole treated tissues increased pepsinogen secretion by factors of 10.0 with $3x10^{-7}$M carbachol and 9.1 with 10^{-9}M cholecystokinin (both $p<0.01$). Thus, basal and secretagogue-stimulated pepsinogen secretion appear independent of acid secretion in intact guinea pig mucosa.

REFERENCES

1. Howes EL, Flood CA, Mullins CR. The influence of pepsin and hydrochloric acid on the healing of gastric defects. Surg Gynecol Obstet 62: 149-157, 1936.

2. Schiffrin MJ, Warren AA. Some factors concerned in the production of experimental ulceration of the gastrointestinal tract in cats. Am J Dig Dis 9: 205-209, 1942.

3. Kivilaakso E, Silen W. Pathogenesis of experimental gastric mucosal injury. N Engl J Med 301: 364-369, 1979.

4. Walker V, Taylor WH. Pepsin I secretion in chronic peptic ulceration. Gut 21: 766-771, 1980.

5. Samloff IM, Stemmerman GN, Heilbrun LK, Nomura A. Elevated serum pepsinogen I and II levels differ as risk factors for duodenal ulcer and gastric ulcer. Gastroenterology 90: 570-576, 1986.

6. Hirschowitz BI, Sachs G. Atropine inhibition of insulin-, histamine-, and pentagastrin-stimulated gastric electrolyte and pepsin secretion in the dog. Gastroenterology 1969; 56:693-702.

7. Hendel L, Henrikson FW, Sivertsen WFU, Worning H. Effect of pentagastrin, secretin and cholecystokinin on gastric secretion of pepsin in man. Scand J Gastroenterol 1981; 16:289-293.

8. Bunce KT, Grewal M, Parsons ME. The effect of cimetidine on basal and stimulated pepsin secretion in the isolated whole stomach of the rat. Br J Pharmac 1981; 73:41-46.

9. Berstad A, Ryning A, Kolstad B, Frislid K. Reduction of postprandial gastric acidity and pepsin concentration by ranitidine and antacids in healthy volunteers. Scand J Gastroenterol 1981; 16(Suppl 69):67-72.

10. Sewing K-Fr, Billian A, Malchow H. Comparative study with ranitidine and cimetidine on gastric secretion. Scand J Gastroenterol 16 (Suppl 69):45-47.

11. Hirschowitz BI, Fong J. Effects of KCl and insulin on benzimidazole-inhibited canine gastric secretion. Am J Physiol 1983; 245:G739-G744.

12. Sandvik AK, Kleveland PM, Waldum HL. Stimulated pepsin secretion after omeprazole-induced acid suppression in the totally isolated, vascularly perfused rat stomach. Scand J Gastroenterol 1987; 22:362-366.

13. Hersey SJ, Miller M, May D, Norris SH. Lack of interaction between acid and pepsinogen secretion in isolated gastric glands. Am J Physiol 1983; 245:G775-G779.

14. Basson MD, Zucker KA, Adrian TE, Zdon MJ, Ballantyne GH, Modlin IM. Pepsin release by prostaglandin E1 analogue: A potential therapeutic problem. Arch Surg 1988; 123(4):431-433.

15. Basson MD, Adrian TE, Modlin IM. Physiologic significance of cholecystokinin in mammalian pepsinogen secretion. Surg Forum 1987; 38:116-118.

16. Rutten MJ, Ito S. Morphology and electrophysiology of guinea pig gastric mucosal repair in vitro. Am J Physiol 1983; 244:G171-182.

17. Rehm WS. A theory of the formation of HCl by the stomach. Gastroenterology 1950; 14:401-417.

18. Starlinger MJ, Hollands MJ, Rowe PH, Matthews JB, Wilen W. Chloride transport of frog gastric fundus: effects of omeprazole. Am J Physiol 1986; 250:G118-126.

19. Zdon MJ, Ballantyne GH, Schafer DE, Tyshkov M, Cambria RP, Modlin IM. Proton pump inhibition - the ultimate control of acid secretion? J Surg Res 1986; 40:353-361.

20. Fellenius E, Berglindh T, Sachs G, Olbe L, Elander B, Sjostrand S, Wallmark B. Substituted benzimidazoles inhibit gastric acid secretion by blocking $(H^{+}+K^{+})$ ATPase. Nature (London) 1981; 290: 159-161.

21. Koelz HR, Hersey SJ, Sachs G, Chew CS. Pepsinogen release from isolated gastric glands. Am J Physiol 1982; 243:G218-G225.

22. Hersey SJ, May D, Schyberg D. Stimulation of pepsinogen release from isolated gastric glands by cholecystokinin-like peptides. Am J Physiol 1983; 244:G192-G197.

23. Raufman J-P, Sutliff VE, Kasbekar DK, Jensen RT, Gardner JD. Pepsinogen secretion from dispersed chief cells from guinea pig stomach. Am J Physiol 1984; 247:G95-G104.

24. Bynum TE, Johnson LR. Stimulation of human pepsin output by topical hydrochloric acid. Dig Dis Sci 1975; 20:607-612.

25. Johnson LR. Pepsin stimulation by topical hydrochloric and acetic acids. Gastroenterology 1972; 62:33-38.

26. Norris SJ, Hersey SJ. pH dependence of pepsinogen and acid secretion in isolated gastric glands. Am J Physiol 1983; 245:G730-738.

27. Kapadia CR, Donaldson RM. Macromolecular secretion by isolated gastric mucosa fundamental differences in pepsinogen and intrinsic factor secretion. Gastroenterology 1979; 74:535-539.

28. Byrnes DJ, Henderson L, Borody T, Rehfeld JF. Radioimmunoassay of cholecystokinin in human plasma. Clin Chim Acta 1981; 111: 81-89.

29. deMuckadell OBS, Fahrenkrug OB, Holst JJ, Lauritsen KB. Release of vasoactive intestinal peptide (VIP) by intraduodenal stimuli. Scand J Gastroenterol 1977; 12: 793-799.

30. Hirschowitz, BI. The control of pepsinogen secretion. Ann NY Acad Sci 1967; 140:709-723.

31. Colin-Jones DG. There is more to healing ulcers than suppressing acid. Gut 1986; 27:475-480.

32. Venables CW. Mucus, pepsin, and peptic ulcer. Gut 1986; 27:233-238.

33. Basson MD, and Modlin IM. Pepsinogen: prolate ellipsoid or unrecognized pathogen? J Clin Gastroenterol 1987; 9:475-479.

CHAPTER 15

Effects of Cholecystokinin and Cholinergic Receptor Blockade on Guinea Pig Pepsinogen Secretion

INTRODUCTION

Cholecystokinin is one of many peptides that stimulate mammalian pepsinogen secretion in pharmacologic doses in vitro. Although cholecystokinin has been reported to stimulate pepsinogen secretion in isolated rabbit and rat gastric gland and cell preparations [1,2,3], the concentrations employed have generally been high. Furthermore, isolated intact frog esophageal mucosa, which has been an important model for pepsinogen secretion, does not respond to CCK at all [4]. CCK has been variously reported to stimulate [5], to have no effect on [6], and even to inhibit [7] pepsinogen secretion in man. The disparity and inconsistency of these findings has cast doubt on the role of cholecystokinin in the physiologic regulation of mammalian pepsinogen secretion.

In separate studies, cholecystokinin has been reported to decrease pentagastrin-stimulated pepsinogen secretion in humans, possibly by a competitive inhibition [6,7]. Questions of specificity of action and primary role for CCK have therefore been addressed. Thus, Praissman and Walden [8] found significant differences in the affinity of labelled CCK and gastrin for guinea pig fundic glands. In addition, they noted differences in binding and dissociative behavior by Scatchard plot analysis. They inferred the existence of a common binding site with differences in affinities rather than two separate receptors. The demonstration of apparent competitive inhibition of pentagastrin in vivo by CCK together with the possibility of a single common receptor might therefore lead to the speculation that CCK stimulates pepsinogen secretion through gastrin receptors as opposed to specific CCK receptors.

L364,718 is a potent new non-peptide cholecystokinin antagonist which is highly specific for peripheral cholecystokinin receptors [9]. The purpose of this study was two-fold: first, to determine whether intact guinea pig fundic mucosa would secrete pepsinogen in response to CCK-8 in a dose dependent manner, and second to determine whether pepsinogen secretion could be inhibited by specific blockade of the CCK-8 receptor using a high affinity, high specificity antagonist, L364,178. To answer these questions, I used isolated intact sheets of guinea pig fundic mucosa mounted in Ussing chambers, a newly developed model for mammalian pepsinogen secretion [10,11]. The effects of carbamylcholine, atropine, pentagastrin and gastrin-I were also studied to further validate the model and to demonstrate the viability and responsiveness of the preparations and confirm the specificity of the CCK and antagonist responses.

METHODS

Preliminary Investigations

Six unfasted Hartley strain guinea pigs weighing 150-200 grams were decapitated and 1 cm^2 pieces of gastric fundic mucosa, stripped of its serosa and smooth muscle, were extracted by homogenization and freeze thawing in a solution of 0.1% Triton X dissolved in 0.01M HCl with a pH of 2.0. After centrifugationat 1700 g and decanting, the supernatant extracts were assayed for peptic activity as were the gastric contents of each animal. In addition, samples of gastric juice from these animals were titrated with 0.1 M NaOH to a pH of 3, 5 and 7 and assayed after 40 minute incubations at 37°C in order to investigate the pH stability of guinea pig pepsin.

Mucosal Preparations (Previously described in detail)

Each preparation was allowed to equilibrate until potential difference, tissue resistance and acid secretion had achieved a steady plateau for at least 30 minutes. (This generally required about two hours.) All fluids were then changed and 0.3% casein hydrolysate (Sigma, St. Louis) was added to the luminal side to saturate protein binding sites. Samples (100ul) of the luminal solution were taken every twenty minutes thereafter. Experimental agents were added to the submucosal solution at twenty minute intervals after the first two time points (forty minutes).

In extensive preliminary investigations (data not shown), I have demonstrated that these preparations regularly maintain steady electrical characteristics and a constant rate of acid and pepsinogen secretion for up to 180 minutes after the post-equilibration fluid change.

EXPERIMENTAL DESIGN

Cholecystokinin-8 (Sincalide) was obtained from Squibb Diagnostics (New Brunswick, NJ), carbamylcholine and gastrin-I from Sigma (St. Louis) and pentagastrin (Peptavulon) from Ayerst (NY, NY). Dose response curves for pepsinogen secretion were generated for each secretagogue using concentrations from 10^{-10}M to 3 x 10^{-8}M for CCK-8, from 10^{-7}M to 3 x 10^{-6}M for carbachol, and from 10^{-9}M to 10^{-6}M for pentagastrin. In addition, gastrin-I was tested at 10^{-6}M. Each preparation was studied in the unstimulated state, after addition of dithiothreitol (vehicle for CCK, gastrin-I and pentagastrin preparations) and for up to four secretagogue concentrations in increasing doses. Sufficient experiments were done with varying concentrations of secretagogues so that at least seven observations were available for each

secretagogue dose.

In addition, to evaluate the possibility that tachyphylaxis might interfere with the efficacy of the secretagogue doses in this sequential dose response model, the effects of 10^{-9}M cholecystokinin in six preparations not previously treated with carbachol were compared with the effects of the same dose of CCK in preparations used in the sequential dose response experiments. A similar comparison was made between three preparations treated with 10^{-6}M carbachol 20 minutes after 3 x 10^{-7}M carbachol and three not so pretreated.

Six preparations were then treated with 10^{-5}M L364,718 (gift from Dr. V.J. Lotti, Merck, Sharpe and Dohme, West Point, Pa.). This was followed twenty minutes later by the addition of 3 x 10^{-9}M CCK (near-maximal) and twenty minutes after that by 3 x 10^{-7}M carbamylcholine (ED_{50}). The bathing fluids were not changed during this experimental period. An additional six preparations were similarly treated with 10^{-5}M atropine,followed at twenty minute intervals by 3 x 10^{-7}M carbamylcholine and 10^{-9}M cholecystokinin.
Dithiothreitol (BioRad Laboratories, Richmond,CA) was added to the nutrient solution to a concentration of 0.001% in all experiments in which cholecystokinin, gastrin-I, and pentagastrin were used to prevent oxidation of these agents [13].

Pepsinogen Assay (Previously described in detail)

DATA ANALYSIS

Acid secretion was measured continuously using pH stat technique and is reported here as the peak rate of acid secretion over each twenty minute interval. Pepsinogen secretion, on the other hand, was measured incrementally by subtracting each sampled pepsinogen concentration from that in the sample taken twenty minutes previously. Pepsinogen secretory rates therefore reflect mean rates over each twenty minute period rather than peak rates as with acid secretion. The twenty minute intervals for measurement were used after each addition of secretagogue or inhibitor as well as during basal measurements.

The rates of acid and pepsinogen secretion in each preparation were then expressed as multiples of the basal (unstimulated) rate in each experiment. This was measured during the first forty minutes of the experimental period. Non-normal data distribution required nonparametric data analysis. Dose response data (7-13 experiments per data point) were ranked and analysis of variance was performed on the ranks to demonstrate that these groups of experiments contained statistically meaningful data. Least significant difference

(LSD) tests were then performed at a 95% confidence level on the ranked data [14]. A computerized statistical analysis package (Statistical Analysis System, SAS Institute, Cary, North Carolina) was used for statistical computation. ED_{50} doses for carbachol and CCK were estimated from best fit linear regressions performed on scatterplots including all measured secretory rates for all dose response experiments.

For inhibitory experiments (6 experiments each), data within each sequence of experiments was handled similarly to the dose response data. Two sample Wilcoxon rank-sum tests were then used to compare inhibitor-treated agonist stimulation with uninhibited agonist stimulation from separate experiments. A two sample Wilcoxon rank-sum test was also used to assess the statistical significance of the response to the single dose of gastrin-I tested.

RESULTS

The pepsinogen content of the guinea pig gastric mucosa was 700 ± 50 units gm^{-1} wet weight (n=6, mean ± SEM) or 21.6 ± 1.6 units cm^{2} (mean ± SEM). The peptic activity of six guinea pig gastric juice specimens averaged 23.5 ± 4.2 units ml^{-1}. Initial studies of the pH stability of the peptic activity in guinea pig gastric juice demonstrated significant inactivation at neutral pH. After titration to pH 5, the peptic activity recoverable from the same samples was only 18.7 ± 3.1 units ml^{-1}. Titration to pH 7 further diminished this activity to11.5 ± 3.6 units ml^{-1}.

Preliminary experiments also demonstrated a 30% increase in recovered peptic activity when 0.3% casein hydrolysate was added to the luminal solution to prevent non-specific binding to glass surfaces. This had no effect on acid secretory rates or the electrical characteristics of the membrane.

All further experiments were accordingly performed with 0.3% casein hydrolysate in the luminal saline and with the pH of the luminal saline maintained at 5 by pH stat technique. Under these conditions, the basal rate of pepsinogen secretion was 0.068 ± 0.017 units cm^{-2} hr^{-1} (n=58, mean ± SEM). The basal acid secretory rate was 0.56 ± 0.01 ueq H^{+} cm^{-2} hr^{-1}. The equilibrium transmucosal PD ranged from 20 mV to 35 mV for these preparations and the tissue resistance from 30 ohm-cm^{2} to 110 ohm-cm^{2}.

Dose-response experiments demonstrated pepsigogic stimulation by CCK-8 with an ED_{50} of 10^{-9}M and maximal activity at 10^{-8}M (Fig. 1). Analysis of variance yielded $p<0.0001$. LSD testing showed all doses of CCK tested (but not DTT alone) to be significantly different from unstimulated secretion with 95% confidence. CCK-8 had no discernible effect on acid secretion

or the electrical characteristics of the membranes. The dithiothreitol in the nutrient solution had no significant effect on basal acid or pepsinogen secretion or the electrical characteristics of the membrane. Tachyphylaxis studies with 10^{-8}M CCK-8 demonstrated that 6 preparations not previously treated with a lower dose of CCK-8 stimulated pepsinogen secretion by a factor of 9.3 $\pm$ 3.1, not significantly different from the 12.1 $\pm$ 3.3 fold increase seen in the sequential dose response experiments.

Carbamylcholine stimulated both pepsinogen secretion and acid secretion maximally at 10^{-6}M (Fig. 2,3). Analysis of variance confirmed the significance of these results to $p<0.0001$. LSD testing with 95% confidence confirmed that all doses of carbachol except 10^{-7}M caused significantly different acid and pepsinogen secretion from basal. Of 11 mucosal sheets exposed to this concentration, seven increased acid and pepsinogen secretion while four were unaffected. Carbachol at 3 x 10^{-7}M concentrations produced a mean decrease of -2 mV in the potential difference ($p<0.1$) and no measurable alteration in tissue resistance.

At 10^{-6}M and 3 x 10^{-6}M concentrations, the mean decreases in potential difference were -4 mV and -7 mV respectively (both $p<0.05$) with increases in tissue resistance by a factor of 1.4 $\pm$ 0.3 and 1.8 $\pm$ 0.5 respectively (both $p<0.05$).

In tachyphylaxis studies using carbachol at 10^{-6}M concentrations, three preparations treated initially with this concentration of carbachol stimulated acid secretion by factors of 3.5, 6.4 and 4 respectively (mean 4.7) while three preparations pretreated with 3x10^{-7}M carbachol increased acid secretion by factors of 5.0, 4.5 and 4.1 (mean 4.5). Similarly, pepsinogen secretion increased by factors of 11.1, 30 and 22.3 over basal (mean 21.1) in preparations treated initially with 10^{-6}M carbachol while preparations pretreated with 3x10^{-7}Mcarbachol then increased pepsinogen secretion by factors of 18.2, 28.5 and 14.3 (mean 20.3) over basal.

Pentagastrin had no measurable effect on acid or pepsinogen secretion or electrical characteristics in concentrations from 10^{-9}M through 10^{-6}M (data not shown). Gastrin-I at 10^{-6}M increased acid secretion by a factor of 1.12 $\pm$ 0.04 ($p<0.05$) but had no measureable effect on pepsinogen secretion or on the electrical characteristics of the tissues.

L364,718 at 10^{-5}M had no significant effect on basal acid secretion or on transmucosal potential difference or resistance. Preparations pretreated with L364,718 continued basal rates of pepsinogen secretion and were uniformly unable to increase pepsinogen secretion in response to a near maximal dose of CCK-8 (Fig. 4). The same tissues which had been

pretreated with L364,718 and which were unresponsive to CCK-8 subsequently responded to 3 x 10^{-7}M carbamylcholine with pepsinogen secretory activity not significantly different from control preparations stimulated with carbamylcholine (Fig. 4). L364,718-treated preparations also responded to 3 x 10^{-7}M carbachol by increasing acid secretion by a factor of 5.0 $\pm$ 1.3. This was not significantly different from the acid secretory response of control preparations on stimulation with 3 x 10^{-7}M carbachol (5.9 $\pm$ 1.8 fold). Each preparation exhibited unchanged electrical parameters consistent with viability throughout these experiments. Analysis of variance yielded $p<0.0001$ for both acid and pepsinogen secretory patterns while LSD testing showed only carbachol stimulation to yield secretion significantly different from basal secretion.

Atropine at 10^{-5}M did not alter acid or pepsinogen secretion or tissue electrical characteristics. After treatment with 10^{-5}M atropine, preparations did not increase the secretion of acid (1.0 $\pm$ 0.1 times unstimulated) or pepsinogen (1.0 $\pm$ 0.2 times unstimulated) when treated with 3 x 10^{-7}M carbamylcholine (Fig. 5). The subsequent addition of 10^{-8}M cholecystokinin to these preparations increased pepsinogen secretion by a factor of 10.1 $\pm$ 2.7 (not significantly different from control preparations which increased by 12.1 $\pm$ 3.3) without changing acid secretion (Fig. 5). Again, electrical parameters remained steady throughout. Analysis of variance confirmed these results with $p<0.0001$ as did LSD analysis with 95% confidence.

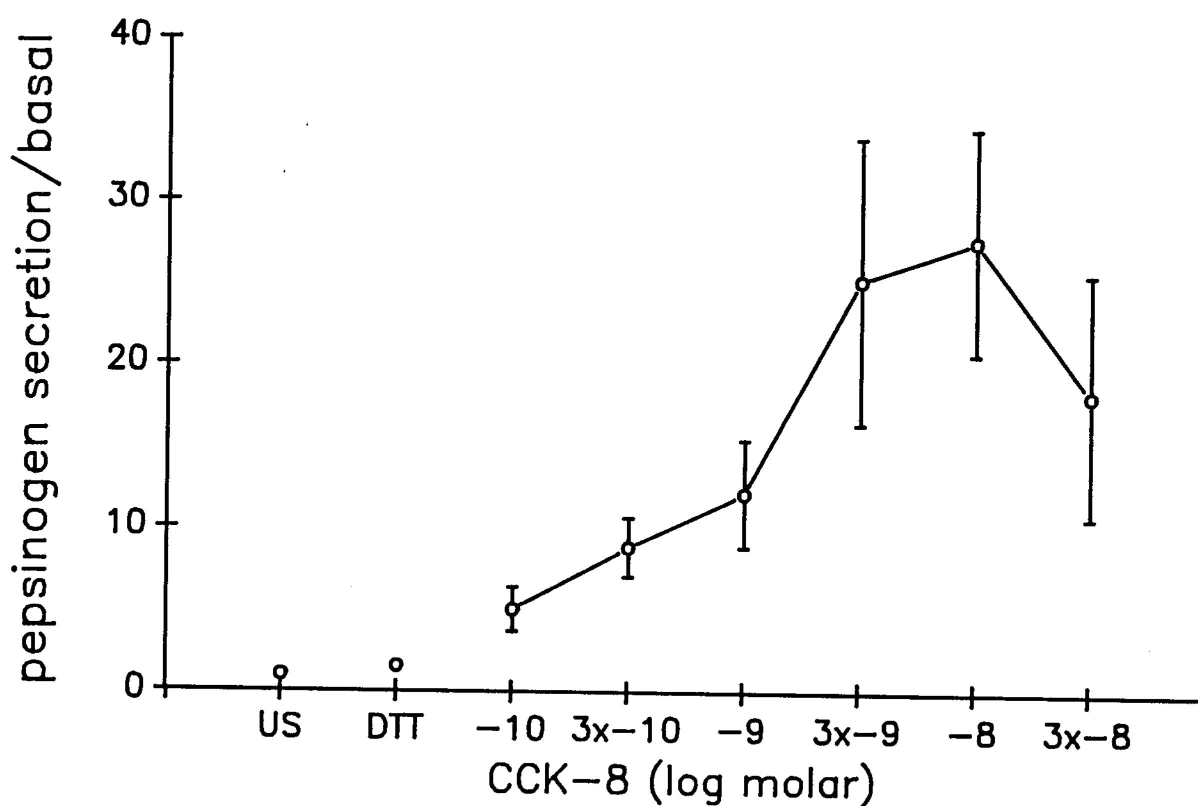

Figure 1. Increasing concentrations of CCK-8 stimulate pepsinogen secretion. Values shown represent mean ± SEM for seven to thirteen preparations and are all significantly different from unstimulated (US) ($p<0.05$) except for the dithiothreitol (DTT) control.

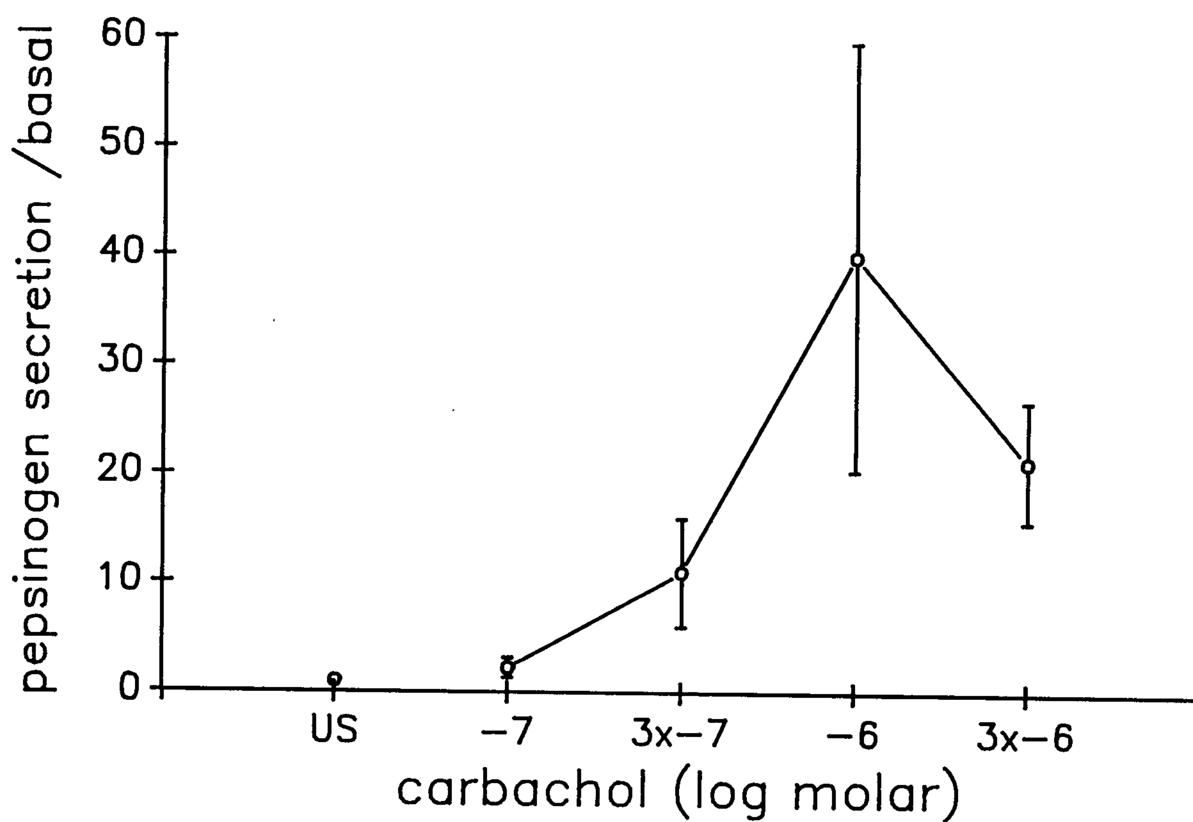

Figure 2. Carbamylcholine stimulates pepsinogen secretion. All values differ significantly from unstimulated (US) ($p<0.05$) except that for carbachol at 10^{-7}M. Values shown represent means ± SEM for 7 to 13 preparations.

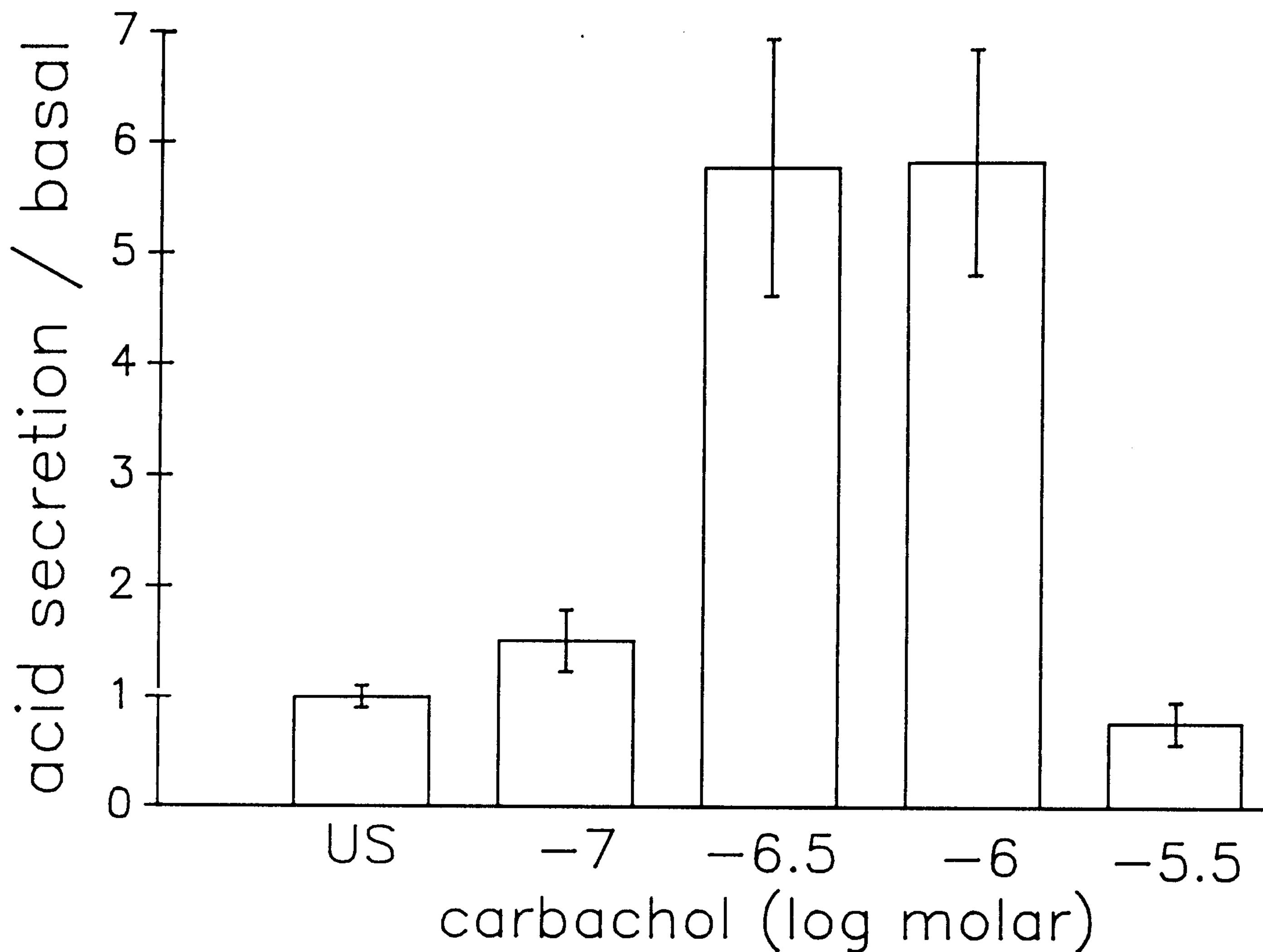

Figure 3. Carbamylcholine stimulates acid secretion. All values differ significantly from unstimulated (US) ($p<0.05$) except that for carbachol at 10^{-7}M. Values shown represent means ± SEM for 7 to 13 preparations.

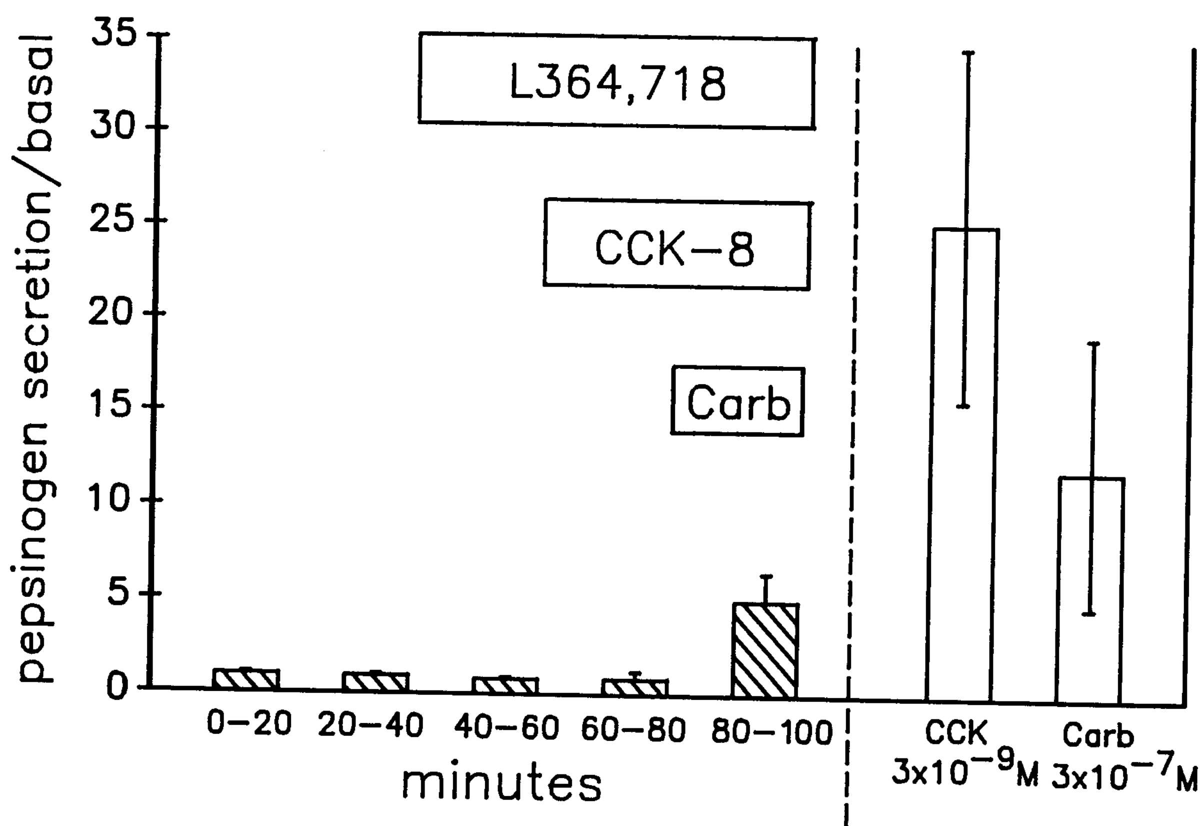

Figure 4. Preparations treated with 10^{-5}M L364,718 do not secrete pepsinogen in response to 3 x 10^{-9}M CCK-8 but do respond to 3 x 10^{-7}M carbamylcholine. (Values are means $\pm$ SEM, n=6). The values shown for CCK and carbachol alone come from separate control preparations, normalized to their basal secretory rates. (n=6) Non-parametric analysis demonstrates no difference between the responses to carbachol in control and experimental preparations but significant differences ($p<0.01$) between responses to CCK-8.

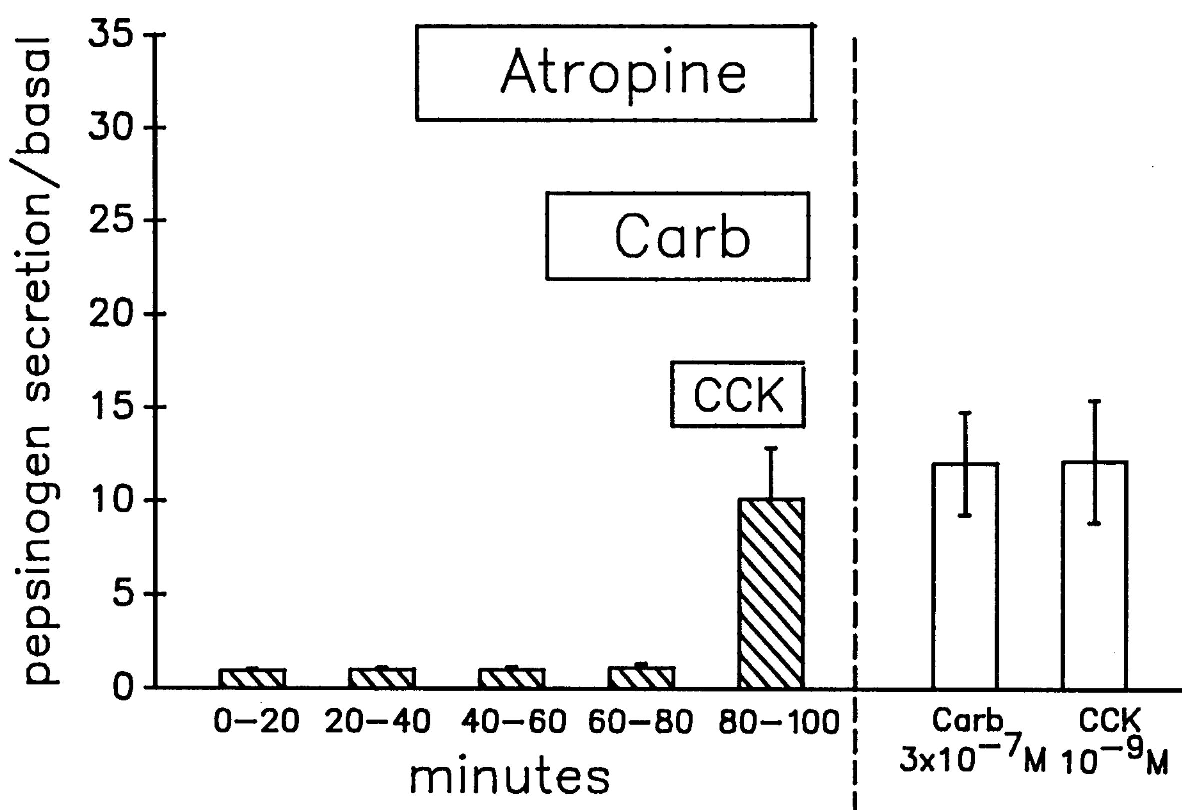

Figure 5. Mucosae treated with 10^{-5}M atropine do not secrete pepsinogen when exposed to 3 x 10^{-7}M carbamylcholine but respond to 10^{-9}M CCK-8. (Values are means $\pm$ SEM, n=6). Values for carbachol and CCK-8 shown come from separate control preparations (n=6). CCK responses are statistically equivalent with or without atropine while the carbachol responses differ significantly ($p < 0.01$). US represents unstimulated or basal secretion.

DISCUSSION

Ussing chamber preparations of amphibian gastric mucosa are an established model for the study of gastric physiology [15,16] and preparations of frog esophagus have been shown to secrete pepsinogen in similar conditions [4,17]. There are, however, to my knowledge no studies of pepsinogen secretion in intact mammalian tissues in the Ussing chamber.

Rutten and Ito [12] have described the preparation of guinea pig gastric mucosa for the Ussing chamber. The viability and utility of this model for the study of acid secretion [18] and gastric erosion [19] have been demonstrated. The basal electrical characteristics and acid secretory rates of the tissues used for these experiments were similar to those previously described and have previously been well characterized [12,19]. The present study extends this model to the measurement of pepsinogen secretion. Sampling intervals of twenty minutes were chosen based on time sequence data [20,21] which suggests maximal secretion over this period after pepsigogic stimulation in guinea pig gastric glands and isolated chief cells. The luminal pH of 5 was chosen for these experiments to avoid the inactivation of pepsin at high pH and the underestimation of acid secretion by autotitration at low pH (5).

The mucosa of the frog esophagus has been reported to contain 0.52 - 2.32 mg/cm^2 of pepsinogen depending on the season [17]. If the Sigma pepsinogen preparation used to standardize these reports was of approximately the same purity as that used by us, then the guinea pigs studied here had less than one hundredth of the pepsinogen present in the frog esophagus.

The pepsinogen assay used for these experiments was therefore adapted to the detection of nanogram quantities of pepsinogen as secreted by guinea pig fundic mucosa. The assay was sensitive and precise under these conditions to 0.005 units of peptic activity/ml and was useful from 0.01 units/ml to 10 units/ml. The ^{125}I-albumin assay is considerably more sensitive and precise than the colorimetric hemoglobin-based method of Anson and Mirsky [23] frequently used to measure secreted pepsinogen. In addition, large numbers of samples can be easily processed using this technique. Albumin bound to ^{131}I has been used [22] for the measurement of peptic activity in human urine but ^{125}I-albumin has a longer half life (60 days) and it can be measured with relative simplicity by gamma counting. An assay has also been described based on 14-C methylated hemoglobin [24]. Although this technique might be similarly modified to adjust its sensitivity, the albumin-based assay is less expensive and does not require liquid scintillation counting. In addition, albumin precipitates more easily than hemoglobin with trichloracetic

acid so that a brief centrifugation may be substituted for filtration to remove the undigested label.

The stable electrical characteristics and carbachol-responsive acid secretion of the guinea pig mucosae mounted in Ussing chambers indicate that these tissues were viable. However, unstimulated guinea pig mucosae secreted approximately 0.3% of their total pepsinogen content per hour. This is only slightly lower than and roughly comparable to the 0.4-3.0% per hour reported for guinea pig gastric glands [20], guinea pig chief cells [21] and rabbit gastric glands [24].

Frog esophageal mucosa, on the other hand has been reported to release 1.6-1.9% of total pepsinogen content per hour in Ussing chambers [17]. The etiology of this higher amphibian basal secretory rate is unclear.

The present study shows that CCK-8 can stimulate pepsinogen secretion in vitro at concentrations as low as 10^{-10}M while carbachol was clearly effective at 3 x 10^{-7}M in this preparation and appeared variably effective at 10^{-7}M. The variable efficacy of 10^{-7}M carbachol is consistent with previous studies on acid secretion in guinea pig mucosal sheets [18].

Raufman [21] has characterized pepsinogen secretion in dispersed guinea pig chief cells in response to CCK-8 and carbachol. CCK-8 produced detectable stimulation of pepsinogen secretion at 30 pM and maximal stimulation at 3 uM in Raufman's data while carbachol was initially effective at 0.3 uM and appeared maximal at 30 uM. Although such doses are similar to those employed in this study, maximal stimulation in the guinea pig chief cells produced secretion only six-fold over basal, comparable to studies in isolated rabbit and rat glands [24,25,26] but significantly lower than the twenty eight to forty-fold increases over basal demonstrated in our preparation. This may stem from some combination of an increased responsiveness to pepsigogic stimuli and lower basal rates of pepsinogen release from undamaged mucosae. Isolated gastric gland and cell suspensions require prolonged mechanical and enzymatic disruption which may damage membrane receptors or otherwise injure the chief cell. The sequential use of increasing agonist concentrations in the same preparation to study dose responsiveness raises the possibility that tachyphylaxis might have interfered with the accuracy of the carbachol and cholecystokinin data reported here. However, 10^{-6}M carbachol was not less potent when administered after $3x10^{-7}$M carbachol than when administered separately and 10^{-9}M CCK was not less potent in sequential dosing than when administered alone.

In addition, the equivalent effects of $3x10^{-7}$M carbachol and

10^{-8}M and 10^{-8}M CCK in dose response studies and when used as positive controls for inhibitor studies also argues against significant tachyphylaxis. Furthermore, even if EC50 values had been underestimated by this technique, this would have no implication for the fundamental findings of receptor specificity which this investigation is intended to address. Frog esophageal mucosa mounted in Ussing chambers has been reported not to respond to cholecystokinin at all [4]. This may represent a species difference since the histology of pepsinogen secretion differs considerably between amphibians and mammals. Frog stomachs, for instance, secrete both acid and pepsinogen from a single cell type, the oxynticopeptic cell [27]. Such anatomic variation may signal important physiologic differences as well. The interpretation of amphibian studies and their extrapolation to mammalian physiologic processes may therefore require caution.
In these studies, L364,718 blocked CCK-stimulated pepsinogen secretion in the intact mammalian mucosal sheets without interfering with another calcium-mediated pepsigogue, carbachol. L364,718 has previously been shown in vitro and in vivo to be highly specific for peripheral CCK receptors, binding neither to central nervous system CCK receptors nor to receptors for gastrin and other peptide hormones [9]. That the observed L364,718 suppression of CCK-responsiveness represents a toxic effect is highly unlikely given the maintenance of normal acid secretion, basal levels of pepsin secretion, and unchanged electrical characteristics of the L364,718-treated tissues as well as their unimpaired ability to secrete acid and pepsinogen in response to cholinergic stimuli.

The demonstrated atropine blockade of cholinergic stimulation of acid and pepsinogen secretion without alteration of the secretagogue effect of cholecystokinin further validates this model for the study of pepsinogen secretion. In addition, this confirms that the carbamylcholine release of pepsinogen used as a control for viability in the L364,718 experiments is a receptor-mediated effect rather than a toxic lysis of chief cells.

The inability of this preparation to produce a measurable response to pentagastrin, a potent secretagogue in vivo, or to gastrin-I, which did stimulate acid secretion slightly but had no effect on pepsinogen secretion, is disappointing but not unexpected. Pentagastrin and gastrin have been reported by several investigators to be at best minimally effective in rabbit isolated glands [2,24,28]. Pentagastrin produced only a slight increase in acid secretion in another study of guinea pig gastric mucosal sheets [18]. The question of why in vitro preparations are not more responsive to gastrin remains unanswered. However, cholecystokinin is unlike gastrin and clearly does stimulate pepsinogen secretion in vitro.

Furthermore, this response is blocked by a CCK-receptor specific blocking agent.

Our data, therefore, supports the hypothesis that cholecystokinin stimulates pepsinogen secretion in this model via its own receptor rather than through cross reactivity with a gastrin receptor. Unlike its frog esophageal counterpart, the intact guinea pig fundic mucosal sheet is highly sensitive to cholecystokinin. (The Ussing chamber-mounted guinea pig gastric mucosa also seems much more responsive to CCK-stimulated pepsinogen secretion than other in vitro mammalian models, perhaps because dispersed glands and cells may "leak" pepsinogen from damaged chief cells and artifactually increase basal measurements.) These data suggest that cholecystokinin potently stimulates pepsinogen secretion in mammals at relatively low concentrations. Furthermore, the pepsigogic effect of CCK seems mediated by a specific peripheral CCK receptor. Cholecystokinin is likely to play a role in the physiologic regulation of pepsinogen secretion in guinea pigs and may be hypothesized to be important in humans as well.

SUMMARY

Although cholecystokinin (CCK) has been reported to stimulate pepsinogen secretion, this action has been poorly characterized. To assess the ability of CCK to regulate mammalian pepsinogen secretion, guinea pig fundic mucosa was incubated in Ussing chambers with CCK-8, carbamylcholine and pentagastrin, and with cholinergic and CCK antagonists. CCK-8 stimulated pepsinogen secretion at 10^{-10}M, with an ED_{50} of 10^{-9}M and maximally (26-fold over basal) at 10^{-8}M. Carbachol stimulated pepsinogen and acid secretion with an ED_{50} of $3x10^{-7}$M and maximally at 10^{-6}M. Pentagastrin (10^{-9}M-10^{-6}M) did not affect acid or pepsinogen secretion while gastrin-I (10^{-6}M) stimulated acid secretion slightly but did not alter pepsinogen secretion. L364,718 (10^{-5}M), a specific CCK peripheral receptor antagonist, abolished all pepsigogic effects of $3x10^{-9}$M CCK-8 without altering basal acid or pepsinogen secretion or mucosal electrical characteristics. L364,718-treated tissues unresponsive to CCK-8 nevertheless secreted pepsinogen and acid in response to $3x10^{-7}$M carbachol identically to control carbachol-treated preparations. Atropine (10^{-5}M) blocked response to $3x10^{-7}$M carbachol without inhibiting 10^{-9}M CCK stimulation. These results support a specific receptor-mediated role for cholecystokinin in the physiologic regulation of guinea pig pepsinogen secretion.

REFERENCES

1. Ballantyne GH, Zdon MJ, Schafer DE, Fratesi GR, Roberts JR, Tyshkov M, Modlin IM: Evidence for dual modulation of pepsinogen secretion using isoproterenol, carbachol, CCK-8, forskolin, 8 bromo-cAMP and A23187 probes. Ann Surg 204:559-565,1986

2. Hersey SJ, May D, Schydberg D: Stimulation of pepsinogen release from isolated gastric glands by cholecystokininlike peptides. Am J Physiol 244:G192-G197,1983

3. Yahav J, Lee PC, Lebenthal E: Ontogeny of pepsin secretory response to secretagogues in isolated rat gastric glands. Am J Physiol 250:G200-204,1986

4. Shirakawa T, Hirschowitz BI: Interactions between stimuli and their antagonists on frog esophageal peptic glands, Am J Physiol 249:G668-G673,1985

5. Wormsley KG: Gastric response to secretin and pancreozymin in man. Scand J Gastroent 3:632-636,1968

6. Petersen H, Berstad A: Effect of commercial cholecystokinin CCK-PZ) on acid and pepsin secretion in man. Acta Hepato-Gastroenterol 20:330-337,1973

7. Hendel L, Henriksen FW, Sivertsen Weis Fogh U, Worning H: Effect of pentagastrin, secretin and cholecystokinin on gastric secretion of pepsin in man. Scand J Gastroenterol 16:289-293,1981

8. Praissman M, Walden M: The binding characteristics of ^{125}I-Gastrin and ^{125}I-CCK8 to guinea pig fundic gastric glands differ: Is there more than one binding site for peptides of the CCK-gastrin family? Biochem Biophys Res Comm 123:641-647,1984

9. Chang RSL, Lotti VJ: Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. Proc Natl Acad Sci USA 83:4923-4926,1986

10. Basson MD, Adrian TE, Modlin IM: A new model for the study of mammalian pepsinogen secretion. Gastroenterology 92:1800,1987

11. Basson MD, Adrian TE, Modlin IM: Physiologic significance of cholecystokinin in mammalian pepsinogen secretion. Surg Forum 38:116-118,1987

12. Rutten MJ, Ito S: Morphology and electrophysiology of guinea pig gastric mucosal repair in vitro. Am J Physiol 244:G171-G182,1983

13. Bacarese-Hamilton AJ, Adrian TE, Chohan P, Anthony T, Bloom SR: Oxidation/reduction of methionine residues in CCK: A study by radioimmunoassay and isocratic reverse phase liquid chromatography. Peptides 6:17-22,1985

14. Steel RGD, Torric JH: "Principles and Procedures of Statistics with Special Reference to the Biological Sciences," New York, McGraw-Hill, pp 99-113, 1960,

15. Kivilaakso E, Cross R, Allison JG, Ito S, Fromm D, Silen W: Mechanism of the inhibitory action of the electric current on H^+ secretion by frog gastric mucosa. Gastroenterology 76:759-803,1979

16. Machen TE, McLennon WL: Na^+-dependent H^+ and Cl-transport in <u>in vitro</u> frog gastric mucosa. Am J Physiol 238:G403-G413,1980

17. Shirakawa T, Hirschowitz BI: Seasonal fluctuations in pepsinogen secretion from frog esophageal peptic glands. Am J Physiol 250:G484-G488,1986

18. Finke U, Rutten M, Murphy RA, Silen W: Effects of epidermal growth factor on acid secretion from guinea pig gastric mucosa: in vitro analysis. Gastroenterology 88:1175-1182,1985

19. Rutten MJ, Ito S: Structural and functional changes by ethanol on in vitro guinea pig gastric mucosa. Am J Physiol 251:G518-G528,1986

20. Berger S, Raufman J-P: Prostaglandin-induced pepsinogen secretion from dispersed gastric glands from guinea pig stomach. Am J Physiol 249:G595-G598,1985

21. Raufman J-P, Sutliff VE, Kasbekar DK, Jensen RT, Gardner JD: Pepsinogen secretion from dispersed chief cells from guinea pig stomach. Am J Physiol 247:G95-G104,1984

22. Floch MH, Montalvo G, Crosby P: Determination of uropepsin by means of albumin I^{131}. Am J Clin Pathol 37:350-354,1962

23. Anson ML, Mirsky AE: The estimation of pepsin with hemoglobin. J Gen Physiol 16:59-63,1932

24. Kasbekar DK, Jensen RT, Gardner JD: Pepsinogen secretion from dispersed glands from rabbit stomach. Am J Physiol 244:G392-G396,1983

25. Raufman, JP, Kasbekar DK, Jensen RT, Gardner JD: Potentiation of pepsinogen secretion from dispersed glands from rat stomach. Am J Physiol 245:G525-G530,1983

26. Koelz HR, Hersey SJ, Sachs G, Chew CS: Pepsinogen release in isolated gastric glands. Am J Physiol 243:218-225,1982

27. Ito S: Functional gastric morphology. In Physiology of the Gastrointestinal Tract, Johnson LR (ed). New York, Raven Press, pp 517-550, 1981

28. Defize J, Pals G, Frants RR, Westerveld BD, Meuwissen SGM, Eriksson AW: Pepsinogen synthesis and secretion in isolated gastric glands. J Clin Pathol 37:531-536,1984

CHAPTER 16

Pepsinogen Release and Acid Secretion from Human and Guinea Pig Gastric Mucosa Compromised by Hypoxia, Endotoxin or Critical Illness

INTRODUCTION

Stress gastritis and acute gastric ulceration represent fundamental problems for critically ill patients. 60-100% of patients with life-threatening illnesses, major burns or severe injury develop gastric erosions or ulcerations [1,2,3]. About 20% have clinical bleeding [3,4]. Acid control via antacids and H_2-blockers does not always prevent these phenomena [3,4,5] and significant side effects and complications are associated with such prophylaxis [6,7,8,9]. In addition, critically ill or injured patients do not generally secrete abnormally large quantities of acid [2,4,10,11]. Thus, although acid may contribute to stress gastritis and ulceration, acid hypersecretion itself may not be the primary or only cause of these lesions.

Pepsin is a powerful proteolytic enzyme with potent mucolytic and barrier-breaking properties [12,13]. Both epidemiologic [14,15,16,17] and experimental [18,19,20] evidence has linked pepsin to ulcerogenesis. Furthermore, pepsin secretion is independent of acid secretion [21,22,23,24,25] and recent evidence suggests that certain isozymes of pepsin may degrade gastric mucus even at a neutral pH [16,26]. Gastric antacids may thus not block all peptic activity.

It was my hypothesis that chief cells exposed to noxious stimuli would release stored pepsinogen, thereby producing a potentialcofactor for the development of acute gastric lesions in the critically ill, septic or hypoxic patient. Gastric acid and pepsin release were therefore investigated in vitro in response to hypoxia, a common problem in severely ill patients [27], and to gram negative endotoxin at concentrations similar to those recorded in clinical sepsis [28,29]. These studies utilized intact guinea pig fundic mucosal sheets mounted in Ussing chambers, a model for gastric acid and pepsinogen secretion [25]. To suggest the clinical relevance of these observations, I also present data on peptic activity in the gastric juice of critically ill patients. Although preliminary and difficult to interpret because of biological and ethical complexities, the finding of substantially increased peptic activity in clinical as well as in vitro situations may lend cogency to the hypothesis.

METHODS

Ussing Preparations

Fundic mucosa from 200 gram Hartley female guinea pigs was stripped of its serosa and mounted in Ussing chambers as previously described [25,30]. For endotoxin studies, the submucosa was bathed in mammalian Ringers solution (122 mM

NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.3 mM $MgSO_4$, 2.0 mM $CaCl_2$, 1.0 mM KH_2PO_4, 20 mM glucose) and gassed with 95% O_2 and 5% CO_2 (buffered pH 7.4, osmolarity 293 mosm/l). The luminal side of the tissue was gassed with pure oxygen and bathed in 154 mM NaCl (308 mosm/l) to which was added 0.03% casein hydrolysate(Polypep, Sigma, St. Louis) to saturate protein binding sites in the chamber. Luminal pH was maintained at 5.0 by titration with 5 mM NaOH via an autotitrator (Radiometer Copenhagen). Acid secretion was calculated from the rate of alkaline autotitration. The luminal solution was sampled (100 ul) at twenty minute intervals for pepsinogen. Potential difference (PD) and tissue resistance were monitored using a voltage/current clamp (DVC 1000 World Precision Instruments, New Haven CT) connected to the chamber by silver chloride electrodes in series with 4 M KCl half cells and 4 M KCl 5% agar bridges. All fluids were maintained at 37°C by a circulating heated water bath. For studies on the effects of hypoxia, the submucosa was bathed in a crystalloid solution without bicarbonate (130 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$,2.0 mM $CaCl_2$, 1.1 mM KH_2PO_4) with 20 mM glucose and 10 mM HEPES (pH 7.35, osmolarity 291 mosm/l). Both sides of the membrane were initially gassed with pure oxygen.

EXPERIMENTAL DESIGN

Hypoxia Studies

Eighteen preparations were stripped, mounted and incubated with pure (100%) oxygen. The submucosal side of the tissues was bathed in HEPES-buffered balanced salt solution while normal saline with casein hydrolysate was placed on the luminal side. Tissues were allowed to recover until PD, resistance and acid secretion had been steady for at least thirty minutes (typically 120 to 180 minutes). All solutions were then changed and experiments were initiated after an additional 20minute equilibration period. Six control preparations were then maintained on pure oxygen for 100 minutes. Six were switched to pure nitrogen after 40 minutes and six to room air (approximately 78% nitrogen and 22% oxygen). In each case, the rate of gas circulation was kept constant by gas flow regulators (Cole-Parmer, Illinois). All gases were similarly humidified by passage through distilled water. Tissues were studied for one hour after the gas change.

Endotoxin Studies

Eight preparations were allowed to equilibrate for at least 120 minutes after mounting, until the electrical characteristics and acid secretion of the tissue had been steady for at least 30 minutes. Solutions were then changed and experiments commenced after 20 additional minutes of

equilibration. After 40 minutes of basal measurements, 0.01 units/ml of endotoxin (Shigella flexnerii lipopolysaccharide, Sigma, St. Louis) were added to the submucosal solution. Forty minutes later, 0.1 units/ml of endotoxin were added to the submucosal solution and measurements were made for an additional 40 minutes.

At the conclusion of hypoxia and endotoxin experiments, tissues were fixed in the chamber with buffered formalin and 3% buffered glutaraldehyde. Light microscopy was performed after staining with hematoxylin and eosin. Tissues for transmission electron microscopy were oriented using a dissecting microscope, embedded in expox 812 (E.F. Fullam, Inc., Latham, NY, USA) and sectioned at one micron thickness. These one micron thick sections werestained with basic fuschin and toluidine blue and examined by light microscopy for orientation and subselection of representative areas for ultrathin section. Ultrathin sections on copper grids were stained with osmium tetroxide and uranyl acetate and examined using a Phillips 300 electron microscope.

Human Studies

Gastric juice from the nasogastric tubes of twenty randomly selected critically ill patients were assayed for pepsin and tested for pH and occult blood (by guaiac testing). All these patients received standard doses of H_2 blockers and antacids. Gastric fluid was also sampled from 20 randomly selected routine surgical patients with nasogastric tubes who were also receiving antacids and H_2 blockers. Each routine patient appeared clinically to be convalescing well and subsequently recovered without complication. Nasogastric sampling was performed over 48 hours after surgery to obviate any effect of anesthesia and samples were taken at least two hours after antacid administration. Patient diagnoses are shown in Table 1.

Pepsinogen Assay

Pepsinogen was assayed in samples from the Ussing chamber by ^{125}I-albumin digestion as recently described [25]. Briefly, samples were acidified by the addition of a 0.031 M HCl / 0.05 M KCl buffer, incubated at 37°C for twenty four hours with ^{125}I-albumin, and then precipitated using 5% trichloroacetic acid (TCA) (0.33 M). Digested fragments of labelled albumin remaining in the supernatant were quantitated by gammascintigraphy after centrifugation at 1500 g and decanting. Each assay was standardized using purified porcine pepsinogen (Sigma) of known activity (3400 u/mg). One unit of peptic activity is defined as the amount needed to produce a change in absorbance at 280 nM of 0.001 per minute at pH 2.0 at 37°C, measured as TCA-soluble products, using hemoglobin as

substrate.

Fresh gastric juice was centrifuged at 1500 g for 15 minutes to remove mucus and debris. The assay was modified for the higher peptic activity of gastric juice by shortening the incubation with labelled substrate to 40 minutes. 2% unlabelled albumin was also added to the reaction mixture to slow the degradation of ^{125}I-albumin. Similar modifications of this assay have recently been described for measurement of pepsin in guinea pig gastric juice [25] and in isolated gastric gland preparations [24].

STATISTICAL ANALYSIS

Acid secretion was measured continuously in Ussing chamber studies, using pH stat technique, and is reported here as peak acid secretion over each 20 minute interval. Pepsinogen secretion, however, was calculated incrementally by subtracting each sampled pepsinogen concentration from that of the prior sample. Pepsinogen secretory rates therefore reflect mean rates over each 20 minute period rather than peak rates as with acid. Statistical analysis for hypoxia studies was by unpaired t-testcomparing experimental values to those observed in control tissues. Statistical analysis for endotoxin experiments was by paired t-test in which each preparation served as its own control. Electrical characteristics and rates of acid and pepsinogen release for each time period were compared with basal parameters and rates recorded over the first forty minutes after equilibration of each preparation. Although secretory rates expressed as multiples of basal levels of secretion are discussed in the text and portrayed graphically, raw data is shown with its statistical analysis in Table 2.

Human data was analyzed by Wilcoxon rank sum tests to compare age, gastric pH, and pepsinogen concentration between ICU and "control" patients. A chi square test was used to compare the incidence of heme positive nasogastric aspirates between the two groups. Finally, Pearson correlation coefficients were determined for all 40 patients to seek correlations between gastric bleeding and intragastric pH and peptic activity. A computerized statistical package (Statistical Analysis System, SAS Institute, Cary, North Carolina) was used for statistical computations.

RESULTS

Hypoxia Studies

Initial equilibrium potential differences (PD) of the eighteen oxygenated preparations for hypoxia studies were -24.5 ± 2.7

mv(range -16 mV to -35 mV). Equilibrium tissue resistance averaged 64 $\pm$ 7 ohm-cm^2. Basal acid secretion was 0.65 $\pm$ 0.06 eq $H^+cm^{-2}hr^{-1}$. Basal pepsinogen release was 0.09 $\pm$ 0.06 units-cm^{-2}hr^{-1}. Control tissues maintained steady acid (Fig. 1) and pepsinogen release (Fig. 2) and constant electrical characteristics (data not shown) for the full 100 minute period.
Tissues exposed to pure nitrogen decreased PD to 0 within 5 minutes while resistance increased 7.2-fold (both $p<0.001$). These values remained constant for the remainder of the experimental period (Table 2). Acid secretion became unmeasurable within five minutes after nitrogen anoxia (Fig. 1). Luminal pepsinogen release over the three serial twenty minute intervals of nitrogen perfusion averaged 16.4 $\pm$ 2.9, 15.1 $\pm$ 3.4 and 15.0 $\pm$ 3.2 times basal levels of pepsinogen release respectively (Fig. 2).

Tissues gassed with room air also decreased PD and increased resistance within minutes of the change from oxygen (Table 2). However, electrical characteristics generally became steady at -8.2 $\pm$ 3.1 mv and 244 $\pm$ 21 ohms-cm^2 within 20 minutes ($p<0.001$). The PD then slowly declined by 3-4 mv over the remainder of the experimental period. Resistance did not change significantly from its initially increased value. Acid secretion continued at a decreased ($p<0.001$) rate (Fig. 1) while pepsinogen release rose to 4.5 $\pm$ 1.4 times basal ($p<0.025$) after 20 minutes of room air perfusion and then to 11.1 $\pm$ 5.1 and 13.5 $\pm$ 7.8 times basal over the next two 20 minute intervals (both $p<0.005$) (Fig. 2).Light and transmission electron microscopy of control (pure oxygen) preparations demonstrated essentially normal gastric histology except for some separation between glandular units. The lumens of the gastric glands were clear and lined by normal microvilli on electron microscopic study. Cell borders within glands were well apposed with intact tight junctions. Random glandular units from control and experimental preparations are shown in Figure 3 to illustrate morphologic differences. Room air-treated preparations were more edematous by light microscopy with focal areas of nuclear and even cellular disruption. Parietal cells appeared more consistently damaged than granule-containing cells (mucus cells and chief cells). Electron microscopy demonstrated diminished numbers of luminal microvilli as well as some luminal debris. Although the tight junctions were intact, significant spreading was observed between the cells, with an appearance somewhat like acantholysis. There were occasional degenerative cytoplasmic vacuoles with flocculent electron densities in retiform patterns (Fig. 3b). Nitrogen-treated tissues did not demonstrate the marked edema characteristic of endotoxin- and room air-treated preparations but were characterized by widespread loss of tissue architecture and cellular disruption. Electron microscopy demonstrated changes similar

to those observed in room air treated tissues but diffusely throughout the tissue as well as total degeneration of some cellular membranes, disruption of intercellular junctions, and abundant luminal debris including intact cellular organelles. Even relatively normal appearing cells had almost no microvilli and exhibited frequent nuclear clearing of chromatingranules. The damage was so widespread that differential patterns of damage to parietal cells and granule-containing cells could not be determined for nitrogen-gassed tissues (Fig. 3c).

Endotoxin Studies

Before exposure to endotoxin, mucosae exhibited PD of -35.5 ± 1.6 mv (ranging from -28 mv to -39 mv) and resistances of 112 ± 14 ohm-cm^2. Tissues secreted acid at 0.89 ± 0.06 ueq-$cm^{-2}hr^{-1}$ and pepsinogen at 0.17 ± 0.07 units-cm^{-2}-hr^{-1}. The effects of endotoxin are shown in Table 2. Pepsinogen release increased 3.3-fold during exposure to 0.01 u/ml of endotoxin ($p<0.05$). The addition of 0.1 u/ml of endotoxin increased pepsinogen release to 6.1-fold over basal ($p<0.05$) (Fig. 4). Acid secretion decreased to 82.1% of the basal rate with the 0.01 u/ml dosage and was further inhibited to 67.4% of basal with 0.1 u/ml of endotoxin (both $p<0.001$) (Fig. 5). PD decreased dose-dependently with endotoxin exposure, achieving statistical significance ($p<0.001$) at the higher dose. Resistance increased with endotoxin, almost doubling at the high dose ($p<0.001$) (Table 2).

Histologic study of the endotoxin-treated tissues demonstrated marked tissue edema with scattered foci of pyknosis and cell lysis. As in the room air-treated tissues, a significant amount of debris was observed in some glandular lumens as well as some loss of microvilli. Some nuclear clearing was observed. The majority of the mucosal cells, however, retained intact nuclei and appeared histologically normal (Fig. 3d).

Human Studies

Demographic characteristics and data from the human studies are shown in Table 3. Despite some overlap (Fig. 5), mean intragastric peptic activity was approximately 18-fold higher in the critically ill patients ($p<0.0001$). Furthermore, the presence of occult blood correlated with intragastric peptic activity ($r=0.59$, $p<0.0001$) but not with gastric pH ($r=0.04$, $p=0.6$).

DISCUSSION

The intact guinea pig fundic mucosal sheet is an established model for the study of gastric mucosal injury [30] and acid

[31] and pepsinogen secretion [25]. Basal electrical characteristics, and acid and pepsinogen secretory rates for endotoxin-treated tissues compare with previous data [25]. Controls for hypoxia experiments received no CO_2 to maintain comparability with the room air and nitrogen conditions. Without CO_2, these control tissues secreted less acid and had lower PD but increased resistance. Pepsinogen secretion was also slightly lower without CO_2 but this did not achieve statistical significance (Table 2). The changes in acid secretion and electrical parameters without CO_2 are consistent with amphibian data [32,33] while the less significant change in pepsinogen secretion suggests that parietal and chief cells may have different CO_2 requirements.

Hypoxia and endotoxin each produced dose-responsive increases inpepsinogen release and electrical resistance while inhibiting acid secretion and decreasing PD. The observed alterations in ion transport are expected since acid secretion and chloride transport (reflected by PD) are energy dependent, requiring aerobic metabolism and sensitive to toxic stimuli. Similar changes in ion transport have previously been documented in anoxic amphibian gastric mucosa although pepsinogen secretion was not measured in that study [34]. Endotoxin also inhibits acid production by feline gastric fistulas [35] while the PD of ex vivo canine gastric flaps is lower during gram negative septicemia [36].

Pepsinogen release has not been previously studied in vitro under these conditions. The histologic appearance of the hypoxic, anoxic and endotoxin-treated tissues, together with the decrease in proton and chloride transport, suggests that the increased pepsinogen release after hypoxia or endotoxin exposure is likely to reflect leakage of intracellular pepsinogen stores from damaged chief cells. The pattern of increasing pepsinogen release with decreasing acid secretion may represent a general and non-specific response of gastric mucosa to noxious stimuli.

Extrapolation to the clinical setting is desireable but must be cautious. The endotoxin concentrations used for these studies are found in septic human patients [28,29]. Although oxygen concentrations bubbled through a salt solution in vitro cannot be directly compared with interstitial oxygen tensions in vivo, visceral hypoxia is common in critical illness [27]. Gastricfundic mucosa is also more sensitive to hypoxia than many other tissues [37]. The in vitro conditions reported here may therefore be relevant to patient care.

The data from human samples, which demonstrates an 18-fold increase in intragastric peptic activity in critically ill patients, is obviously preliminary. Its interpretation involves assumptions about the validity of patient selection

and sampling and the possibility of alternative mechanisms in whole organisms.

The peptic activity in the gastric juice of my "control" patients is higher than that reported by Domschke [38] in the gastric juice of normal volunteers but lower than the normal range reported by Hirschowitz [39]. Differences in assay technique and standardization between laboratories may account for these variations, while clearly additional investigations will require comparison with truly asymptomatic normal volunteers assayed in my laboratory as well as serial samples from each patient.

Nevertheless, even the preliminary data now available strongly suggests that pepsinogen concentrations are abnormally elevated in the gastric juice of some critically ill patients. Other studies have documented elevated intragastric peptic activity in neurosurgical patients [40] and those with liver dysfunction [41]. Stress [42,43], adrenergic stimulation [44,45,46] and alterations in gastric emptying and fluid secretion might alter measured peptic activity, but seem unlikely to produce an increase of this magnitude. Thus, while the mechanism of theobserved increase in intragastric pepsinogen concentration in critical illness requires further investigation, both the in vitro results and the human data are consistent with the hypothesis that noxious stimuli may damage chief cells and release stored pepsinogen.

Finally, if pepsinogen is indeed released under these circumstances, then the clinical relevance of gastric pepsinogen in acid-blocked patients must be addressed. Walker and Taylor [47] have demonstrated by several methods that from 28%-80% of gastric juice peptic activity is retained at neutral pH. At least part of this "alkali-resistance" is believed due to a specific pepsin isozyme which retains activity at pH over 5.0 and is highly mucolytic [12,13,26]. This isozyme has been shown to be secreted disproportionately by patients exposed to trauma [48] or with peptic ulcers [16] as well as in response to vagal stimulation [44]. In addition, the neutralization of the gastric lumen with nasogastric antacids is likely to have less effect in the immediate microenvironment of the gastric mucosa. Behind the gastric mucus layer, the gastric pits may still be acid. Finally, Ohe [49] has suggested that released pepsinogen may be activated within the interstitium of the gastric mucosa and thus cause pathology even before its release into the gastric lumen.

The proton is probably a significant cofactor in acute gastric mucosal pathology in critically ill patients, but acid hypersecretion is not the primary defect. Ethical imperatives prohibited interfering with antacid and H_2-blocking therapy forformal acid secretory studies but luminal pH did not

correlate with gastric bleeding in this study. Furthermore, gastric aspirates from patients with trauma, major burns, neurosurgical problems and sepsis prior to the introduction of current antiulcer prophylaxis also contained less acid than normal [10,41,50].

Conventional prophylaxis against gastritis may derive some of its potency from peptic inhibition. Elevating intragastric pH does inhibit or denature some pepsin isozymes [51]. Furthermore, H-2 blockers inhibit pepsin secretion in vivo in man [38,39] while antacids bind and thus inhibit luminal pepsin [52]. However, anti-acid prophylaxis may be undesireable. Gastric neutralization is associated with significant bacteriologic problems [7,8,9] while H-2 blockade itself has complications [6]. Intervention directed specifically at blocking pepsinogen release or binding luminal pepsin may prevent erosions with less side effects. The present results suggest that gastric fundic mucosa responds to noxious stimuli by decreasing active ion transport but increasing pepsinogen release. Although the latter probably reflects a pathologic rather than physiologic process, pepsinogen released in this manner may contribute to the development of acute gastric mucosal lesions in critically ill or severely stressed patients. Pepsin blocking agents such as bismuth [53,54] or sucralfate [55] may improve current prophylaxis against acute gastric mucosal lesions while decreasing its morbidity.

Table 1

Diagnoses of Patients Studied

ICU Patients	Routine Patients
Mandibulectomy, rad neck dissection	Hemicolectomy
Massive MI, intraaortic balloon pump	Sigmoid resection
Colectomy, portal 5-FU infusion	Neg neck exploration
Head injury	Appendectomy
Thoracic aortic dissection	Appendectomy
Severe pancreatitis	2 weeks after esophagectomy
Esophagectomy for cancer	Colostomy closure
Colectomy	Cholecystectomy
Thymectomy, respiratory failure	Lower GI bleeding
Head injury	Hemicolectomy
Emergency portocaval shunt	Lower GI bleeding
Radical neck dissection	Lower GI bleeding
Emergency coronary bypass after myocardial infarction	2 weeks after gastrojejunostomy
Cholecystectomy, sepsis epinephrine, dobutamine drips	Coma
Abdominal aortic aneurysm resection	S/P inc. ing. hernia
Hemicolectomy, pneumonia, sepsis	Anterior resection
Drug overdose	Cholecystectomy
Pneumonia, Ogilvie's syndrome	Elective colostomy
Removal infarcted small bowel	Partial small bowel obstruction

Table II

	PD (-mv)	RESISTANCE (ohms/cm2)	PEPSIN (u/cm2/hr)	ACID (ueqH+/cm2/hr)
ENDOTOXIN EXPTS (5% CO2)				
0 u/ml endotoxin	35.5±1.6	112±13	0.17±0.07	0.89±0.06
0.01 u/ml endotoxin	34.1±1.4	135±16**	0.57±0.21*	0.74±0.07**
0.10 u/ml endotoxin	29.9±1.3*	190±26**	1.04±0.55*	0.60±0.09**
OXYGEN EXPTS (0% CO2)				
100% O2	24.5±2.7	64±7	0.09±0.07	0.65±0.06
Room air	3.1±2.8**	244±21**	0.33±0.08**	0.20±0.03**
100% N2	0.5±0.5**	460±37**	0.57±0.10**	0.008±0.005**

*$p<0.05$ **$p<0.001$

Table III

	Age	M/F	pH	Peptic Activity	Occult Bld
Routine	58+6	17/3	6.1+0.4	315+101 u/ml	2/20
Critical	59+4	18/2	5.7+0.4	5490+101 u/ml**	10/20*

(**p<0.0001 *p<0.01)

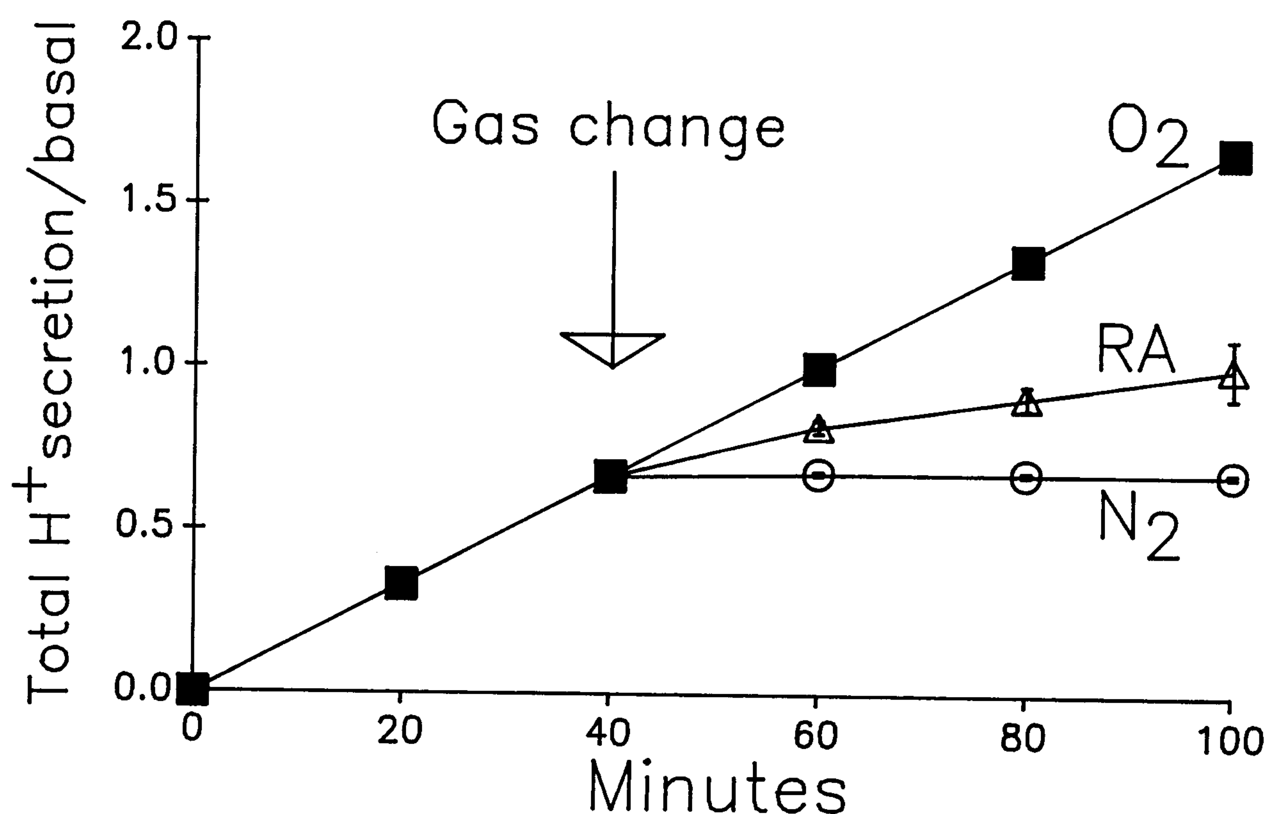

Figure 1. Eighteen tissues were initially equilibrated on pure oxygen. Six were then continued on oxygen (indicated by filled squares) while six were then treated with room air (indicated by open triangles) and six were gassed with pure nitrogen (indicated by open circles). Values shown represent total acid secretion per preparation normalized to each preparation's basal acid secretory rate (mean ± standard error).

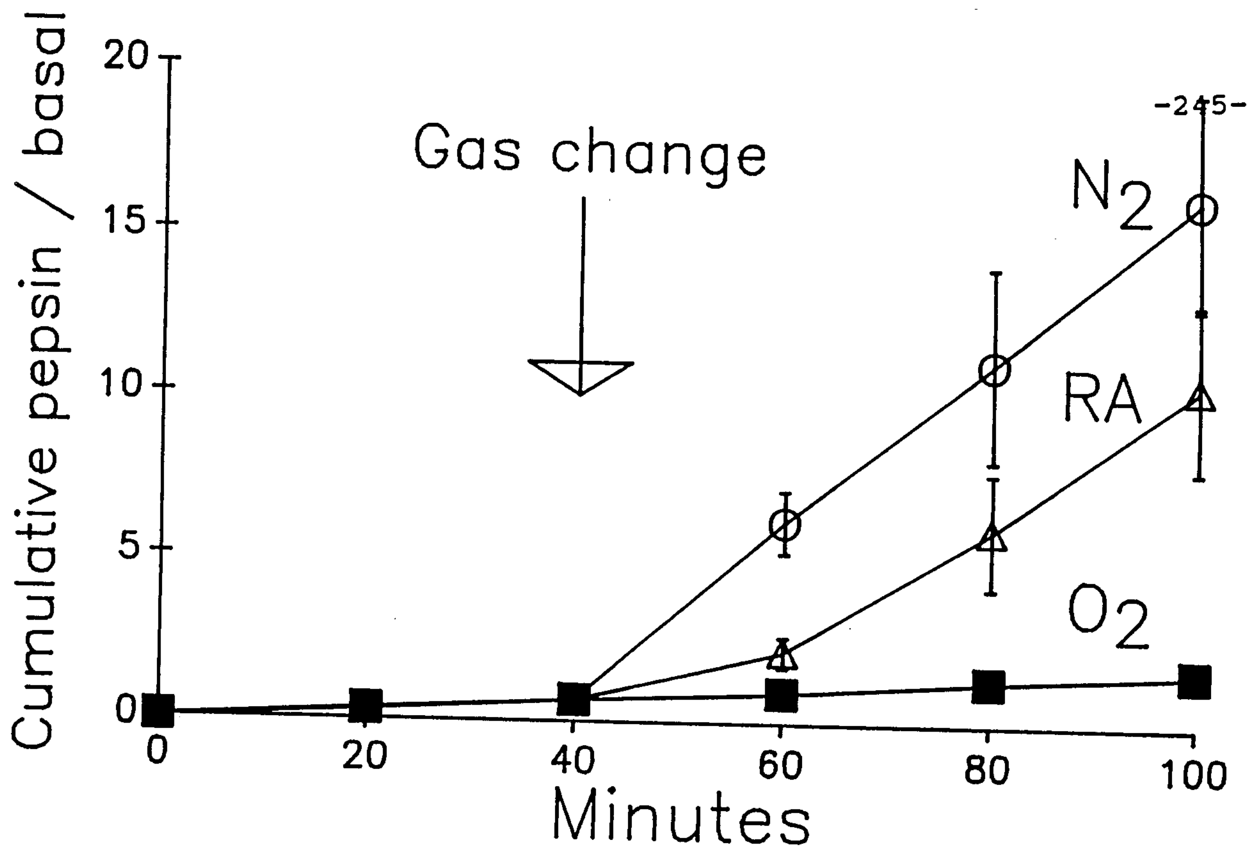

Figure 2. Eighteen tissues were initially equilibrated on pure oxygen. Six were then continued on oxygen (indicated by filled squares) while six were then treated with room air (indicated by open triangles) and six were gassed with pure nitrogen (indicated by open circles). Values shown represent peptic activity in the luminal solutions of these preparations normalized to each preparation's basal pepsinogen secretory rate (mean ± standard error).

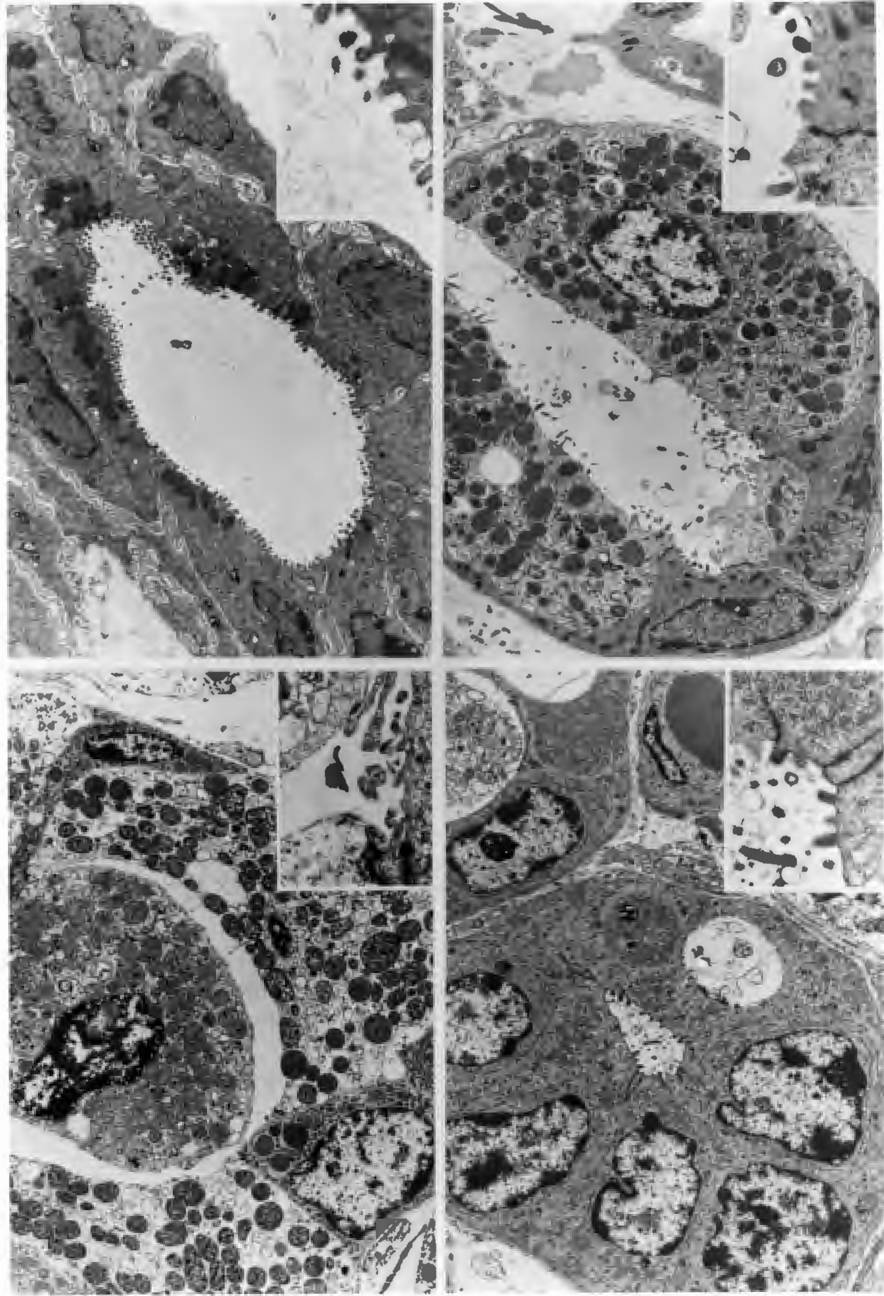

Figure 3. Transmission electron micrographs of representative glandular units from oxygenated (a), room air treated (b), nitrogen treated (c), and endotoxin treated (d) mucosal sheets. (original magnification 7,300X) Insets (22500X) demonstrate luminal microvillar morphology. (a) Transmission electron micrograph of mucosa maintained on pure oxygen demonstrates viable glandular histology with minimal edema and numerous microvilli lining the lumen. The cells shown are mostly surface and mucus neck cells. (b) Electron micrograph of room air-gassed tissue illustrates cellular damage seen in some areas of the mucosa. Although nucleii and cell membranes appear basically intact and tight junctions persist, some separation is seen between cell membranes basal to the tight junction. Microvilli are reduced but present. Numerous mitochondria suggest parietal cells. (c) Electron micrograph of nitrogen-gassed tissue reveals loss of normal cytoskeletal structure with nuclear clearing, vacuolization and cellular debris in the gland lumen. Microvilli are absent from the lumen. Preserved mitochondrial structures suggest that the cells seen are parietal cells. (d) Endotoxin-exposed tissues appear less edematous with smaller glandular lumens and less interstitial space. Nuclei appear morphologically normal in most cells and cytoskeletal structure is generally intact. However, some cellular damage is observed. Microvilli are decreased but present. These may represent surface cells.

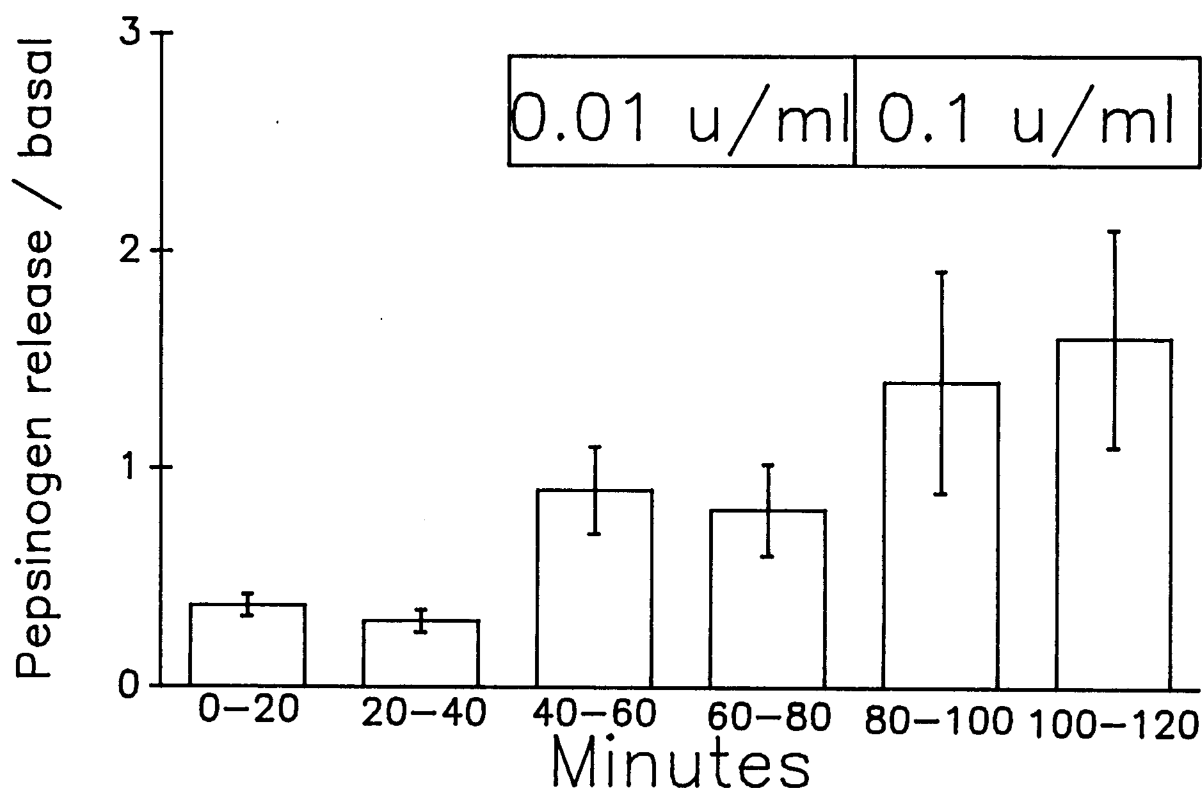

Figure 4. Mucosal preparations (n=8) were monitored under basal conditions for forty minutes, then treated with 0.01 units/ml of endotoxin for forty minutes, and finally exposed to 0.1 units/ml of endotoxin for an additional forty minutes. Values shown represent the increase in pepsinogen secretion observed in each twenty minute interval expressed as multiples of the basal secretory rate (mean ± standard error).

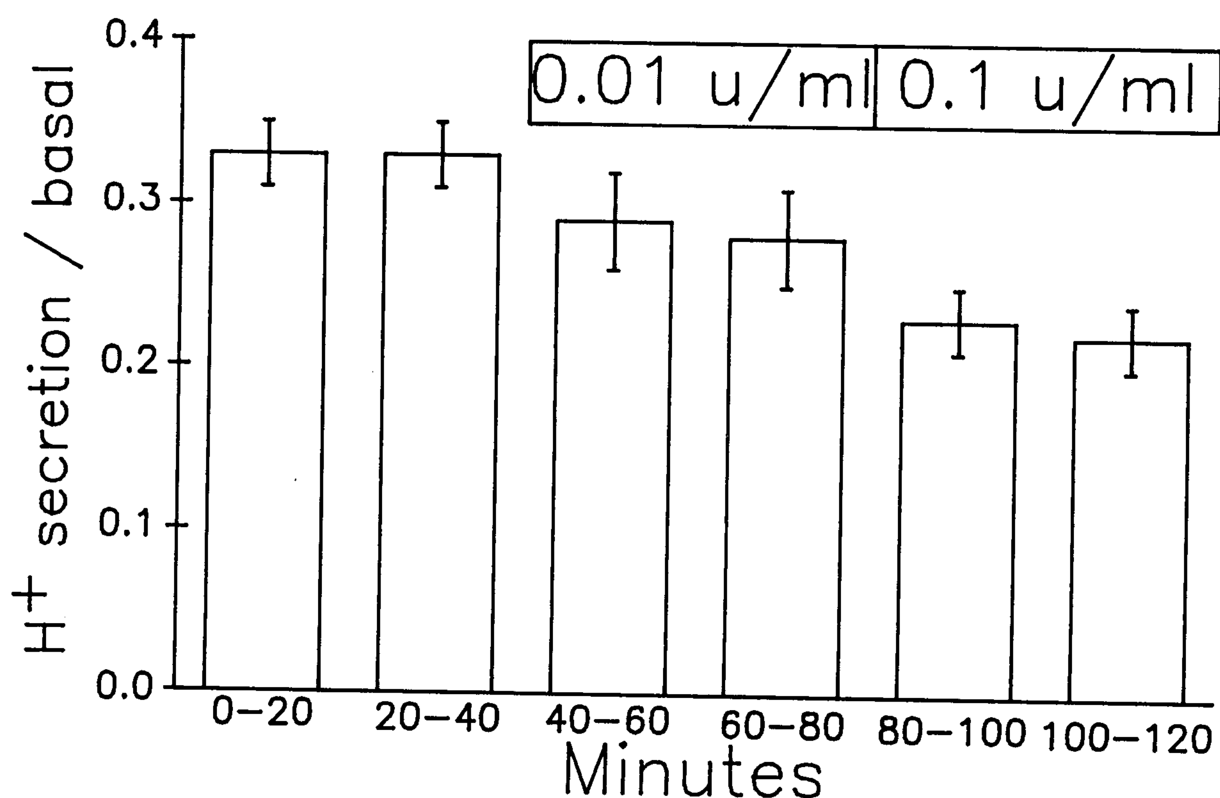

Figure 5. Mucosal preparations (n=8) were monitored under basal conditions for forty minutes, then treated with 0.01 units/ml of endotoxin for forty minutes, and finally exposed to 0.1 units/ml of endotoxin for an additional forty minutes. Values shown represent the increase in acid secretion observed in each twenty minute interval expressed as multiples of the basal secretory rate (mean ± standard error).

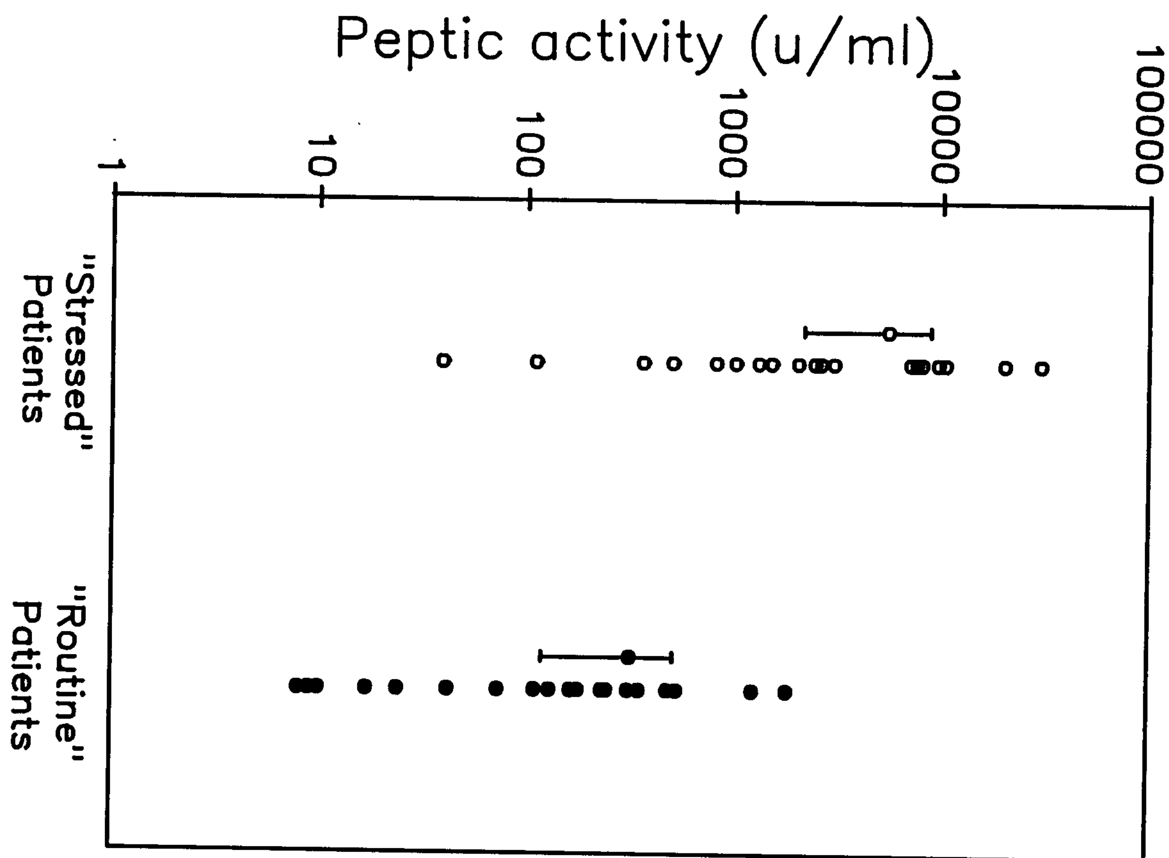

Figure 6. Intragastric peptic activity of twenty critically ill patients is compared to that of twenty routinely convalescing patients. Each data point represents the peptic activity of a gastric sample from a single patient assayed in triplicate. Means and 95% confidence intervals for each group are also depicted.

SUMMARY

Despite blockade and neutralization of gastric acid, acute gastric lesions cause substantial morbidity and mortality in critically ill patients. Pepsinogen release in response to noxious stimuli such as hypoxia and endotoxin might contribute to mucosal damage. Guinea pig fundic mucosa was mounted in Ussing chambers. Acid secretion, pepsinogen release, potential difference (PD) and resistance were monitored. Gassing with room air or nitrogen diminished acid secretion and PD but increased pepsinogen release 9.7- and 15.5-fold respectively (both $p<0.001$). Similarly, endotoxin (0.01 and 0.1 units/ml) dose-dependently inhibited acid secretion and PD but increased pepsinogen release 3.3- and 6.1-fold (both $p<0.05$). Endotoxic and air-gassed tissues were edematous with scattered cellular damage by light and transmission electron microscopy; nitrogen-exposed membranes appeared necrotic. Pepsin release may therefore have resulted from cell damage rather than exocytosis. Intragastric peptic activity in critically ill H_2-receptor blocked patients (n=20) was 5490 $\pm$ 1701 u/ml. The gastric juice of H_2-blocked convalescing surgical patients (n=20) contained 315 $\pm$ 101 u/ml. ($p<0.0001$) Occult blood correlated with intragastric peptic activity ($r=0.59$, $p<0.0001$) but not with gastric pH ($r=0.04$, $p=0.6$) These data suggest that the complex of pathophysiologic abnormalities common in critical illness cause substantial pepsin release. The efflux of this potent mucolytic barrier breaker may damage gastric mucosa in severely stressed patients.

REFERENCES

1. Czaja, A.J., McAlhany, J.C., and B.A. Pruitt, Jr. 1974. Acute gastroduodenal disease after thermal injury: An endoscopic evaluation of incidence and natural history. N. Engl. J. Med. 291:925-929.

2. Halloran, L.G., Zfass, A.M., Gayle, W.E., Wheeler, C.B.,and J.D. Miller. 1980. Prevention of acute gastrointestinal complications after severe head injury; A controlled trial of cimetidine prophylaxis. Am. J. Surg. 139:44-49.

3. Peura, D.A., and L. R. Johnson. 1985. Cimetidine for prevention and treatment of gastroduodenal mucosal lesions in patients in an intensive care unit. Ann. Int. Med. 103:173-177.

4. Zinner, M.J., Zuidema, G.D., Smith, P.L.,and M. Mignosa. 1981. The prevention of upper gastrointestinal tract bleeding in patients in an intensive care unit. Surg. Gynec. Obstet. 153:214-220.

5. Hastings, P.R., Skillman, J.J., Bushness, L.S., and W.S. Silen. 1978. Antacid prophylaxis of bleeding in the criticallly ill. N. Engl. J. Med. 298:1041-1045.

6. McGuigan, J.E. 1981. A consideration of the adverse effects of cimetidine. Gastroenterology 80:181-192.

7. Feretis, C.B., Contou, C.T., Papoutsis, G.G., Contostolis, S.B., Basioukas, P.S. Calaitzopoulos, J.D., Nissiotis, A.S., and N.S. Apostolidis. 1984. The effect of preoperative treatment with cimetidine on postoperative wound sepsis. Am. Surg. 50:594-652.

8. Axon, A.T.R. 1986. Potential hazards of hypochlorhydria in the treatment of peptic ulcer. Scand. J. Gastroenterol. 21(Suppl 122):17-21.

9. Driks, M.K., Craven, D.E., Celli, B.R., Manning, M., Burke, R.A., Garvin, G.M., Kunches, L.M., Farber, H.W., Wedel, S.A.,and W.R. McCabe. 1987. Nosocomial pneumonia in intubated patients given sucralfate as compared with antacids or histamine type 2 blockers. N. Engl. J. Med. 317:1376-1382.

10. Stremple, J.F., Molot, M.D., McNamara, J., Mori, H., and G.B.J. Glass. 1972. Posttraumatic gastric bleeding: Prospective gastric secretion composition. Arch. Surg. 105:177-185.

11. O'Neill, J.A. 1970. The influence of thermal burns on gastric acid secretion. Surgery 67:267-271.

12. Pearson, J.P., Ward, R., Allen, A., Roberts, N.B., and W.H. Taylor 1986. Mucus degradation by pepsin: comparison of mucolytic activity of human pepsin 1 and pepsin 3: implications in peptic ulceration. Gut 27:243-248.

13. Venables, C.W. 1986. Mucus, pepsin, and peptic ulceration. Gut 27:233-238.

14. Rotter, J.I., Sones, J.Q., Samloff, I.M., Richardson, T.T., Gursky, J.M., Walsh, J.H., and D.L. Rimoin. 1979. Duodenal ulcer disease associated with elevated serum pepsinogen I. N. Engl. J. Med. 300:63-66.

15. Samloff, I.M., Stemmermann, G.N., Heilbrun, L.K., and A. Nomura. 1986. Elevated serum pepsinogen I and II levels differ as risk factors for duodenal ulcer and gastric ulcer. Gastroenterology 90:570-576.

16. Venables, C.W. 1971. The secretion of pepsin 1 in peptic ulceration and its relationship to activity. Br. J. Surg. 58:864.

17. Hirschowitz, B.I. 1982. Factors influencing pentagastrin dose response of acid, chloride and pepsin in peptic ulcer. In Gut Peptides and Ulcer. A. Miyoshi, editors. Biomedical Research Foundation, Tokyo, 357-365.

18. Schiffrin, M.J., and A.A. Warren. 1942. Some factors concerned in the production of experimental ulceration of the gastrointestinal tract in cats. Am. J. Dig. Dis. 9:205-209.

19. Kivilaakso, E., Barzilai, A., Schiessel, R., Crass, R., and W. Silen. 1979. Ulceration of isolated amphibian gastric mucosa. Gastroenterology 77:31-37.

20. Wallace, J.L., and B.J.R. Whittle. 1986. Role of mucus in the repair of gastric epithelial damage in the rat: Inhibition of epithelial recovery by mucolytic agents. Gastroenterology 91:603-611.

21. Hirschowitz, B.I. 1967. The control of pepsinogen secretion. Ann NY Acad Sci 140:709-723.

22. Hersey, S.J., Miller, M., May, D., and S.H. Norris. 1983. Lack of interaction between acid and pepsinogen secretion in isolated gastric glands. Am. J. Physiol. 245:G775-G779.

23. Sandvik, A.K., Kleveland, P.M., and H.L. Waldum. 1987. Stimulated pepsin secretion after omeprazole-induced acid suppression in the totally isolated vascularly perfused rat stomach. Scand. J. Gastroenterol. 22:362-366.

24. Basson, M.D., Zucker, K.A., Adrian, T.E., Zdon, M.J., Ballantyne, G.H., and I.M. Modlin. 1988. Pepsin release by prostaglandin E1 analogue: A potential therapeutic problem. Arch. Surg. 123:432-434.

25. Basson, M.D., Adrian, T.E., and I.M. Modlin. 1988. Dissociation of pepsinogen and acid secretion in the guinea pig. Gastroenterology 95:321-326.

26. Pearson, J.P., Ward, R., Allen, A., Roberts, V.B. and W.H. Taylor. 1986. Mucus degradation by pepsin: comparison of mucolytic activity of human pepsin 1 and pepsin 3: implications in peptic ulceration. Gut 27:243-248.

27. Bihari, D., Smithies, M., Gimson, A., and J. Tinker. 1987. The effects of vasodilation with prostacyclin on oxygen delivery and uptake in critically ill patients. N. Engl. J. Med. 317:397-403.

28. Levin, J., Poore, T.E., Young, N.S., Margolis, S., Zauber, N.P., Townes, A.S., and W.R. Bell. 1972. Gram-negative sepsis: Detection of endotoxemia with the Limulus test. Ann. Int. Med. 76:1-7.

29. Winchurch, R.A., Thupari, J.N., and A.M. Munster. 1987. Endotoxemia in burn patients: Levels of circulating endotoxins are related to burn size. Surgery 102:808-812.

30. Rutten, M.J., and S. Ito. Morphology and electrophysiology of guinea pig gastric mucosal repair in vitro. Am. J. Physiol. 244:G171-G182.

31. Finke, U., Rutten, M., Murphy, R.A., and W. Silen. 1985. Effects of epidermal growth factor on acid secretion from guinea pig gastric mucosa: in vitro analysis. Gastroenterology 88:1175-1182.

32. Kidder, G.W. 1975. Electrophysiology of the frog gastric mucosa with sufficient CO_2. Am. J. Physiol. 228:928-933.

33. Sanders, S.S., Hayne, V.B., and W.S. Rehm. 1973. Normal H^+ rates in frog stomach in absence of exogenous CO_2 and a note on pH stat method. Am. J. Physiol. 225:1311-1321.

34. Sen, P.C., Tague, L.L., and T.K. Ray. 1980. Secretion of H^+ and K^+ by bullfrog gastric mucosa: Characterization of K^+ transport pathway. Am. J. Physiol. 239:G484-G492.

35. Impicciatore, M., Hansen, D.G., Rachmilewitz, D., Maitra, S.K., Lugaro, G., and M.I. Grossman. 1980. Comparison of human urine gastric inhibitor (HUGI) and bacterial endotoxin as inhibitors of acid secretion. Eur. J. Pharmacol. 65:365-8.

36. Payne, J.G., and J.C. Bowen. 1981. Hypoxia of canine gastric mucosa caused by Escherichia coli sepsis and prevented with methylprednisolone therapy. Gastroenterology 80:84-93.

37. Menguy, R., and Y.F. Masters. 1974. Gastric mucosal energy metabolism and "stress ulceration". Ann Surg 180:538-548.

38. Domschke, W., Lux, G., and S. Domschke. 1980. Furan H_2-antagonist ranitidine inhibits pentagastrin-stimulated gastric secretion stronger than cimetidine. Gastroenterology 79:1267-1271.

39. Hirschowitz, B.I., Danilewitz, M., and E. Molins. 1982. Inhibition of basal acid, chloride and pepsin secretion in duodenal ulcer by graded doses of ranitidine and atropine with studies of pharmacokinetics of ranitidine. Gastroenterology 82:1314-1326.

40. Albillos, A., Abreu, L., Alvarez-Mon, M., Gea, F., Gonzalo, M.A., Rossi, I., Garrido, A., Barrios, C., and P. Escartin. 1988. Study of the secretion of pepsinogen I in cirrhotic humans with and without portocaval shunt. Am. J. Gastroenterol. 83:37-41.

41. Idjadi, F., Robbins, R., Stahl, W.M., and G. Essiet. 1971. Prospective study of gastric secretion in stressed patients with intracranial injury. J. Trauma 11:681-688.

42. Walker, P., Luther, J., Samloff, I.M., and M. Feldman. 1988. Life events stress and psychosocial factors in men with peptic ulcer disease: II. Relationships with serum pepsinogen concentrations and behavioral risk factors. Gastroenterology 94:323-330.

43. Walker, V., and W.H. Taylor. 1976. Pepsin 1 secretion in peptic ulcer and stress. Clin Sci Mol Med 51:14.

44. Koelz, J.R., Hersey, S.J., Sachs, G., and C.S. Chew. 1982. Pepsinogen release from isolated gastric glands. Am. J. Physiol. 243:G218-G225.

45. Matsumoto, H., Dickinson, K.E.J., Shirakawa, T., Komiyama, K., and B.I. Hirschowitz. 1987. Synergism between cellular messengers and agonist combinations in pepsinogen secretion. Am. J. Physiol. 253:G557-G565.

46. Ballantyne, G.H., Zdon, M.J., Schafer, D.E., Fratesi, G.R., Roberts, J.R., Tyshkov, M., and I.M. Modlin. 1986. Evidence for dual modulation of pepsinogen secretion using isoproterenol, carbachol, CCK-8, forskolin, 8 bromo-cAMP, and A23187 probes. Ann. Surg. 204:559-565.

47. Walker, V., and W.H. Taylor. 1979. Cigarette smoking, chronic peptic ulceration and pepsin 1 secretion. Gut 20:971-976.

48. Wright, C.L., Shaw, B., Sanders, D.J. and J.D. Reed. 1975. Variations in the proportions of individual pepsins secreted by the cat in response to vagal stimulation and hypoglycemia. Clin Sci Mol Med 48:297-305.

49. Ohe, K., Yokoya, H., and Kitaura, T., Kunita, T., and A. Miyoshi. 1980. Increase in pepsin content during the course of aspirin- and taurocholate-induced gastrin ulceration in rats. Dig Dis Sci 25:849-856.

50. Zinner, M.J., Turtinen, L., and N.J. Gurll. 1975. Stress ulceration and the gastric mucosal barrier. Am. Surgeon 41:209-213.

51. Schlamowitz, M., and L.U. Peterson. 1950. Studies on the optimum pH for the action of pepsin on "native" and denatured bovine serum albumin and bovine hemoglobin. J. Biol. Chem. 234:3137-3145.

52. Brouwers, J.R.B.J. 1981. Pathofysiologie en Farmacatherapie van peptische afwijkingen. Thesis, University of Amerstdam. As cited in: Wieriks, J., Jaitly, K.D., Koekkoek, P.H., and U. Lavy. 1982. Pharmacological properties of colloidal bismuth subcitrate (CBS-DE-NOL). Scand. J. Gastroenterol. 17(Suppl 80):11-16.

53. Wieriks, J., Jaitly, K.D., Koekkoek, P.H., and U. Lavy. 1982. Pharmacological properties of colloidal bismuth subcitrate (CBS-DE-NOL). Scand. J. Gastroenterol. 17(Suppl 80):11-16.

54. Baron, J.H., Barr, J., Batten, J., Sidebotham, R., and J. Spencer. 1986. Acid, pepsin and mucus secretion in patients with gastric and duodenal ulcer before and after colloidal bismuth subcitrate (De-Nol). Gut 27:486-491.

55. Samloff, I.M., and C. O'Dell. 1985. Inhibition of peptic activity by sucralfate. Am. J. Med. 79(Suppl 2C):15-18.

Conclusion

This thesis provides a broad historical background of digestion and the contextual relationship of pepsinogen to the understanding of digestive physiology. It describes the specific roles of two persons, Schwann and Langley, in the discovery of a specific ferment in the gastric mucosa and juice. The methodology section includes the validation and development of isolated gastric glands, parietal cells and Ussing chamber systems for use in these studies. In addition, it describes novel techniques--the microtitre plate for measurement of secretion and an isotopic technique for the quantitative of the relatively small pepsinogen signal generated by mammalian mucosa.

The initial chapters of the experimental section serve to validate the system and confirm existing information. The subsequent search for a specific pepsinogen inhibitor, however, proved difficult. Studies with the putative calmodulin antagonist trifluperazine provided data which was probably spurious and reflective of either cell damage or multiple nonspecific effects. The methylated synthetic prostaglandin E1 analogue (misoprostol) proved itself to be an agonist of pepsinogen secretion rather than the contrary, although it inhibited acid secretion as expected. In the subsequent study, it was apparent that blocking the parietal cell proton pump specifically had no inhibitory effect on pepsinogen secretion. Indeed, it required a specific CCK receptor inhibitor (L364718) to inhibit CCK stimulated pepsinogen secretion. Studies with both acid (histamine) and pepsinogen (CCK and carbachol) agonists demonstrated the very specific nature of CCK receptor blockade on chief cell function. The final study of the experimental section was undertaken in an attempt to relate events present in critically ill patients to an experimental mucosal membrane model. Thus endotoxemia and hypoxia were shown to generate substantial release of pepsinogen concomitant with morphological evidence of mucosal damage. Similarly, in a group of critically ill patients, a marked increase in pepsinogen levels in gastric juice was noted. While the latter data are no way conclusive, they are consistent with the possible relationship between pepsinogen release and events noted to occur in the gastric mucosa of critically ill patients.

This thesis demonstrates that chief cell function may be studied using isolated gastric gland preparations and isolated mucosal membrane models. It provides clear evidence that both cyclic AMP and calcium calmodulin systems are involved in the secretion of pepsinogen. It refutes the possibility that a calcium calmodulin antagonist trifluperazine could be of use in the inhibition of pepsinogen secretion and demonstrates that the acid inhibitory antiulcer agent misoprostol (synthetic prostaglandin E1 analogue) increases pepsinogen secretion.

Of particular interest, was the finding of a clear dissociation of chief cell function from parietal cell function. This observation may be of particular relevance in the therapeutic control of pepsinogen secretion. A further novel observation was the demonstration of the efficacy of the CCK receptor antagonist L364718 in the inhibition of CCK stimulated pepsinogen secretion. The possible therapeutic application of this observation may warrant further consideration. Finally, the proposal that events which occur in critically ill patients may in some way augment pepsinogen release has been addressed. Although no ideal model of such a situation exists, the studies described in this thesis indicate a major release of pepsinogen under conditions of hypoxemia and endotoxemia generated in a Ussing chamber mucosal membrane model. Similarly, elevation of pepsinogen levels on the gastric juice in critically ill patients, though not conclusive, may be construed as further evidence in support of the proposal.

Pepsinogen is one of the most potent proteolytic enzymes and is found in substantial quantities in the gastric mucosa. These studies draw specific attention to the important modulatory role of cholecystokinin in its secretion. In addition, they define the separation between the physiological control mechanisms of acid secretion and pepsinogen. Finally, they suggest that substantial release of pepsinogen occurs under pathological conditions in the gastric mucosa consistent with those present in critically ill patients.