QUANTITATIVE CYTOCHEMICAL STUDIES OF ACID SECRETAGOGUE EFFECTS ON THE CARBONIC ANHYDRASE ACTIVITY OF GASTRIC PARIETAL CELL SECTIONS

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To Denise, Lindy and Jeffery

CONTENTS

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ABSTRACT

Acid secretion is mediated by hormonal, paracrine, neurocrine and luminal factors. The major acid secretagogues are gastrin, histamine and acetylcholine. Controversy exists regarding the specific actions and interactions of these agents upon the acid secreting parietal cell. Code (1956, 1977) suggested that histamine is the final common pathway for all acid secretagogues. However Soll, using oxygen consumption (1978 a, b) and (^{14}C)- aminopyrine uptake by isolated canine parietal cells (1980) as an index of acid secretion, showed independent and potentiating effects of these agents. Gastrin was only a weak stimulus for oxygen uptake and did so only in high doses and in the presence of the phosphodiesterase inhibitor isobutyl-methylxanthine. In contrast, Loveridge et al (1974) described a quantitative cytochemical assay, which detects changes in parietal cell carbonic anhydrase (CA) and is sensitive to gastrin in concentrations of 2.5×10^{-15} to 2.5×10^{-12} mol/l. CA is present in the stomach of all acid secreting species. Its function appears to be the maintenance of intracellular acid-base balance and is necessary for stimulated, but not basal acid secretion. Parietal cell CA activity may therefore be regarded as an index of parietal cell function.

Since Loveridge et al (1974) had shown activation of CA by concentrations of gastrin lower than that found in the serum from fasting individuals and as CA activity is an index of parietal cell function, Loveridge's model was adapted and utilised to study the status and function of the secretagogues acting upon the parietal cell.

CA activity was measured in 18 μm sections obtained from fundic mucosa of fasted guinea pigs. CA catalysed the formation of OH^- ions which were trapped with cobalt and precipitated with hydrogen sulphide to form a cobalt sulphide precipitate in the cytoplasm of the parietal cells. The density of the staining, which was measured in 15 - 20 cells per section by quantitative cytochemistry with a Vickers M85 scanning microdensitometer, was a reflection of CA activity. The coefficient of variation in staining between the cells of one section was 2.2 - 6.9%.

Heptadecapeptide gastrin (G17-I) (2.3×10^{-15} to 2.5×10^{-12} mol/l), histamine (5.8×10^{-17} to 1.0×10^{-14} mol/l) and carbamylcholine (1.2×10^{-12} to 1.0×10^{-9} mol/l) caused dose-related increases in CA activity. The optimal response time for all three agents was 90 seconds. The response to G17-I was inhibited by boiling the tissue, by acetazolamide (10^{-5}M) and by anoxia. After an anoxic period there was incomplete recovery of the G17-I response.

Comparing the D_{50}'s (dose causing half-maximal effects), histamine was the most potent of the secretagogues, being 100 times more potent than gastrin which was in turn 400 times more potent than carbamylcholine. The maximum effect of gastrin, however was approximately 50% higher than either histamine or carbamylcholine.

The addition of subthreshold doses of G17-I (2.5×10^{-16}M) to each concentration of histamine and vice versa, of subthreshold doses of histamine (1.0×10^{-17}M) to each concentration of G17-I significantly reduced the minimum effective dose and the D_{50} calculated from the regression lines for histamine and G17-I respectively. Subthreshold doses of neither agent altered the carbamylcholine dose response curves.

Moreover, the addition of a subthreshold dose of carbamylcholine (1.0×10^{-14}M) did not alter the histamine dose response curve but did cause a small reduction in the maximal effect of gastrin. Histamine (1.0×10^{-14}M)-stimulated CA activity was reduced $74 \pm 7.8\%$ by the addition of the histamine H_2-receptor blocking agent, cimetidine, whereas G17-I (2.5×10^{-12}M) was reduced by $23 \pm 4\%$. Cimetidine did not significantly alter carbamylcholine-stimulated CA activity. Atropine (10^{-5}M) in contrast had no significant effect upon either histamine-or gastrin-stimulated CA activity but inhibited carbamylcholine (1.0×10^{-9} mol/l)-stimulated activity by $79 \pm 9\%$.

These studies indicate firstly that CA in guinea pig parietal tissue is activated by G17-I, histamine and carbamylcholine independently and secondly, that G17-I and histamine potentiate each other and act independently of cholinergic pathways.

Lastly to show the independence of action of the secretagogues the effect of the calcium channel blocking agent lanthanum chloride (10^{-6}M) on gastrin,histamine-and carbamylcholine-stimulated CA activity was tested. Lanthanum caused $77 \pm 8\%$ inhibition of the carbamylcholine (1.0×10^{-9} mol/l) effect, $45 \pm 10\%$ inhibition of the gastrin (2.5×10^{-12} mol/l) effect and $2 \pm 9\%$ inhibition of the histamine (1.0×10^{-14}mol/l) effect. This is further evidence in favour of the independent mechanisms of action of the acid secretagogues.

Gastrin comprises a group of circulating peptides of varying molecular forms, including big gastrin (G34), little gastrin (G17) and minigastrin (G14),. all of which may be sulphated or non-sulphated.

Cholecystokinin (CCK) shares a common carboxyterminal pentapeptide sequence and the smaller molecular weight forms may also have an effect in acid secretion. The potency of these peptides in acid secretion is difficult to assess because of varying rates of endogenous secretion and degradation. This could be overcome by measurement of potency at a cellular level by quantitating their effect upon CA activity. Synthetic human G17-I (SHG-17-I), natural human G17-I (NHG-17-I), NHG-17-II, and the carboxyterminal decapeptide had similar effects on CA activity. Pentagastrin and CCK-octapeptide caused dose-dependent increases in CA activity parallel to SHG-17-I with relative potencies of 32% and 4% respectively. G34-I caused a non-parallel dose-dependent increase in CA activity with a similar potency to SHG-17-I. The amino-terminal decapeptide of G17-I had no effect on CA activity and confirmed that the biological activity of gastrin lies at the carboxyterminal.

The last section of this thesis deals with the measurement of the carbonic anhydrase stimulating activity (CASA) of serum in comparison with the gastrin radio-immunoassay. Loveridge et al (1974) described this assay as a cytochemical bioassay for gastrin but since other secretagogues stimulate CA activity this may not be specific for gastrin. However, removal of gastrin from serum by charcoal absorption and affinity chromatography removed all CASA from the serum, suggesting that gastrin may in fact be the major circulating CASA.However, since no specific precautions were taken to prevent degradation of histamine, which occurs very rapidly in serum, one cannot be certain of this.

There was no correlation between the immunoreactive gastrins measured by two antisera and the CASA in either the fasting or post-prandial serum samples. Following a meal the CASA rose more slowly than the immunoreactive gastrins and reached a peak at 60 minutes (compared to 15 minutes for the immunoreactive gastrins) and fell more rapidly to reach baseline by 120 minutes (compared to 180 minutes for the immunoreactive gastrins). It is possible that the effects of CASA in serum may be modulated by circulating inhibitors and other secretagogues whereas the immunoreactive gastrins include a number with varying biological activity. This may account for the disparities noted. Patients with hypergastrinaemia had a significantly elevated level of CASA in their serum although again, the correlation between CASA and immunoreactive gastrins was poor.

This thesis thus presents work designed to study the effects of acid secretagogues upon the parietal cell, in order to gain a greater understanding of their modes of action, and interaction and the role of circulating secretagogues in the mediation of parietal cell function, with the aim of increasing the understanding of the pathophysiology of the world-wide problem of peptic ulcer disease.

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BBG	big big gastrin
BSA	bovine serum albumin
^{o}C	degrees centigrade
CA	carbonic anhydrase
C-amp	3'- 5'cyclic adenosine monophosphate
CASA	carbonic anhydrase stimulating activity
CCK	cholecystokinin
CCK-8	cholecystokinin octapeptide
CNS	central nervous system
CPC	cobalt phosphate complex
cpm	counts per minute
D_{min}	minimal effective dose
D_{50}	dose causing half maximal effect
Fig.	figure
FSH	follicle stimulating hormone
g.	gram
GIP	gastric inhibitory polypeptide
GIT	gastro-intestinal tract
G14	minigastrin
G17	heptadecapeptide or 'little' gastrin.
G17-I	non-sulphated heptadecapeptide gastrin
G17-II	sulphated heptadecapeptide gastrin
G34	big gastrin
HACA	high activity carbonic anhydrase
hr	hour
l	litre
LACA	low activity carbonic anhydrase
LH	luteinizing hormone
M	molar. (moles per litre)
meq.	milliequivalent
mg.	milligram
mm.	millimetre
mM	millimolar
mol	mole
ng	nanogram

NHG-17-I	non-sulphated heptadecapeptide natural human gastrin
NHG-17-II	sulphated heptadecapeptide natural human gastrin
nm	nanometres
p	probability
pg	picogram
PTH	parathyroid hormone
r	correlation coefficient
RNA	ribose nucleic acid
SEM	standard error of mean
SHG-17-I	non-sulphated heptadecapeptide synthetic human gastrin
TSH	thyroid stimulating hormone
VIP	vasoactive intestinal (poly) peptide
µCi	microcurie
µg	microgram
µl	microlitre
µm	micrometre

CHAPTER I.

OBJECTIVES OF THE STUDY

One may embark upon a project aimed at answering a specific question, during the course of which other questions are unearthed, tackled and solved, but the original question is never truly answered. The work to be described in this thesis proceeded in much this manner. Prof. Aaron Vinik, who was Co-director of the Endocrine and Diabetes research group at the University of Cape Town, had for several years been interested in the characterisation and measurements of the various species forms of gastrin-like molecules present in tissue and serum. The physiological role of these gastrins in acid secretion and their possible pathophysiological role in peptic ulcer disease was, and still is, not clear. To study this problem it was necessary to establish a sensitive bioassay for gastrin-like activity and in 1976 Dr. Beverly Napier travelled to London to learn the technique of the cytochemical bioassays in Dr. J. Chayen's laboratory at the Kennedy Institute. Thus, when I joined the group in 1977 my aim was, in collaboration with Dr. Napier, to establish a cytochemical section bioassay for gastrin, to measure the biological activity of gastrins in serum and to compare these with the immunoreactive gastrins, the purpose being to explain some of the discrepancies between acid secretion and serum gastrin levels which had previously been widely reported.

Acid secretion, however, is a complex process involving neurocrine, paracrine and hormonal mechanisms. There are at least three major acid secretagogues, of which gastrin is one, and in addition several putative physiological acid inhibitors.

These secretagogues and inhibitors interact on the parietal cell to cause, in some cases, potentiating effects (Soll 1978b) and as such, the role of an individual substance such as gastrin becomes all the more complicated.

It became obvious that the measurement of bioactive gastrins in serum was not possible as long as the biological end-point of the assay, namely the parietal cell was also under non-gastrin influences. Serum therefore contains "CA stimulatory activity" (CASA) of which doubtless, gastrin is a major, but not the only component. We nevertheless measured the CASA of serum in normal subjects, after a mixed meal and in hypergastrinaemic states and these results have been reported in this work.

However, the findings that the parietal cells in this system responded to all three major secretagogues led this work in a different direction. The discovery of the histamine H_2-receptor blockers by Black and his colleagues (Black, Duncan et al, 1972) and the findings that these agents significantly reduced acid secretion induced by all agents has led to renewed interest in the mechanisms of secretagogue interactions at a receptor level, and their mechanisms of action within the parietal cell. It became apparent that the technique of the cytochemical bioassay provided a unique model of parietal cell activation and the remainder of this work discusses the initial findings in this field.

This work therefore consists of three sections. Section A is a literature review in which the control of acid secretion by the four mechanisms, neurocrine, endocrine, paracrine and luminal factors is discussed, with special emphasis on gastrin, histamine and acetylcholine.

In addition, the mechanism of secretion of acid by the parietal cell has been briefly discussed with a special emphasis on the role of carbonic anhydrase (CA) in parietal cell function.

Section B includes the methods established for measuring CA activity in parietal mucosal sections by quantitative cytochemistry and the validation of these measurements.

This is followed by the effects of the three secretagogues on CA activity, their interactions, inhibition by the histamine H_2-receptor blocker cimetidine and the anticholinergic agent atropine and the effects of lanthanum chloride on secretagogue function.

Section C includes the cytochemical bioassay for CASA in serum of normal subjects, both fasting and postprandial and in hypergastrinaemic patients and the comparison with the radioimmunoassay for gastrin. This is followed by a summary of findings and the appendix in which the method of radioimmunoassay for gastrin, and statistical methods are discussed.

SECTION A: LITERATURE REVIEW

CHAPTER 2 ACID SECRETION IN THE STOMACH

INTRODUCTION

The secretion of acid by the upper gastrointestinal tract first appears among the lowest vertebrates on the phylogenetic tree and has since been retained by most species (Reite 1972).

The major physiological role for this acid seems to be in the facilitation of protein digestion by pepsin while other roles include the promotion of iron absorption by the conversion of dietary iron to the absorbable ferrous form, and the sterilisation of the upper gastrointestinal tract (Davenport 1977). The physiological importance of acid-peptic digestion in man however is probably quite limited while the other "physiological roles" are also relatively non-essential. This is evidenced by the normal nutritional status of persons with achlorhydria.

However, although acid secretion appears to be a dispensable physiological function in man, acid does play a major role in the pathophysiology of peptic ulcer disease and most of the bleeding disorders of the gastrointestinal tract. These diseases cause significant morbidity and mortality world-wide. For instance, in male Massachusetts physicians, the lifetime prevalence of peptic ulcers was 7.7% (Grossman 1979a) and in the U.S.A. about 342,000 cases of peptic ulcer are admitted to hospital each year. (Elashoff & Grossman, 1980). Furthermore the methods of treatment of peptic ulcers are still inadequate, in that there is a high recurrence rate after both medical and surgical treatment. This is partially due to inadequate understanding of the pathophysiology of this group of diseases.

In order to develop more rational forms of treatment, and to initiate appropriate preventative measures for the development of peptic ulcers, we must acquire a far greater understanding of the physiology of acid secretion. This includes its regulation, ionic fluxes across the mucosal barriers and the cellular physiology and biochemistry of the parietal cell per se.

This section reviews current knowledge on aspects of control and mechanisms of acid secretion with special reference to gastrin, histamine, acetylcholine and the role of carbonic anhydrase in acid secretion. The review includes literature published before July 1980.

CHAPTER 3

3: 1 Anatomy of the Parietal Mucosa

The enzymes and hydrochloric acid secreted in the stomach are mainly produced in the glands of the mucous membrane of the fundus and body of the stomach. The anatomy of these glands differs to some extent between species.

In the human the glands extend from the bottom of the gastric pits or foveolae, which appear like tiny openings in the gastric mucosa to the muscularis mucosa. They are straight most of their length but become tortuous near the muscularis mucosa. Each tubular gland consists of three segments; the deepest point is the base, the middle part is the neck and the upper part is the isthmus which is continuous with the pit. Several glands may open into a pit.(Fig. 3:1).

The glands contain four main types of secreting cells. The isthmus contains both surface epithelial cells, which secrete mucous, and parietal cells which secrete acid. The latter are large cells and have relatively clear cytoplasm stained by PA Schiff and haematoxylin methods. (Fig. 3:2). They vary from round to triangular in shape and have dark centrally placed nuclei. (Fig. 3:2). The size varies from 10-16 μ in diameter. The neck of the gland is made up chiefly of cells known as mucous neck cells. These are stuffed with pink mucous and have a foamy appearance. The nuclei of these cells are pressed against their bases where they often have a more or less triangular shape. Individual parietal cells are scattered between groups of mucous neck cells.

The base of the gland is made up mostly of zymogen (chief) cells. These have accumulations of basophilic material in their cytoplasm near their bases and produce pepsin.

The parietal cells are scattered among the zymogen cells. The guinea pig differs from the human in that the parietal cells are far more evenly distributed throughout the gland with a number apparent in both the base and neck area of the glands.

3: 2 The Fine Structure of the Parietal Cell (Lentz 1971)

Parietal cells are pyramidal or triangular in shape. The striking morphological specialization of this cell is secretory canaliculi that extend from the apex of the cell and pass lateral to the nucleus, almost to the base of the cell. Numerous microvilli extend into the lumen of the canaliculus. The canaliculi open into a common outlet that is continuous with the lumen of the gland. Thus the extensive surface area of the canaliculus is exposed to the glandular lumen. Microvilli also occur on the surface of the common opening and apical region of the cell. The lateral surfaces are relatively smooth in contour but the basal surface is thrown into villi or plications. Another unusual feature of this cell is an extensive system of cytoplasmic tubules limited by smooth membranes. These tubules may be continuous with the canaliculi in places, in which case they may be regarded as complex invaginations of plasma membrane rather than the smooth-surfaced endoplasmic reticulum. Mitochondria are relatively large, numerous and spherical or oval in shape. Cristae are closely packed and transverse over half the width of the organelle. Lyosomes are present as well as a few short cisternae of rough surfaced endoplasmic reticulum. Free ribosomes occur in the cytoplasm and a small Golgi apparatus near the base of the cell.

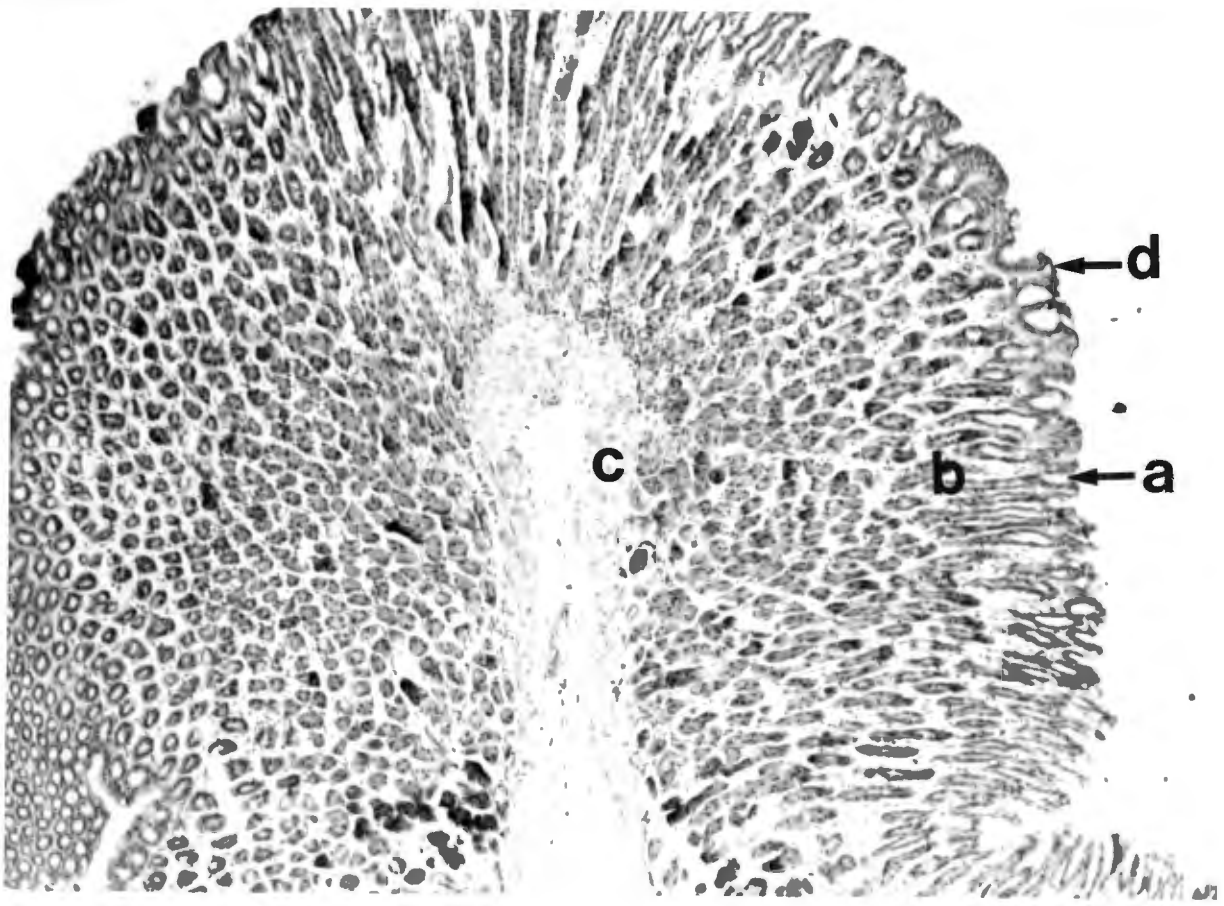


FIG. 3:1 Human Gastric Mucosa

a) Gastric pit

b) Neck of gland

c) Muscularis mucosa

d) Surface epithelium

X 60 Haematoxylin and Eosin

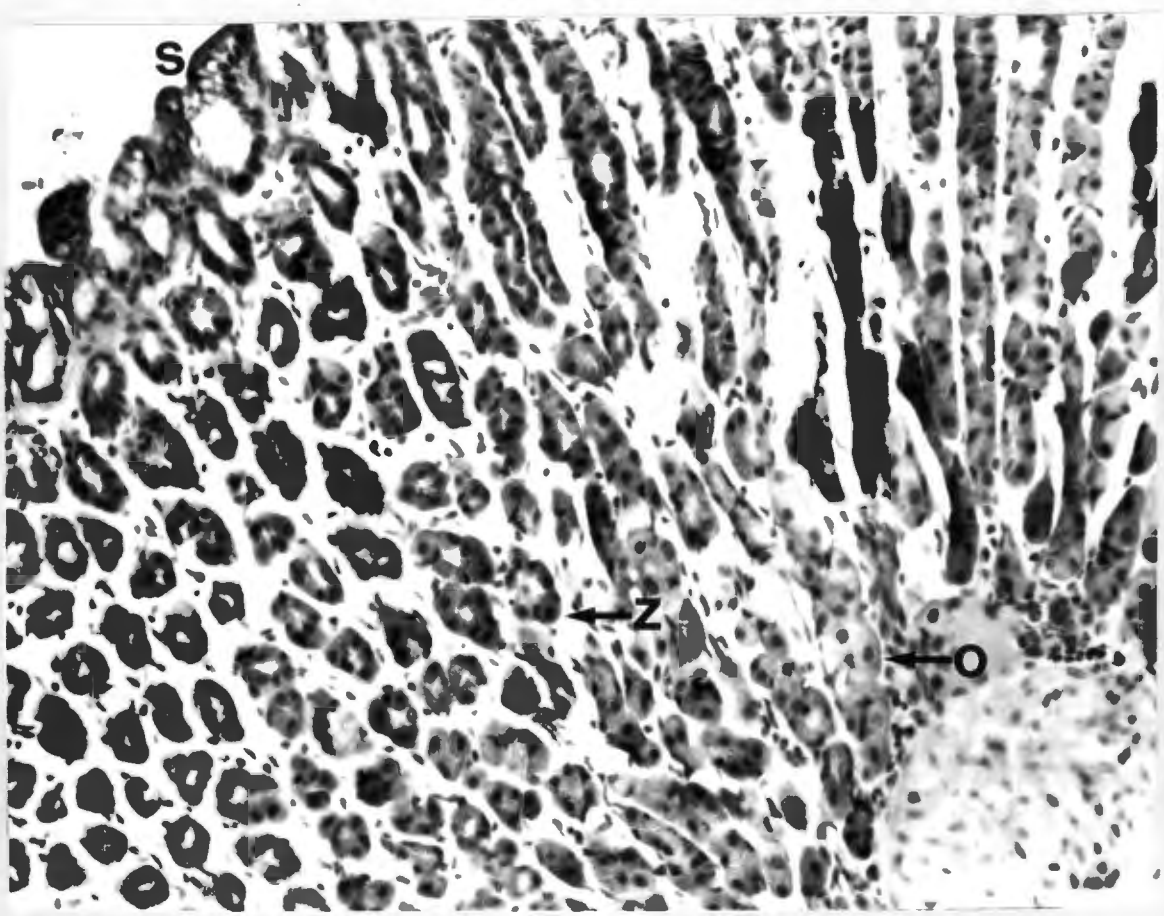


FIG. 3:2 Human Fundic Mucosa

O Oxyntic/parietal cell

Z Zymogen/chief cell

S Surface epithelium

X 150 Haematoxylin and eosin

CHAPTER 4

THE REGULATION OF GASTRIC ACID SECRETION

The regulation of acid secretion ranks as one of the most complex physiological processes in the body. The physiological stimulus for the secretion of gastric acid is the ingestion of food and the response of gastric acid secretion to food can be divided into three phases. Firstly the cephalic phase which may occur in anticipation of eating, secondly the gastric phase which occurs when food reaches the stomach and lastly, the intestinal phase of acid secretion. (Davenport 1977). At the cellular level however, acid secretion during all three phases may be considered to be the result of four main regulatory pathways with both stimulatory and inhibitory components.

4:1 The Four Pathways to the Parietal Cell

All bodily processes, including gastric acid secretion are regulated by chemical messengers which originate in cells responsive to alterations in the environment. These are delivered to target cells by one of three modes; neuronal, endocrine or paracrine. (Soll and Grossman 1978). In response to a physiological stimulus such as a meal, the parietal cell is directly or indirectly regulated through each of these pathways. (Soll & Grossman 1978). In addition, recent studies indicate that in the gut, luminal contents may also have a direct effect on target parietal cells. (Debas and Grossman 1975). The relative importance of each of these pathways in determining the integrated response of the parietal cells to a meal is not yet known. It is likely that these four pathways will not have the same functional inter-relationships under all physiological situations and in all species.

Lastly, it is interesting from a teleological point of view that acid secretion, a function which is apparently physiologically dispensible, is under such redundancy of control.

4: 2 The Neurocrine Pathway of Acid Secretion

Neuronal or neurocrine effects can directly activate the parietal cell to secrete acid by release of acetylcholine and other neurotransmitters from post-ganglionic nerve endings.

The neuronal path can be activated both by vagal efferent fibres and by local reflex arcs within the wall of the stomach. (Debas 1977). Cephalic phase stimuli act solely through vagal efferent fibres, whereas gastric distension can act both through vasovagal reflexes and also somewhat less efficiently through local reflex arcs. (Soll and Grossman 1978). The effects mediated by both vagal efferent fibres and local reflex arcs are inhibited by muscarinic cholinergic antagonists such as atropine. The view that the post-ganglionic neuronal pathway utilises cholinergic transmitters is further supported by observations in laboratory animals that exogenous cholinomimetic drugs such as carbachol produce the same maximum response as vagal stimulation, gastrin or histamine. In man however, there is only a small response to these drugs, (Roland, Berstad et al, 1975) highlighting the wide variability between species in the regulation of gastric acid secretion. It is possible that in man there are also cholinergic inhibitory pathways or non-cholinergic vagal efferent fibres. (Soll and Grossman, 1978).

The release of gastrin too, is in part under neuronal control. In fact, each of the mechanisms described for neuronal activation of the parietal cell can also cause neuronal release of gastrin. However, the administration of exogenous cholinomimetic drugs causes little or no release of gastrin and atropine often enhances rather than inhibits gastrin release. (Walsh and Grossman, 1975).

Thus it is possible that vagal release of gastrin is non-cholinergic or alternatively cholinergic but atropine resistant. (Soll and Grossman, 1978).

Although acetylcholine is the only known neurocrine stimulator of acid secretion there are a number of peptides recently isolated in gut which are also present in brain and may have neuro-modulatory actions. These include somatostatin (Polak, Grimelius et al 1975, Brownstein, Arimura et al 1975), vaso-active intestinal peptide, (V.I.P.) (Bryant, Polak et al 1976, Larsson, Fahrenkrug et al 1976), substance P (Pearse and Polak 1975), (Nilson, Larsson et al 1975) neurotensin (Polak, Sullivan et al 1977a), the endorphins and encephalins (Hughes, Smith et al 1975, Polak, Sullivan et al 1977b) and cholecystokinin (CCK) (Rehfeld 1978). In addition, V.I.P., substance P, the encephalins (Lundberg, Hokfelt et al, 1979)gastrins (Uvnas Wallensten, Rehfeld and Uvnas 1977), and somatostatin (Uvnas Wallensten, Efendik and Luft 1978), are present in the vagus and V.I.P., substance P (Polak and Bloom 1978) and CCK tetrapeptide (Larsson and Rehfeld 1979) have been isolated in the autonomic nerves of the gut. As V.I.P. and somatostatin are known inhibitors of acid secretion, it is conceivable that these may act as neurocrine inhibitors, whereas encephalins and CCK may act as neurocrine stimulators. This is however still speculative and the physiological functions of all these gut-brain peptides is still unknown.

Other known neurotransmitters which may act as inhibitors of acid secretion via a neurocrine pathway include dopamine (Valenzuela 1976) and serotonin (Jaffe, Kopen and Lazan 1977).

4: 3 The Paracrine Pathway of Acid Secretion

Paracrine secretion refers to diffusion of a chemical messenger from its cell of origin across inter-cellular spaces to its target (Creutzveld 1976). Both the concept of paracrine secretion and its potential role in the secretion of acid remain hypothetical. There is however little doubt that histamine secreted locally from mucosal stores has a major effect on parietal cell function and it is of interest that histamine appeared in the upper gastrointestinal tract at the same point in the phylogenetic tree that the ability to secrete acid was acquired (Reite 1972). Histamine is present in large quantities, about 40 μg/ gram wet weight in the parietal mucosa of humans and other mammals (Reite, 1972, Troidl, Lorentz et al, 1975). While histamine is the major paracrine secretagogue postulated, it is now becoming apparent that there are several substances which inhibit acid secretion which may have a physiological role, acting via the paracrine pathway. These include the prostaglandins, a group of ubiquitous compounds synthesized from fatty acid precursors which exhibit a variety of biological functions, including the inhibition of gastric acid secretion (Robert, Nezamis and Phillips, 1968). Prostaglandins of the A and E series are synthesized in the gastric mucosa and are secreted into gastric juice under basal and stimulated conditions. Their physiological role in acid secretion is unknown but they have, in addition to acid inhibition, been postulated to be cytoprotective to the gastric mucosa, an effect not entirely accounted for by their effects on acid secretion (Chaudbury and Jacobson, 1978).

Another substance which may act via the paracrine pathway is somatostatin. In the pancreas the somatostatin D-cells are found in the islets adjacent to the insulin-producing B-cells and the glucagon A-cells. It has been postulated by Unger and Orci (1977) that somatostatin acts as a paracrine substance within the islet, to regulate insulin and glucagon responses to absorbed nutrients. Somatostatin, which inhibits both gastrin release and acid secretion directly (Bloom, Mortimer et al, 1974), is found in high concentrations in the antrum (Kronheim, Berelowitz and Pimstone, 1976). It has been suggested that it may inhibit gastrin secretion in an analogous manner to its suggested mode of action in the pancreas. The mechanism of action of somatostatin on the parietal cell per se or even whether this occurs under physiological conditions is still unknown.

While the roles of the candidate paracrine acid inhibitors are still obscure, the emergence of histamine as a prime acid secretagogue has, with the development of the histamine H_2-receptor blockers, become increasingly evident. (Black, Duncan et al, 1972). This substance will therefore be reviewed in greater detail.

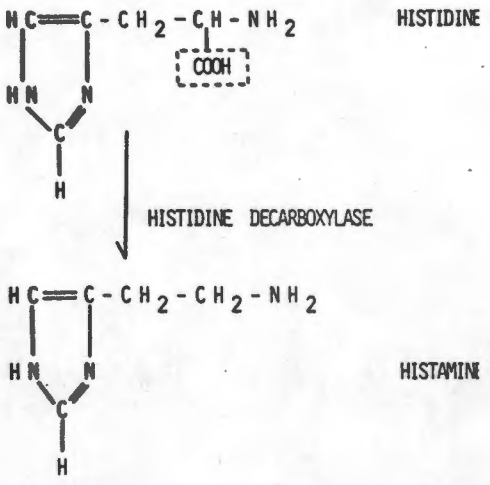
4: 3: 1 Histamine as an Acid Secretagogue

4: 3: 1: 1 History:

Histamine was discovered in 1907 when it was synthesized by Windhaus and Vogt. It was first isolated from intestinal mucosa in 1911 (Barger and Dale) but was only found to stimulate gastric secretion in 1920 by Popielski.

4: 3: 1: 2 Chemistry:

Histamine has a single chemical source. It is derived from histidine by the action of the enzyme histidine decarboxylase.



This enzyme is easy to demonstrate in the gastric mucosa of a rat in which the activity is exceptionally high. However, in all other species the activity of this enzyme is low and difficult to detect (Code, 1977). Histamine is very rapidly inactivated. This occurs through one of two mechanisms; either by deamination of the side chain with the enzyme diamine oxidase or by methylation of the nitrogen of the imidazole ring with the enzyme N-methyl transferase. These enzymes are present both in serum (Beaven and Schaff, 1975) and in tissue (Baylin, Beaven et al, 1972). Code (1977), has said that the inactivation of histamine is "explosive". Thus histamine is not normally detected in serum of guinea pigs, cats, dogs or humans (Beaven, Jacobsen and Horakova, 1972). These workers used an assay with a lower limit of sensitivity of approximately 2nM Small concentrations were noted in rat blood, and the mast cell histamine releaser compound 48/80 increased the histamine in rat serum. However, extracts of most tissues contain measurable histamine concentrations (Beaven, Jacobson and Horakova, 1972).

4: 3: 1: 3 Cells of Origin:

Although histamine is present in the gastric mucosa of most species tested, including man (Code, 1956) the cells of origin have been difficult to identify and characterise. There appear to be inter-species differences and there is some evidence in animals other than the rat, that histamine is contained in a cell that is morphologically indistinguishable from a mast cell. Further evidence for this has been provided by Soll, Lewin and Beaven (1979). However, gastric histaminocytes are physiologically quite distinct from perivascular mast cells - for example perivascular mast cell histamine, but no gastric mucosal histamine is released by compound 48/80 and by antigen-antibody reactions, whereas gastric histamine mobilization is under an entirely different set of controls. Soll, Lewin and Beaven (1979) have suggested that in dog, histamine containing cells are situated in the lamina propria in close proximity to the gastric glands. However, whether these cells are activated by compound 48/80 or whether they control gastric acid secretion has not yet been demonstrated.

4: 3: 1: 4 Mechanisms of Gastric Histamine Mobilisation

The mechanism controlling the release of gastric histamine and the pathways leading to the formation of gastric histamine stores remains unknown. Much of the experimental data in this field has been gathered in rat models. One of the reasons for this is that histidine decarboxylase activity is easily measurable in rats, as it is about 1 000-fold higher than in most other species (Code, 1977). Thus it may be incorrect to extrapolate these findings to other species.

In 1938 McIntosh showed that histamine was present in gastric juice of dogs when secretion was stimulated by the direct stimulation of the vagus or by psychic stimulation. Emmelin and Kahlson (1944) confirmed this, while Code, Hallenbeck and Gregory (1947) found that histamine is present in gastric juice of dogs during their response to a meal of meat. The histamine output occurs during the early phases of gastric secretion. Hill and Code (1959) showed that vagal stimulation in dogs caused accumulation of histamine in the gastric mucosa, as if vagal stimulation had increased its production. However as vagal stimulation is a potent releaser of gastrin in dogs, it is difficult to differentiate this from a gastrin-mediated effect.

It was subsequently shown in rats that the injection of gastrin (or pentagastrin) results in a lowering of gastric mucosal histamine content, reflecting the mobilisation of histamine (Kahlson, Rosengren et al, 1964, Kahlson, Rosengren and Thurnberg, 1967, Hakanson, Larsson et al, 1976), Furthermore, the rate of histamine formation in the rats' gastric mucosa was accelerated following administration of gastrin, through the activation of histidine decarboxylase (Kahlson, Rosengren et al, 1964, Hakanson, Kroesen et al, 1974). Hakanson, Hedenbro et al (1977) suggested that vagal stimulation had an insignificant effect on histamine mobilisation. On the other hand gastrin appeared to be an obligatory intermediate of histamine mobilisation in rats. Hakanson, Larsson et al, (1978) also showed that the mobilised mucosal histamine inhibits further release of histamine by way of an apparent H_2-receptor mediated auto-feedback mechanism in the histamine storage cells. Thus release of histamine in rats appears to be controlled mainly by gastrin, whereas inhibition of release is mediated by a bio-feedback mechanism.

However, as has been mentioned, this cannot be demonstrated in any other species and the mechanism of histamine release is, in most species, still largely unknown.

4: 3: 1: 5 The H_2-receptor and H_2-receptor Blockers:

It had been known for some time that although certain effects of histamine, such as the stimulation of contraction of smooth muscle, could be blocked by the antihistaminics such as mepyramine, these drugs had no effect on acid secretion. Thus, two types of histamine receptors appeared to be present - the H_1-receptor which was mepyramine sensitive and the H_2-receptor which was not.

In 1972, Black, Duncan et al discovered a specific antagonist of the H_2-receptor. The first substance synthesized with these properties was burimamide. The subsequent substance developed, metiamide, was 4 to 6 times as potent as burimamide but the occurrence of granulocytopaenia in a small number of patients limited its use. Cimetidine, the newest H_2-receptor blocker synthesized, is free of this side-effect and has been successfully marketed in this country for the treatment of duodenal ulceration. Cimetidine is an effective inhibitor of both basal acid secretion as well as histamine and pentagastrin-stimulated secretion (Richardson, 1978). It is generally less active against cholinergically stimulated secretion but does have some inhibitory effect (Brimblecombe, Duncan et al 1978). Thus it appears that a specific H_2-receptor antagonist has a rather non-specific effect on acid secretion. In order to understand the reasons for this, one needs to consider the interactions of the acid secretagogues. The various hypotheses of acid secretagogue interaction will be discussed below.

4:4 The Endocrine Pathway of Acid Secretion

The only hormone known to stimulate acid secretion under physiological conditions is gastrin (Walsh and Grossman, 1975). It is likely that another hormone is involved in the intestinal phase of gastric acid secretion but this has not yet been isolated (Grossman, Brown et al, 1974).

The encephalins, the natural ligands for opiate receptors, have recently been detected by immunocytochemistry throughout the tissues of the digestive system (Polak, Sullivan et al, 1977b). Konturek, Pawlik et al, (1978) have shown that met-encephalin stimulates acid secretion. However, the mechanism by which this occurs and the physiological significance is still obscure, although there is some evidence that the encephalins may act directly on the parietal cell (Solomon, 1980).

It is possible that in pathological states non-gastrin endocrine acid stimulators circulate in serum. Bugat, Walsh et al (1976), using a rat stomach bioassay, found that the serum from duodenal ulcer patients contained increased acid secretagogue activity in the face of normal serum gastrins. Watson, Vinik et al, (1978) showed that bile duct ligation in the pig resulted in a remarkable increase in acid secretion in the presence of normal serum gastrins. The nature of these acid stimulators is unknown.

There is evidence too, that inhibition of gastric acid secretion may be regulated via endocrine pathways. Acidification of the duodenum appears to release an inhibitory hormone which has been named bulbogastrone, but has not been fully identified (Andersson, Nilsson and Uvnas, 1967).

Furthermore, the presence of fat in the duodenum has for some years been known to cause acid inhibition. This is mediated by a hormone called enterogastrone, but this too has not yet been isolated. Certain hormones which have been isolated have been shown to inhibit gastric acid secretion, but whether this is a primary physiological action of these hormones is still unknown.

Secretin is released in response to acid in the duodenum, but the release of secretin during a meal is small (Schaffalitsky, de Muckadell and Fahrenkrug, 1978). Secretin inhibits gastrin-stimulated acid secretion (Brooks and Grossman, 1970), but whether this is a physiological or pharmacological effect is still unknown. Similarly, gastric inhibitory polypeptide (G.I.P.), inhibits both the endogenous release of gastrin and gastric acid secretion (Pederson and Brown, 1972). This peptide was shown to be released in response to glucose and fats and was proposed as an entero-gastrone (Brown, Dryburgh, et al 1975). However G.I.P. release in response to fat is small and late and thus this proposal seems unlikely (Soll and Walsh, 1979). Vaso-active intestinal peptide (V.I.P.) and somatostatin which have already been discussed, may well yet be found to act via the endocrine pathway.

Other peptides which have been demonstrated to inhibit acid secretion include glucagon (Konturek, Biernat et al, 1975), and calcitonin (Hesch, Hufner et al, 1971). Urogastrone or human epidermal growth factor, which was shown to inhibit gastric acid in the 1930's (Gray, Wieczorowski and Ivy, 1939), has recently been localised in the Brunner's glands of human duodenum (Heitz, Kasper et al, 1978), and submandibular glands (Elder, Williams and Gregory, 1976). This substance too, is therefore a candidate gastric endocrine inhibitor.

There are thus many possible endocrine modulators of acid secretion. This study will deal mainly with the secretagogues, with a particular emphasis on gastrin, and its actions on the parietal cell. It is therefore appropriate that this group of peptides be reviewed in greater depth.

4: 4: 1 The Gastrins

4:4:1:1. History:

In 1905, the year that Starling introduced the word "hormone" to designate a class of chemical messengers which may be released by one part of the body and control activities in a different part of the body, Edkins announced that extracts of antral mucosa stimulate acid secretion when injected intravenously into anaesthetised cats. He named the active principle Gastrin. However, his extracts contained histamine as well as gastrin and for many years a controversy raged over whether they contained a stimulant other than histamine.

In 1938, Komarov produced histamine-free extracts that still stimulated acid secretion, but a number of workers could not verify this finding. Thus the controversy was still not resolved. All doubt was dispelled in 1964 when Gregory and Tracy isolated pure gastrin from hog antral mucosa and with Kenner and co-workers determined its structure (Gregory, Hardy, et al 1964) and synthesized it (Anderson, Barton et al, 1964).

4: 4: 1: 2 Chemistry:

The compounds isolated from hog antral mucosa by Gregory and Tracy were heptadecapeptides. Two species were found; one form was sulphated on the tyrosine residue in position 6 from the carboxy-terminal and was named Gastrin II; the other form was non-sulphated (Gastrin I). In all other respects the two species were identical. Gastrin heptadecapeptides have been purified from antral mucosa of several species including man, (Bentley, Kenner and Sheppard, 1966) pig, cat, sheep and cow and found to differ by only one or two amino-acids in the middle of the linear peptide chain (Kenner and Sheppard, 1973).

In 1970, Yalow and Berson described a larger molecular form of gastrin and named it "big gastrin". Big gastrin has been purified from human gastrinoma extracts (Gregory and Tracy, 1972) and from hog antral mucosa. The amino-acid sequences have been determined by Gregory and Tracy (1974a). Big gastrin (abbreviated in terminology to G34), contains 34 amino-acids. By convention the amino-acids of gastrins are numbered from the amino-terminal. Of the 34 amino-acids, residues 18-34 are identical to the gastrin heptadecapeptide (G17). (Table 4:1).

Subsequently, minigastrin (G14), identical to residue 21-34 of G34 or 4-17 of G17 was isolated in the serum of a Zollinger-Ellison syndrome patient (Gregory and Tracy, 1974b). Thus the carboxy-terminal, which is the biologically active portion of the molecule, is common to all three forms of gastrin, G34, G17 and G14. All occur in both sulphated and non-sulphated forms, usually in approximately equimolar quantities. Tridecapeptide, amino-terminal of G17, has also been identified in the serum and mucosal extracts of a patient with gastrinoma (Dockray and Walsh, 1975). This was biologically inactive. Furthermore, large quantities of N-terminal material has been found in the sera of several normal patients (Malagalada, 1978a).

Recently Rehfeld and Larsson (1979), have suggested that the gastrin found in the largest molar quantities in gut is the carboxy-terminal tetrapeptide, which is common to both cholecystokinin and gastrin. This has been designated CCK4 and is apparently present in the autonomic nerves of the gastrointestinal tract.

Table 4:1

SOME CHEMICALLY IDENTIFIED GASTRINS

(a)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Pyr	Leu	Gly	Pro	Gln	Gly	His	Pro	Ser	Leu	Val	Ala	Asp	Pro	Ser	Lys	Lys	Gln	Gly	Pro	Trp	Leu	Glu	Glu	Glu	Glu	Glu	Ala	Tyr	Gly	Trp	Met	Asp	Phe-NH_2
																												I					
																												R					

(b)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Pyr	Gly	Pro	Trp	Leu	Glu	Glu	Glu	Glu	Glu	Ala	Tyr	Gly	Trp	Met	Asp	Phe-NH_2
											I					
											R					

(c)

1	2	3	4	5	6	7	8	9	10	11	12	13
Leu	Glu	Glu	Glu	Glu	Glu	Ala	Tyr	Gly	Trp	Met	Asp	Phe-NH_2
							I					
							R					

(a) Human big gastrin (G-34) MW 3839

(b) Human heptadecapeptide (little) gastrin (G-17, 18-34 of G-34) MW 2098

(c) Human minigastrin (G-13, 22-34 of G-34, 5-17 of G-17) MW 1647

gastrin I, R = H

gastrin II, R = SO_3H

Pyr = pyroglutamyl

Other forms of gastrin have been found in tissues in the circulation. Two of these are big-big gastrin (BBG) of Yalow and Berson (1972), and component I of Rehfeld (Rehfeld, Stadil and Vikelsoe, 1974). There is controversy over the existence of these forms, which emerge from Sephadex gel-filtration columns with high molecular weight material. Although it has been suggested that (BBG) constitutes a major fraction of plasma gastrin in normogastrinaemic subjects, Rehfeld, Schwartz and Stadil (1977), maintained that BBG is not a true entity in normal serum but is attributable to interference in the gastrin assay by normal serum proteins. There is however, no doubt that in certain gastrinoma patients, gastrin macromolecules, consisting of G17 covalently coupled in its amino-terminal to proteins of varying length, do exist. The biological activity of these large molecular weight gastrins is unknown.

Studies of protein biosynthetic mechanisms have shown that peptide synthesis proceeds from N-terminal to C-terminal amino-acid (Habener and Potts, 1978). It thus seems likely that gastrin is synthesized as the 34-amino-acid, big gastrin, or a larger molecule and then converted enzymatically into the heptadecapeptide and other smaller gastrin fragments. Recently a preprohormone form of gastrin consisting of 110-140 aminoacids has been postulated following detection and partial sequence analysis of gastrin messenger RNA (Noyes, Mevarech et al, 1979). In this way gastrin appears to resemble other hormones such as insulin (Steiner and Oyer, 1967), glucagon (Tager and Steiner, 1973) and parathyroid hormone (Habener, Kemper et al, 1975). There is as yet no evidence for conversion of G34 to G17 in the circulation and the conversion seems likely to occur in the G cell.

Internal cyclization of the N-terminal glutamic acid residue leads to the formation of a pyroglutamyl group in G34 and G17 and prevents digestion by aminopeptidases.

All known forms of gastrin are linear peptides that contain no sulphide bonds. At neutral pH G17 exists as random coils but at pH less than 4.5 the five glutamic residues form an alpha helix. (Piskiewicz, 1974).

4: 4: 1: 3 Structure-Activity Relations

The carboxy-terminal portion of the gastrin molecule has all the biologic actions of the whole molecule (Tracy and Gregory, 1964). Traces of activity are seen with fragments as small as a C-terminal dipeptide amide. The tripeptide amide has distinct activity (Lin, 1972) and the tetrapeptide amide is about one sixth as potent as G17 on a molar basis. Removal of the C-terminal amide to form the free acid results in complete loss of activity but one of the hydrogens of this amide may be replaced by a NH_2 or CH_3 without loss of activity (Morley, 1968).

Pentagastrin is a commercially available synthetic pentapeptide consisting of the C-terminal tetrapeptide amide of gastrin plus beta-alamine and an N-terminal blocking group (tertiary-butyl-oxycarbonyl). Its potency is comparable to that of the C-terminal pentapeptide of gastrin. Expressed in terms of exogenous dose needed to give a certain fraction of maximal response, molar potency increases with chain length from G14 to G34. (Walsh, Debas and Grossman, 1974, Debas, Walsh and Grossman, 1974a, Walsh, Isenberg et al, 1976).

Expressed as a blood level needed to give a certain fraction of maximal response, G17 is six times more potent on a molar basis than G34 (Walsh, Isenberg et al, 1976), and equal in potency to G14 (Carter, Taylor, et al 1979). The half-life disappearance time of G17 after intravenous infusion in human subjects is 5 to 6 minutes (Walsh, Isenberg et al, 1976). The half-life disappearance time of G14 in dogs is 2.8 minutes, which did not differ significantly from that of G17 (1.7 mins.) (Carter, Taylor et al, 1979). This has not yet been confirmed in humans.

Walsh, Isenberg et al (1976), showed that after stimulation of gastrin release by the instillation of a peptone solution into the stomach, G34 comprises about 75% of the total molar concentration of circulating gastrins. However, considering the differences in potency and half-life disappearance times of G34 and G17, they calculated that G34 contributes less than 50% of the acid stimulatory activity. More recently however, Taylor, Dockray and Walker (1979) estimated that about 50% of the increase in serum gastrin after a meal was due to G17, whilst Lamers, Harrison et al (1979) reported that the levels of G34 double, whereas G17 quadruples after a meal, to end up with a ratio of 1:1. In 1978 Feldman, Walsh et al, using a specific anti-G17 antiserum showed that the gastrin-induced acid secretion, in response to an intragastric peptone infusion could be entirely accounted for by G17 release only. The reasons for these discrepant findings were not discussed, but it is possible that the circulating gastrins do not have their expected biological activities.

It appears that gastrin and cholecystokinin have developed in evolution from a common precursor molecule. The C-terminal pentapeptide amide sequence of both molecules are identical and this fragment has all the biological actions of both hormones.

The crucial difference between gastrin and cholecystokinin (CCK) is the location of the sulphated tyrosine residue. In gastrin it is in the position 6 from the C-terminus, while in CCK it is is position 7. In CCK this fragment is, unlike gastrin, always sulphated. Removal of the sulphated group causes it to revert to a gastrin-like pattern of activity. CCK-like peptides, that is those with sulphated tyrosines in position 7, have high relative potency for gall bladder contraction and pancreatic enzyme secretion and lower relative potency for gastric acid secretion. The C-terminal heptapeptide amide of CCK is the smallest fragment that shows the CCK pattern of preferential potency on gall-bladder and pancreas (Ondetti, Rubin et al, 1970). This heptapeptide fragment is more potent on a molar basis than whole CCK but the ratio of its potency for the gall bladder and pancreas to its potency on acid secretion is not as great as that of whole cholecystokinin. Thus, whereas the octapeptide fragment of the hormone and the related peptide caerulein have a very high potency for gall bladder and pancreas, they do not have the degree of specificity for these organs that whole CCK has. Among the gastrin-like peptides the ratio of potency for gall bladder and pancreas to potency on acid secretion does not vary with chain length, being essentially constant for G14 through G34.

Cholecystokinin-like peptides stimulate acid secretion in all species, but in some species (rat, cat) they are full agonists (i.e., the maximal response is as great as to gastrin) whereas in others (man, dog) they are partial agonists, producing maximal responses about one fifth as great as gastrin (Johnson and Grossman, 1971).

4: 4: 1: 4 Distribution of Gastrin

The cells that contain gastrin in the gut are called G cells and are found in the pyloric glands of the distal stomach (antrum) and the proximal duodenum (McGuigan and Greider, 1971). About 5 to 50 µg G17 can be recovered from boiling water extracts per gram of antral mucosa. G34 and other molecular variants represent less than 10% of antral gastrin content (Berson and Yalow, 1971). In proximal duodenum the gastrin concentration is 10 to 50% of that in antral mucosa but in view of the greater bulk of duodenal mucosa it has been estimated that the total duodenum content may be similar to the antral content (Nilsson, Yalow and Berson, 1973). Lower concentrations of gastrin are found in the distal duodenum and jejunum.

The duodenal gastrin is made up predominantly of G34 (Berson and Yalow, 1971). Small amounts of human gastrin have been extracted from human pancreas by some workers but this has not been confirmed. Gastrin has also been identified in the pituitary gland (Rehfeld, 1978), while the gastrin-like peptide described in brain (Vanderhaeghen, Signeau and Gepts, 1975) has been identified as cholecystokinin-octapeptide (Dockray, 1976). In the serum, the predominant gastrin that is present in the fasting state is BBG (Yalow and Wu, 1973), although this is disputed by Rehfeld, Schwartz and Stadil (1977). In fed normal subjects, or hypergastrinaemic patients (e.g., those with gastrinomas or pernicious anaemia) G34 is the predominant circulating form comprising 70 to 90% of total immunoreactivity, (Walsh and Grossman, 1975). In gastrinoma patients, as has been discussed, many other gastrin variants are found in serum.

4: 4: 1: 5 The Release of Gastrin

As with acid secretion, gastrin secretion is mediated via several pathways. These too, include endocrine, neurocrine, luminal and paracrine pathways. However, in the case of gastrin the luminal pathway tends to predominate and amino-acids in the chyme which bathes the microvilli at the luminal surface of the G-cells are the most potent releasers of gastrin (Richardson, Walsh et al, 1976, Walsh, Isenberg et al, 1976, Richardson, Walsh et al, 1977).

Strunz, Walsh et al (1978a), showed that the release is stimulated chiefly by four amino-acids, namely cysteine, phenylalanine, tryptophan and hydroxyproline. Konturek, Tasler et al (1977) in contrast found that phenylalanine was a weak releaser of gastrin, whereas serine, valine and tryptophan caused greater release. The reason for this discrepancy is unknown. Glucose and fat release gastrin but at much lower levels.

Inhibition of gastrin release occurs where the pH of the stomach lumen falls. At a pH of 1.0 maximal suppression of gastrin release occurs while even at a pH of 2.5, gastrin release in response to a meal is reduced. (Walsh, Richardson et al, 1975). In contrast long term hypo- or achlorhydria is a stimulant to gastrin release. Thus in atrophic gastritis and pernicious anaemia there is a markedly elevated fasting serum gastrin, with excessive release after food.

The neurocrine pathway to gastrin secretion is mediated via the vagus. Vagal excitation in dogs by sham feeding (Nilsson, Simon et al, 1972 , Tepperman, Walsh and Preshaw, 1972) or insulin

hypoglycaemia (Csendes, Walsh and Grossman, 1972) stimulates the release of gastrin from the G-cells. This release is abolished by vagotomy. Atropine enhances the serum gastrin response to food in low doses but in high doses blocks the response (Impicciatore, Walsh and Grossman, 1977). In man the cholinergic control of gastrin secretion is of much less importance than the dog. Sham feeding in man causes a slight rise in serum gastrin (Richardson, Walsh et al, 1977), while gastric distension has no effect (Idem).

The endocrine and paracrine pathways to gastrin release are less well defined. Blood-borne stimulants include calcium (Reeder, Becker and Thompson, 1974, Christiansen, 1974) epinephrine (Stadil and Rehfeld, 1973), and parathyroid hormone (Bolman, Cooper et al, 1977), but these only appear to act as gastrin releasers in pathological situations such as parathyroid adenomas (Christiansen, 1974) or phaeochromocytomas (Hayes, Ardill et al, 1972).

Bombesin, a peptide isolated from frog skin, releases antral gastrin when injected intravenously in man or dog (Bertaccini, Melchiori et al, 1974). This peptide has recently been shown to be present in endocrine cells of the duodenum (Polak, Ghatei et al 1978) and may play a role in the release of gastrin by an endocrine route. All the peptides which have been shown to inhibit acid secretion also inhibit gastrin release. These include the four chemically related peptides, glucagon (Becker, Reeder and Thompson, 1973) secretin (Thompson, Reeder et al, 1972), V.I.P and G.I.P. (Pedersen and Brown, 1972) as well as calcitonin (Becker, Reeder et al, 1974, Bolman, Cooper et al, 1977) and

somatostatin (Bloom, Mortimer et al, 1974; Barros D'sa, Bloom and Baron, 1978). However, whether these are pharmacological effects or whether they modulate gastrin release physiologically via the endocrine or paracrine pathway is still unknown.

4:4:1:6: The Target

Gastrin when injected intravenously, has a wide range of effects ranging from water and electrolyte secretion in the small bowel and pancreas to stimulation of the smooth muscle of the gall-bladder (Walsh and Grossman, 1975). Most of these effects are probably pharmacological. The primary physiological effect of gastrin is unquestionably the stimulation of acid secretion, but the manner in which this is achieved is still controversial.

Peptide hormones usually act at membrane-bound receptors, and gastrin is probably no exception. Gastrin binding has been demonstrated on gastric plasma membranes (Lewin, Soumarmon et al, 1976, Brown and Gallagher, 1978) and binding to isolated parietal cells of guinea pig (Del Mazo and McGuigan, 1976) and rat (Soumarmon, Cheret and Lewin, 1977) has been described. However, all these workers have failed to demonstrate biological specificity and sensitivity and their findings to date remain controversial (Soll and Grossman, 1978). Takeuchi, Speir and Johnson (1979a, b, 1980) have recently described an improved membrane preparation which satisfies some of the criteria for hormone receptor interaction.

Using an auto-radiographic technique, Salgalnik, Bersimbaev et al, (1976), have shown binding of labelled (3H)-pentagastrin to the histamine producing α-like endocrine cells and chief cells, rather than to

the parietal cells in rats, while Hedenbro, Fink and Fiddian-Green (1978 a,b) have demonstrated by immunofluorescence Leu-gastrin binding to the luminal surface of the parietal cells. Recently it was demonstrated that insulin has in addition to membrane receptors, intracellular binding sites. Kirsch, Vinik et al (1975) have demonstrated gastrin binding to the cytosol fraction of liver homogenate, so the possibility of gastrin having intracellular binding sites also arises. However, demonstrating that a hormone will bind to a particular cell or cellular constituent per se is not sufficient evidence that the binding represents occupation of a receptor by the hormone. Even if it is established that hormone binding correlates with changes in some cellular biochemical reaction, it cannot be assumed that this reaction is a reflection of the interaction of the hormone with the same receptor that causes the hormone response, until it has been established that the change measured is an integral step in the sequence of events leading to the hormone response. Thus in the case of gastrin, where the sequence of events leading to acid secretion is still unknown, the importance of the demonstration of gastrin-binding to various cells and cell components has yet to be shown.

4:5 The Luminal Pathway of Acid Secretion

Debas and Grossman (1975) showed that protein digests stimulated acid secretion when instilled into isolated denervated fundic (Heidenhain) pouches. This was independent of gastrin release. Konturek, Tasler et al (1977), showed that amino-acids phenyl-alanine, alanine and glycine were the most potent in stimulating acid secretion, although they were weak releasers of gastrin.

The L-isomers of all the amino-acids tested produced acid secretion, although certain of these amino-acids were potent releasers of gastrin. Undigested protein such as serum albumin does not stimulate acid secretion (Debas and Grossman, 1975).

Gastrin and other gut hormones may also act via the luminal pathway. Knight, Fiddian-Green and Vinik, (1978) showed that gastrin was released into gastric juice in response to albumin. Fiddian-Green, Farrel et al,(1978) also showed that gastrin added to neutral gastric juice stimulated acid secretion, although this was disputed by Hengels, Muller et al, (1979). Lastly, Hedenbro, Fink and Fiddian-Green (1978b) have demonstrated Leu-gastrin binding to the luminal surface of gastric parietal cells. However, whether this is biologically effective is still not clear. Thus the concept of luminal action of gut hormones is a novel and exciting but still controversial approach.

4:6: The Interaction Between the Pathways

The regulation of acid secretion would be adequately confusing, were the mechanisms enumerated acting independently. However, to further complicate matters, the main gastric acid secretagogues do not act independently but show a marked interdependence. This interdependence is most dramatically evidenced clinically by the effects of the histamine-H_2-receptor antagonists and anticholinergic agents on acid secretion. H_2-receptor blockers such as cimetidine and metiamide reduce not only histamine-stimulated acid secretion, but also basal and gastrin or cholinergic-mediated acid secretion.

Thus these agents cause a reduction in basal acid secretion by about 50 to 95% (Sewing, Hagie et al, 1978, Richardson, 1978) while acid secretion in response to all other stimuli is reduced by 80 to 95% (Grossman and Konturek, 1974, Richardson, Bailey et al, 1975, Barbezat and Bank, 1978). Furthermore, anticholinergic agents inhibit the acid response to histamine and gastrin as well as to cholinergic stimuli (Konturek, Oleksy and Wysocki, 1968, Hirschowitz and Sachs, 1969, Feldman, Richardson et al, 1977).

Vagotomy and antrectomy are often used in surgical treatment of peptic ulcer disease. Each of these surgical manoeuvres alone inhibits the acid response to all forms of stimulation. (Bergegardh, Broman et al, 1976, Konturek, Wysocki and Oleksy, 1968). Interactions have also been demonstrated by administering two secretagogues simultaneously. Johnson and Grossman (1969) showed potentiation of the effects of gastrin and histamine in dogs. However, Brooks, Johnson and Grossman (1970) could not demonstrate the same in man. Hakanson,Hedenbro et al (1977) have shown that gastrin is an obligatory intermediate in the postprandial mobilisation of gastrin histamine. This however cannot be demonstrated in other species (Grossman, Beaven et al, 1978). Thus the interactions between the secretagogues are further complicated by species differences.

4: 6: 1 Theories of Secretagogue Action and Interaction

The interdependence of the gastric secretagogues has been of interest to physiologists for several years. Two major theories have been proposed to explain secretagogue interaction. Code in 1956 proposed that histamine acted as the final common effector for all secretagogues on gastric acid secretion.

According to this theory gastrin and acetylcholine release histamine, which acts on the parietal cell to induce acid secretion. As has previously been discussed, the evidence that gastrin causes the release of histamine has been confined to the rat and has been shown in no other species to date. (Grossman, Beaven et al, 1978).

A second theory is that proposed by Soll (1978a, b) namely that the parietal cell has specific receptors for histamine, gastrin and acetylcholine, and that potentiating interactions occur between these secretagogues at the parietal cell itself. Soll showed in a series of elegant experiments using isolated canine parietal cells that histamine, gastrin and carbachol (an acetylcholine agonist) each increased O_2 consumption by, and (^{14}C)-aminopyrine accumulation in the parietal cells. (Soll, 1978a, b, 1980).

As these two indices may be regarded as indicative of acid stimulation, the experiments seem to show that each secretagogue has an individual receptor on the cell. This theory was further strengthened by the use of a histamine H_2-receptor blocker and atropine which blocked the histamine and the carbachol effects respectively, without affecting the gastrin effect (Soll, 1978a, b, 1980). Soll reported histamine-gastrin potentiation as well as histamine-carbachol potentiation. The potentiation effect was blocked by the respective inhibitors, leaving a residual effect which was equal to the effect of the secretagogue alone.

A major flaw in Soll's work is the insensitivity of the system. Thus gastrin only stimulated O_2 uptake when used in a concentration

of 10^{-7}M and in order to bring out the differences in secretagogue action he found it necessary to augment the effects of the secretagogue by a phophodiesterase inhibitor, itself a potent secretagogue.

Using histamine and isobutylmethylxanthine together with the gastrin, the lowest concentration of gastrin to cause an increase in O_2 consumption was 10^{-10}M which is the upper limit of the physiological range of gastrin. (Soll, 1978b). As gastrin alone has an effect only at 10^{-7}M concentration, this enormous concentration of gastrin may have been causing pharmacological rather than physiological effect; thus it may not necessarily mean that gastrin-mediated acid secretion occurs via the gastrin receptor on the parietal cell. Indeed Sonnenberg, Berglindh et al, (1979), using isolated rat parietal cells, could show no effect of gastrin at all in their system, and could offer no explanation for this discrepancy. Thus there are several questions still open regarding the interactions of secretagogues which require other techniques for answers.

CHAPTER 5

CELLULAR ASPECTS OF ACID SECRETION

5: 1 Mediators of Hormone Action in the Cell

If one assumes that the acid secretagogues act by binding cell membrane receptors one must postulate a "second messenger system" which "switches on" acid secretion. Since the discovery of mediation of hormone action by cyclic-AMP, there has been a great deal of interest in cyclic nucleotides as possible second messengers for the secretagogues in the parietal cell.

Much of the data on this is contradictory and this has been attributed to differences in the biological preparations that have been studied and the rapidly changing technology of assay procedures for adenylyl-cyclase, cyclic-AMP and the cyclic nucleotide, phosphodiesterase. This was recently reviewed by Thompson, Rosenfeld and Jacobson (1977). They showed that in three out of four species tested, histamine had no effect on gastric mucosal adenylyl cyclase activity. In contrast, Wollin, Soll and Samloff (1979) showed that the same concentration of histamine which caused an increased oxygen consumption in isolated canine parietal cells, caused an increase in cyclic AMP within these cells. Simon and Kather (1977) showed an increase in cyclic-AMP in human gastric mucosa in vitro in response to histamine, an effect which was inhibited by a histamine H_2-receptor blocker. In contrast again, Levine, Schwartzel et al, (1977) failed to show significant increases in mucosal or gastric juice cyclic-AMP levels in response to betazole, a histamine agonist.

Recently Carlisle, Chew and Hersey (1978), showed that the changes in parietal cell surface membranes which accompanied acid secretion appeared to be dependent on c-AMP. These changes however, could still occur in the presence of anoxia or thiocyanate which inhibits acid secretion.

Before one can seriously consider cyclic-AMP as a candidate for mediating histamine action in the parietal cell, the effects of secretin and the prostaglandins on cyclic-AMP production must be considered. Both secretin and the prostaglandins inhibit gastric acid secretion, yet stimulate adenylyl cyclase (Thompson, Chang et al, 1977, Dousa and Dozois, 1977) and cause a rise in cyclic-AMP production by unenriched isolated canine fundic cells. (Wollin, Soll and Samloff, 1979). The latter reported that these effects on cyclic-AMP production occurred in non-parietal cells and only at high concentrations (Wollin, Soll and Samloff, 1979, Thompson, Chang et al, 1977). It thus remains unclear whether these effects are physiological or solely pharmacological.

There are no solid candidates for the intra-cellular mediators of the effects of gastrin or acetylcholine. Narumi and Maki (1973) showed an increase in tissue cyclic-AMP in rats after stimulation with gastrin and carbachol. However, these agents were given in large doses and in addition, cause histamine release in this particular species.

Náfrády and Wolleman (1977) however, showed that pentagastrin stimulated the adenylyl cyclase of rat stomach in the presence of histamine H_2-receptor blocker. However, the data of Wollin, Soll and Samloff (1979) and Thompson, Rosenfeld and Jacobson (1977) are not in agreement with this.

It was suggested by Eichhorn, Salzman and Silen (1974) that the mediator of acetylcholine is cyclic-AMP. Griessen (1978) however, could not confirm this. Soll (1979) showed in his isolated canine parietal cell preparation that lanthanum, which displaces calcium from cell membranes and stops calcium fluxes across membranes (Hellman, Sehlin and Taljedal, 1976) and the presence of a low extra-cellular calcium concentration, both completely inhibited O_2 consumption and (^{14}C)-aminopyrine uptake in response to carbachol and partially in response to gastrin. This was confirmed by Sonnenberg, Berglindh et al, (1979). In contrast, removal of Ca^{++} from histamine or dibutyryl cyclic-AMP treated cells had only slight effect. These studies suggest an important role for calcium in acetylcholine-stimulated acid secretion, possibly via an effect on calcium plasma membrane pathways.

5:2 Cytoplasmic Events during Acid Secretion

The primary process of acid secretion is one that raises hydrogen ion concentrations from 0.00005 mN in the plasma to 150 - 170mN in gastric juice. This requires a minimum of 6.4 Kjoules/litre of gastric juice secreted (Davenport, 1977). The sources of metabolic energy for this secretion are uncertain, although there is a clear increase in oxygen consumption that is proportional to acid secretion and activation of the Krebs tricarboxylic cycle (Hersey, 1974). There are conflicting studies regarding the source of the substrate pool. Studies in frog and rabbit gastric mucosa with labelled precursors suggest a preferential metabolism of fatty acids (Hersey, 1977) whereas in dog gastric mucosa major increases in glycolytic intermediates were found without significant changes in the glycogen stores, which suggests metabolism of glucose (Sachs, Chang et al, 1977).

The differences in these results may reflect enhancement of more than one metabolic pathway, with variation in the pattern under different experimental conditions and in different species.

The second major question is whether these metabolic events are a major primary effect of the gastric secretagogues: viz. the mobilisation of substrates (thus constituting a rate-limiting metabolic step) or whether these events are secondary to the increase in demand for energy. Increased energy utilization or uncouplers of oxidative phosphorylation such as dinitrophenol normally results in a shift of the respiratory-chain components to a more oxidized form (Hersey, 1974). In contrast, in vitro studies with amphibian gastric mucosa indicate a transition of these respiratory-chain components to a more reduced form following secretagogue administration (Hersey, 1974). These redox changes occur shortly after the onset of stimulation and before actual secretion of acid commences. These observations combined with the increase in oxygen consumption therefore are most consistent with a primary mobilisation of substrate by the secretagogues (Hersey 1974, 1977).

Two final related questions regard the source of the hydrogen ions for the secretion of acid and the means of delivery of metabolic energy to the hydrogen ion pump. Two theories have been proposed to deal with these questions. The first theory, the redox theory, suggest that hydrogen ions are directly generated from the oxidation-reduction process and delivered to the secretory surface via the electron transport system.

The second theory, for which evidence is increasing, the ATP hypothesis, proposes that ATP generated from substrate metabolism provides the necessary energy via a specialized membrane ATP-ase.

5:3 Membrane Events during Acid Secretion

5:3:1 Morphological Transformation

The parietal cell undergoes a very dramatic morphological transformation with stimulation (Forte, Machen and Forte, 1977). In the resting state the cytoplasm of the parietal cell is filled with tubulovesicles, which is specialized smooth endoplasmic reticulum. With stimulation, these tubulovesicles coalesce to form extensive secretory canaliculi that are lined with microvilli and that communicate with the luminal surface of the cell. This transformation must involve the cytoskeleton of the cell, although the details of the role of microfilaments and microtubules in the transformation and in the equally complex reversion to the resting state remain unknown (Forte, Machen and Forte, 1977). Carlisle, Chew and Hersey (1978) showed that these changes in the parietal cell surface membranes appeared to be dependent on cyclic-AMP.

The acid secretory apparatus is apparently housed in the membranes of the tubulovesicles and secretory canaliculi. The function of the morphological transformation that the parietal cell undergoes with stimulation seems to be to increase the luminal secretory surface.

5:3:2: The H^+ Pump

ATP is an energy source in tissues throughout the body. It is formed mainly by phosphorylation of ADP, a reaction which is tightly coupled to the energy production by the oxidation reactions in the mitochondrial respiratory cytochrome system (Lehninger, 1975).

When ATP is metabolized in a reaction catalysed by ATP-ase there is energy released and the formation of a proton plus ADP.

$$\text{Thus} \quad ATP^{-2} + H_2O \rightleftharpoons ADP^{-1} + P^{2} + H^{+}$$

There are several ATP-ases present in gastric mucosa. However, recently a specialized K^+-stimulated ATP-ase has been described which appears to represent a major component of the concentrating mechanism for the hydrogen ion (Forte and Lee, 1977). This K^+-stimulated ATP-ase has been found in abundance in five species thus far. It is associated with the parietal cells, and in development studies with tadpoles, the appearance of this enzyme coincides with the development of the tubulovesicles and with the appearance of the ability to secrete acid. In recent years techniques have been developed for isolating the vesicles derived apparently from the tubulovesicles of the parietal cell (Sachs, Chang et al 1977, Forte and Lee, 1977).

These vesicles contain the K^+-stimulated ATP-ase and when exogenous ATP is added, they are able to concentrate acid within them. Antibodies made to highly enriched preparations of these vesicles react with the supranuclear region of the parietal cell and inhibit both hydrogen ion concentration by the vesicles and the K^+-ATP-ase (Sachs, Chang et al, 1977). Studies with these vesicles indicated that the behaviour of the hydrogen-ion pump is best described by a model of active H^+/K^+ exchange, with a passive KCl link. (Sachs, Chang et al, 1977). The function of these vesicles is not inhibited by Cl^- removal from the medium and therefore differs from in vitro studies with intact mucosa where removal of serosal Cl^- markedly inhibited acid secretion (Durbin, 1977, Rehm and Sanders, 1977).

Under conditions of Cl^- removal in the latter studies with intact mucosa, hydrogen ion secretion appears to be an electrogenic mechanism rather than a central exchange mechanism as it appears to be in isolated vesicles. The acid secretory mechanism thus appears to be more complex than simply the K^+-stimulated ATP-ase in the apical membrane. Nonetheless this enzyme appears to be a major component of the H^+ pump.

CHAPTER 6

CARBONIC ANHYDRASE - ITS ROLE IN THE PARIETAL CELL

6: 1: Introduction

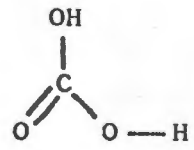
The Carbonic anhydrases (CA) are a group of enzymes that catalyse the reversible reaction:

$$H_2O + CO_2 \rightleftharpoons H_2CO_3$$

In the presence of suitable buffers the overall reaction is:

$$OH^- + CO_2 \rightleftharpoons HCO_3^- \quad \text{(Maren, 1967)}$$

The fundamental change that occurs is from a non-polar gas with a linear structure O = C = O to an acid or its conjugate bases with the coplanar configuration:



The enzyme has a wide distribution in nature. A CA is present in plants where it is thought to be involved in photosynthesis (Burr, 1935). Certain strains of bacteria Neisseria too have been found to possess a CA. These differ in structure from mammalian CA. (Veitch and Blankenship, 1963). Their functions however remain the same. Carbonic anhydrase is present throughout the animal kingdom, both in invertebrates and vertebrates and develops early in the embryo's life. In the human, CA activity in the stomach can be detected as early as the fourth month of gestation. (Berfenstam, 1952). In vertebrates the enzymes are found in association with ion transporting epithelia. Thus in mammals CA activity is found mainly in the stomach, red blood cells, the renal cortex, the

the eye and salivary glands, with smaller amounts present in the choroid plexus of the brain, the pancreas, the intestine, liver and the uterus and lung. (Maren, 1967).

In certain tissues such as the kidneys, the red blood cells and the stomach, the function of CA has been well studied. In other tissues such as the brain and lung, its function is still conjectural. The enzyme differs from other enzymes in general in two respects:

1) The reaction that it catalyses is inorganic.
2) The reaction occurs at a significant rate without enzyme catalysis.

Thus inhibition of CA does not prevent the reaction from occurring. The molecular mechanism by which CO_2 is converted to HCO_3^- at a physiological pH is not known with certainty. Kinetic data for the uncatalysed reaction are consistent with either of two separate or co-existing mechanisms.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- \qquad (A)$$

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \qquad (B)$$

It is not possible to say whether A or B is catalysed by carbonic anhydrase although studies suggest that CA contains a binding site for HCO_3^- rather than H_2CO_3. Enzyme bound OH^- reacts with CO_2 and at a physiological pH the conversion may be directly;

$$CO_2 \rightleftharpoons HCO_3^-$$

With a few exceptions, the work on the physical and chemical properties of CA derives from the red cells of various species. The red cell CA's are Zn^{++} containing proteins of 30,000 daltons with 1 mole of metal bound to one mole of protein.

They are relatively stable, water soluble and retain activity over a pH range of 6 - 10 with maximum activity at pH 8 and have isoelectric points varying from 5.5 - 7.5, depending on the enzyme type. (Maren, 1967). The enzyme loses its activity if the Zn^{++} moiety is removed and addition of Zn^{++} completely restores activity to the apoenzyme. (Lindskog and Nyman, 1964). Certain other divalent ions can restore activity, notably cobalt. This factor may be important in the histochemical staining method for the enzyme. Cobalt, which is a molecular trap for the hydroxyl groups, produced by the action of the enzyme, will not inhibit the enzyme reaction, whereas other divalent cations such as copper and lead, would do so.

In mammals, two major forms (isoenzymes) of CA exist. These have been called the high activity carbonic anhydrase (HACA) and the low activity carbonic anhydrase (LACA). In the guinea pig the HACA enzyme is 18 times as active as the LACA. The mammalian isoenzymes differ from one another in their amino-acid compositions (the difference in serine contents being a consistent finding in a number of species) in their physical properties (isoelectric pH, retention by ion exchange resins, electrophoretic mobility) and their kinetic properties. (Maren, 1967). The CA's of the GIT are indistinguishable from those of the kidney and red blood cells although whereas both kinetic types of CA are found in blood, the stomach contains only the high activity form.

6: 2 Carbonic Anhydrase Localisation in the Stomach:

Reports of the distribution of carbonic anhydrase in the stomach are conflicting. Biochemical measurements by Davenport (1939), indicated that in cats and rats the enzyme was predominantly in the parietal cells which has five to six times the carbonic

anhydrase activity of red blood cells. The surface epithelial cells contained approximately one tenth the activity of the parietal cells.

However, Boass and Wilson (1964), using an elaboration of the manometric technique used by Davenport, indicated that in rats the highest enzyme levels were present in the mucous-secreting columnar epithelial cells and that the oxyntic cells contained less than 25% of the activity of the surface cells. Studies of the distribution of CA by histochemical techniques have indicated that the enzyme is present in the surface epithelial and oxyntic cells, (Vollrath, 1959), the oxyntic cells only (Cross, 1970) and the oxyntic and chief cells (Winborn, Seelig and Girand, 1974). These studies used a relatively less specific technique for the demonstration of carbonic anhydrase.

O'Brien, Rosen et al (1977) using a modification of Hansson's method showed that in bullfrog gastric mucosa the entire fundal gland stains heavily for CA. This was confirmed by an isolated cell preparation of the frog's stomach, where biochemical estimations of CA in various cell fractions were all similar. Histochemical staining of rabbit and cat fundal mucosa however, showed heavy staining in the parietal cells with no activity in the chief cells. There was also quite conspicuous staining in the neck and superficial cells. The antral areas showed fairly marked staining throughout. Thus, it would appear that in mammals CA in the fundal area is limited to the surface and parietal cells. Lonnerholm, (1977), confirmed that in guinea pig the surface and parietal cells stained most heavily for CA.

In terms of subcellular distribution, CA is predominantly a cytoplasmic enzyme. Narumi and Kanno (1973) prepared subcellular portions of gastric mucosa and found activity in the microsomal and mitochondrial portions. However, the greatest activity was in the cytosol fraction.

6:2:1: Methods of Histochemical Localisation of Carbonic Anhydrase

The first published technique for the demonstration of carbonic anhydrase (CA), was that of Kurata (1953). It has, however, been found to give unsatisfactory results by most workers (Hausler, 1958, Fand, Levine and Erwin 1959, Pearce, 1968). Hausler (1958), described a histochemical technique with sections floating on the reaction medium which was slightly modified by Waldeyer and Hausler (1959). This method is dependent on the dehydration of HCO_3 by the enzyme thus:

$$HCO_3^- \rightarrow OH^-$$
$$\searrow CO_2$$

With the release of CO_2 into the atmosphere, the OH^- is trapped by Co^{++} in the medium and cobalt salts are precipitated in the areas of enzyme activity. The precipitated cobalt is then made visible as black CoS by dipping the sections into H_2S or $(NH_4)_2S$. The immediate precipitation of basic Co^{++} salts in the medium is prevented by adding SO_4 to the medium and by adjusting its pH to around 7.4. The drawback to this method was that the incubation times of 20 to 120 minutes were so long that diffusion of the enzyme or the reaction product could take place. Furthermore, inhibition of the staining required the presence of acetazolamide, an inhibitor with a K_1 of about 10^{-8}M, in millimolar concentrations.

This made it doubtful that the staining which occurred was specifically catalysed by CA, and difficult to differentiate from the non-catalysed reaction which occurs simultaneously. The method was improved by Hansson in 1967 and this method now seems to be generally accepted to be both specific and sensitive (Lonnerholm, 1975). Hansson's basic change to the old method was the addition of phosphate to the medium. This changed the composition of the reaction product and allowed much shorter incubation times i.e., 1 to 15 minutes.

A further more important difference was that the specific inhibitors of CA at micromolar concentrations reduced the density of the staining. The composition of the new reaction product was believed to be a cobalt-phosphate complex. Hansson found that by altering the concentration of phosphate in the medium the appearance of uncatalysed staining could be delayed. Lonnerholm (1975) showed that the staining reaction time correlated well with ^{32}P deposition in the sections, confirming the probable formation of this cobalt-phosphate complex.

In Hansson's method, sections are floated on the surface of a freshly prepared incubation medium containing $NaHCO_3$, $CoSO_4$ and KH_2PO_4. The dehydration of HCO_3^- to CO_2 and OH^- is still the basis of the staining reaction. Continuous local OH^- formation at sites of CA activity causes deposition of the basic cobalt phosphate complex (CPC).

The entire reaction that is believed to occur is;

$$HCO_3^- + H_2O \rightleftharpoons H_2CO_3 + OH^-$$

$$H_2CO_3 \rightleftharpoons H_2O + CO_2 \uparrow$$

$$OH^- + CO^{++} + H_2PO_4 \longrightarrow CPC$$

$$CPC + H_2S \longrightarrow CoS \text{ (Black precipitate)}$$

The exact nature of the cobalt-phosphate complex is unknown. The release of CO_2 from the reactions is rate-limiting. If there is no free flow of CO_2 off the sections or if the reaction is carried out in a CO_2 environment, staining does not occur. For this reason Hansson suggested floating the sections on the surface of the reaction medium. Furthermore, he found that if the sections dipped below the surface of the medium, staining was inhibited. Hansson's technique has been criticised by Muther (1972), but Lightfoot and Cassidy (1973) and Rosen and Musser (1972) argued in favour of the validity of the method. Lonnerholm (1975) provided strong evidence in favour of the specificity of the technique. He showed that the staining was enzyme-dependent by allowing solutions of the 2 isoenzymes of carbonic anhydrase to infiltrate filter paper. Using the same histochemical staining technique he showed staining of the filter paper at the sites of infiltration. The activity of these isoenzymes was estimated by the highest dilution of the enzyme to cause visible staining of the filter paper. These measurements correlated well with the relative activity of the enzymes as measured by biochemical methods.

Furthermore, Lonnerholm (1975) showed that staining of the filter paper did not occur with the Zn^{++} free apoenzyme, but addition of Zn^{+++} restored activity - both in the staining reaction on filter paper and by biochemical estimations. Co^{++} added to the apoenzyme restored about 50% of activity by both criteria while Ca^{++} had no effect.

Other studies in which Hannson's method has been used successfully include those of Hansson (1968), Laurent, Dunel and Barets (1969) Pesetsky (1969), Lauwers, Boedts and Geerinckx (1970), Rosen (1970, 1972 a, b) Musser and Rosen (1973 a, b), Rosen and Friedly (1973), Battercherjee (1971, 1972), Lonnerholm 1971, 1972, 1973, 1974) and O'Brien, Rosen et al (1977) and Lonnerholm (1977). Distinct and consistent staining patterns were reported by these authors. Furthermore, available biochemical data was found to correlate well with histochemical staining.

Loveridge, Bloom et al (1974) and Loveridge, Hoile et al (1978) used a modification of this technique in the cytochemical assay for gastrin. In this technique the CA activity assessed in gastric parietal cells by measuring the density of the histochemical stain was found to relate directly to the concentration of gastrin used to treat the tissues. The histochemical staining of carbonic anhydrase in the parietal cells may therefore not only reflect localisation of the enzyme but quantitation of enzyme activity. This will be further discussed below.

6: 3: The Function of Gastric Carbonic Anhydrase:

When Davenport first showed in 1939 that carbonic anhydrase (CA) was present in the acid producing cells of the stomach, he suggested that the function might be to catalyse the hydration of CO_2 furnishing hydrogen ions for secretion. However, in 1946 he reviewed the literature to that date and concluded that the spontaneous rate of hydration of CO_2 was just sufficient to account for the observed rates of acid secretion and that CA was not necessary for acid secretion. He based this conclusion on three groups of experiments and on the rate constant (K_1) for the uncatalysed hydration of CO_2 which had been calculated by Roughton and Booth to be 0.13m/sec. (1946). Davenport therefore calculated that the velocity of the uncatalysed reaction would account entirely for the hydrogen ion production by the cat stomach. Secondly, Davenport quoted the work of Feldberg, Keilen and Mann (1940) who showed that the CA inhibitor sulphanilamide increased histamine-stimulated acid secretion rather than inhibiting it. This he confirmed himself in 1941. Unfortunately however, he did not distinguish between stimulated and basal acid secretion. Furthermore, sulphanilamide is a poor inhibitor of CA (Maren, 1963a). In 1948 Davenport and Jensen published the results of an experiment utilizing the isolated mouse stomach as a model. They again showed no decrease in acid secretion after the CA inhibitor thiopentane-2-sulphonamide. In the light of current knowledge, Davenport's conclusions are now open to criticism.

Firstly, the K_1 for the uncatalysed hydration of CO_2 at $37^{\circ}C$ is now thought to be in the region of 0.43m/sec (Maren, 1963b).

Maren (1967), recalculated Davenport's data on the basis of the corrected K_1 value and found that only approximately one third of the actual rate of acid secretion could be furnished by the uncatalysed reaction. Secondly, Davenport does not distinguish between basal and stimulated acid secretion. In certain species basal acid secretion is low and the rate of acid secretion may well be totally accounted for by the uncatalysed reaction alone. A CA inhibitor in these animals might therefore be expected to have little or no effect.

Feldberg, Keilen and Mann (1940) used histamine to stimulate acid secretion. However, the CA inhibitor was administered 1 - 2 hours before the histamine, during which time metabolic acidosis may have developed, secondary to the effect of the agent on the kidney. Byers, Jordan and Maren (1962), showed that metabolic acidosis itself caused an increase in acid secretion and that CA inhibitors may have a biphasic effect, firstly inhibiting and then enhancing acid secretion. This would have accounted for the increase in acid secretion noted by Feldberg et al. Davenport and Jensen's mouse stomach model was unusual in several respects, making the data difficult to interpret. Firstly, the rate of acid secretion achieved even after secretagogues was low. Secondly, histamine did not cause an increase in acid secretion although acetylcholine did. Under these circumstances the viability of the model must be seriously questioned. The CA inhibitor had no effect but this may be related to the low rate of acid secretion which occurred.

Davies and Roughton in 1948 disagreed with Davenport's position. Using a higher gastric secretion rate than Davenport but keeping below maximal rates, they concluded that the uncatalysed

hydration of CO_2 was too slow to account for the physiological process of acid secretion.

Janowitz, Colcher and Hollander (1952) showed that 20 mg/kg acetazolamide reduced H^+-ion concentration and output after histamine stimulation of dogs with vagally denervated (Heidenhain) pouches. Byers, Jordan and Maren (1962) showed essentially the same as Janowitz et al. However, they used 5 mg/kg acetazolamide and produced greater inhibition of acid secretion than Janowitz et al. This may again have been due to the acid-base changes and metabolic acidosis induced by the CA inhibitor in the higher doses. Powell, Robbins et al, (1962) using the intact dog stomach and maximum histamine stimulation, yielding acid secretory rates of 20 meqH^+/hr demonstrated a fall in acid secretion to about 4meqH^+/hr after 60mg/kg acetazolamide. Maren (1967) has calculated that in all three instances quoted above, the acid secretory rate recorded after full CA inhibition is in good agreement with the theoretical acid production which could be produced by the uncatalysed dehydration of CO_2. In making this calculation he took into account the total parietal cell bulk (10 to 15% of total mucosal cells) and the known K_1 of this reaction at 37°C.

Further evidence in this field was provided by Emas (1962)
He found firstly that histamine-and gastrin-induced acid secretion was inhibited by acetazolamide to approximately the same degree in cats. Secondly, there was a smooth dose-response curve from 10mg/kg acetazolamide (no effect) to 50 mg/kg (maximal) and thirdly, at low levels of H^+ secretion (about 0.8 meq/hr) maximum drug effect reduced secretion about 50%. At high levels (about 2.6 meq/hr), the drug reduced secretion about 80%.

The residual secretion after complete inhibition was always 0.4 - 0.6 meq/hr.

If the theoretical uncatalysed rate of CO_2 dehydration is calculated for the cat, the production of H^+-ions is very close to the acid secretion recorded, i.e., 0.32 meq/hr. Finally, Maren (1967) reported that the rate of acid secretion during full carbonic anhydrase inhibition could not be increased by additional histamine. In man, inhibition of acid secretion by acetazolamide has been noted by Texter and Barborka (1955), Janowitz, Dreiling et al, (1957), Hirshowitz, London and Wiggins (1959) and Gailitis and Schreiber (1960). All of these authors made use of different doses of acetazolamide, given by different routes. As such, duration and extent of inhibition of acid secretion varied with the different authors. Two groups, Mauro and Liffredo (1959), and Poller (1956), concluded that the slight degree of acid inhibition noted was not significant. The rate of induction of metabolic acidosis secondary to the drug, with its stimulant effect on acid secretion is the probable reason for such conflicting reports. The degree of metabolic acidosis would vary with the varied conditions described above.

Thus there is now overwhelming evidence that acetazolamide, which is a CA inhibitor, inhibits acid secretion. There is also good evidence that the acid secretion which occurs after total CA inhibition in approximately equal to the acid which would result from the uncatalysed hydration of CO_2. Lastly, acid secretion cannot be further stimulated in the presence of CA inhibition.

From the data discussed one could conclude that Davenport's original suggestion may be correct, viz. that the function of carbonic anhydrase may be to provide the hydrogen ions for secretion by catalysing the dehydration of CO_2. Most or all of the hydrogen ions secreted in the basal state could be accounted for by the uncatalysed reaction while high rates of acid secretion would require the reaction to be catalysed.

The question of the origin of the hydrogen ion and acid secretion has been discussed previously. If the hydrogen ion originated directly from the dehydration of CO_2 one would expect that inhibition of CA would lead to an immediate cessation of acid secretion. Hersey and High (1971), showed in frog gastric mucosa, that stimulated acid secretion continued at the same rate for about 5 to 15 minutes after acetazolamide administration, before there was evidence of inhibition of secretion. However, following the administration of diamox, there was an immediate rise in intracellular pH, which reached an apparently stable value in 3 to 5 minutes, and remained relatively stable for at least one hour.

This alkaline shift in the cell would be consistent with the hypothesis that carbonic anhydrase maintains the intracellular milieu by maintaining intracellular neutrality. (O'Brien, Rosen et al, 1977). Thus for every hydrogen ion secreted, a hydrogen ion must replace it in the intracellular ionic pool. Secondly, every hydrogen ion secreted on the luminal surface of the cell is accompanied by the excretion of a bicarbonate ion on the serosal side. This accounts for the phenomenon of the alkaline tide associated with acid secretion. (Davenport, 1977).

The bicarbonate ion is formed by the action of carbonic anhydrase and is secreted at the serosal surface of the cell in exchange for a chloride ion by a system of neutral exchange (Berglindh, 1977). (Fig. 6:1).

The chloride is then secreted at the luminal surface of the cell by a system of active transport (Berglindh, 1977). This occurs separately from the H^+ ion secretion, but balances the latter. The proposed function of CA in relation to acid secretion is represented by Fig. 6:1.

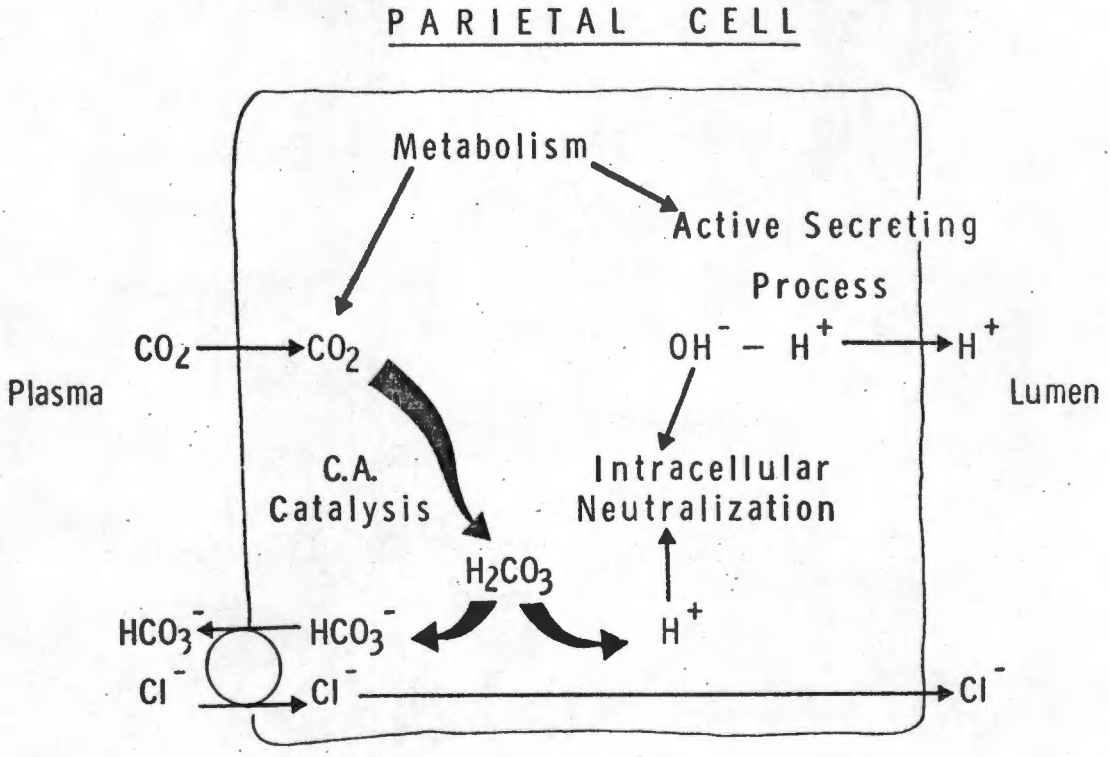


FIG. 6:1 FUNCTION OF CARBONIC ANHYDRASE IN THE PARIETAL CELL

The carbonic anhydrase activity (dark arrows) catalyses the formation of HCO_3^- + H^+ which have a role in the maintenance of intracellular pH homeostasis.

CA is also present in high concentrations in the surface cells of the stomach (Lonnerholm, 1977, O'Brien, Rosen et al, 1977). Schiessel Matthews et al (1980) and Kivilaakso and Silen (1979) have proposed that the function of the surface cell CA is the neutralization of influxing luminal H^+ and re-extrusion of the acid and CA is thus important in the maintenance of mucosal integrity. This proposal is still unproven and the relationship between surface cell and parietal cell CA is not known.

The conclusions therefore are that CA is an enzyme present in the cytoplasm of the surface and parietal cells of fundic mucosa. In the parietal cells, although CA is not directly responsible for acid secretion i.e., it is not the proton donor, stimulated acid secretion cannot occur for long in the absence of active CA. Furthermore, the activity of CA in the parietal cell as determined by the rate of formation of hydrogen ions by the dehydration of CO_2 should balance the rate of hydrogen ion secretion in the stimulated state and thus may be regarded as an index of stimulated acid secretion. This led to this work whereby CA activity, measured by quantitative cytochemistry was used to investigate acid secretagogue status and functions.

CHAPTER 7

THE MEASUREMENT OF THE GASTRINS AND THE CYTOCHEMICAL BIOASSAYS

7:1: Introduction:

The scientific method is concerned firstly with the formulation of a theory and secondly with the proof of the theory by observation. Endocrinology in the broader sense deals with the function of biologically active substances (hormones) in many tissues of the body. In order to observe the effects of perturbations of these hormones, one requires methods of assay which are sufficiently sensitive, accurate, reproducible and specific in order to make a correct observation.

7:2: Measurements of Gastrin:

The measurement of gastrin, as with many other hormones, started with a bioassay. In the case of gastrin the response in gastric acid secretion evoked by administration of a test substance to an experimental animal was compared with that produced by a standard reference preparation of known biological activity. Animals used in bioassay techniques which were described, included the cat, rat and dog, either in the anaesthetized or conscious state, Histamine was used as the reference standard before preparations of gastrin were available, but there is considerable disadvantage in using a reference standard which differs from the test substance. More recently, pure or synthetic human gastrin has been used.

For a reliable bioassay certain basic requirements must be met: The animal must be sensitive to gastrin; it must respond predictably to different doses of the test substance; the reference standard must be of a constant potency and it must behave as gastrin (Emas and Uvnas, 1973).

The first method of gastrin bioassay that fulfilled these requirements was described by Uvnas and Emas (1961) in cats with gastric fistulae. They too initially used histamine as the standard reference preparation, but subsequently showed that the sensitivity of the assay was 0.08-0.2 µg synthetic human gastrin (Emas and Uvnas, 1973). Other bioassays described included those of Blair, Harper and Reed (1962), later refined by Blair, Keelyside et al (1968), also in the cat, and Ghosh and Schild (1958) and Lai (1964) who used anaesthetized rat models. An improved bioassay for gastrin using the perfused rat stomach was described by Smith, Lawrence, et al (1970). This assay had a sensitivity of 10 - 20 ng of synthetic human gastrin and the steepest part of the dose-response curve lay between 40 and 200 ng of synthetic gastrin. This assay was used to measure gastrin activity in the plasma and tumour extracts of a patient with the Zollinger-Ellison syndrome.

It is apparent that until now the techniques of bioassay have posed many limitations. Only a very small number of unknown or test specimens and in some cases only one, could be assayed at a time. Most methods are tedious and time-consuming to execute. Variations in the methods and animals used made comparison between different assays difficult. However, the greatest limitation was probably the insensitivity of these methods with the lowest detectable level in the range of 1 ng (Walsh and Grossman, 1975). This is not sensitive enough to measure normal circulating gastrin levels, which are normally less than 100 pg/ml. Bioassay techniques were thus until recently thought to be useful only in the measurement of serum gastrin levels in hypergastrinaemic patients (Walsh and Grossman 1975).

However, bioassays can be performed with good precision, so that interassay variations are often less than 10% (Walsh and Grossman, 1975, Loraine and Bell, 1971). A difficulty with gastrin bioassays is that they all measure acid secretion by one or other method. However, as has been extensively reviewed, acid secretion is the net effect of several stimuli acting together on the parietal cell. Thus it is difficult to measure the gastrin-like activity present in biological fluids as these may contain a variety of other acid secretagogues. Thus these assays cannot be said to be specific for gastrin, except when they are used to measure the biological activity of purified gastrin extracts.

Once pure gastrin preparations became available, the technique of radioimmunoassay of gastrin could be developed (Yalow and Berson 1970a, McGuigan and Trudeau, 1970a). This technique is about a thousand times more sensitive than the old bioassays, allowing detection of gastrin levels as low as 1 picogram (Walsh and Grossman, 1975) and large numbers of serum gastrin estimations can be performed simultaneously with high sensitivity and relative specificity (Loraine and Bell 1971, Walsh and Grossman 1975). However it soon became obvious that radioimmunoassay not only of gastrin, but of several other peptides too, had serious limitations. It was found that heterogenous forms of many peptides with different molecular weights and biological activity occurred (Yalow and Berson, 1970, 1971, Rehfeld, Stadil and Vikelsoe, 1974). These include degradation products, prohormone forms, many of which are biologically inactive as well as various related biologically active molecules. Measurements of these hormones therefore often depend upon the relative specificity of the antiserum for the different forms.

In addition, protein interference in the assays gave rise to entities such as "big-big gastrin" (Yalow and Berson, 1972), which have subsequently been shown to be artifactual (Rehfeld, Schwartz and Stadil, 1977). The result of this problem of antigenic variability is that the immunoreactive measurements do not necessarily reflect biological activity and thus physiological function.

Thus as long ago as 1967, a special meeting convened by the World Health Organization recommended the development of "Biological microassays" that would have the same sensitivity as the equivalent radioimmunoassays and should be run in parallel with them. In 1974, Chayen and his colleagues described a group of cytochemical bioassays which were in many cases more than 1000 times sensitive then immunoassays. Thus, although these assays like other bioassays, were tedious and time-consuming in their execution. for the first time biological activity of hormones in serum could be measured and compared to radioimmunoassay measurements.

7:3 The Cytochemical Bioassays:

These assays first described by Chayen and colleagues (Chayen, Daly et al 1976) revert conceptually to the functional analysis: since a hormone shows its activity by causing a specific biochemical effect in its target cell, it should be possible to measure the biological activity of a hormone by measuring a specific biochemical response in the target cells.

This has been done in assays using dispersed cells (Sayers, Swallow and Giordano, 1971, Van Damme, Robertson and Diczfalusy) but for various reasons, including the tissue dilution artifact (Chayen, Jones et al, 1961), the difficulty in achieving a sufficient bulk of cells, and the damage to cells during the separation process, Chayen's group believed it preferable to be able to study the response of the target cells in their fully differentiated state, held in their natural stroma in their natural state. This was done by the cytochemical demonstration of a biochemical reaction within the cells. Quantitation of the reaction was made possible by the development of the scanning and integrating microdensitometer designed by Deeley (1955), that allowed the precise measurement of reaction products within cells even though the products were distributed inhomogeneously.

Using this technique assays were established for ACTH (Daly, Loveridge et al, 1974; LH (Rees, Holdaway et al, 1973; TSH (Bitensky, Alaghband-Zadeh and Chayen, 1974;) gastrin (Loveridge, Bloom et al 1974) and PTH (Chambers, Dunham et al, 1978 and Fenton, Somers and Heath, 1978). All assays were initially done on segments of guinea pig tissue but it became obvious that the procedure could be streamlined by using unfixed frozen sections of tissue without loss of sensitivity (Alaghband-Zadeh, Daly et al 1974; Loveridge, Hoile et al, 1978; Gilbert, Besser et al, 1977 and Buckingham, Chayen et al, 1979). All the assays were several hundred-fold more sensitive than their respective immunoassays and serum had to be diluted 1:100 and 1:1000 for measurement in the assays.

Thus for the first time gastrin-like biological activity in serum could be measured parallel to the immunological activity. However, although Loveridge, Hoile et al (1978) claimed that histamine did not alter carbonic anhydrase activity, it was found as will be discussed, that both histamine and carbamylcholine caused dose-dependent increases in CA activity. Thus like other bioassays, this assay could not be said to be a specific gastrin bioassay but rather responded to total CA stimulatory activity (CASA) of serum.

This work describes the establishment of the technique for the measurement of CASA in plasma by quantitative cytochemistry and shows how this technique may be modified to act as a model to investigate acid secretagogue interactions and their possible role in acid secretion.

QUANTITATION OF CARBONIC ANHYDRASE ACTIVITY IN PARIETAL CELL SECTIONS

CHAPTER 8 MATERIALS AND METHODS

8:1 Chemicals:

Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) (Sigma Chemical Co., St. Louis M.O., U.S.A.)

Cobalt Sulphate (Analar grade, B.D.H. Chemicals, Poole, England).

Sodium hydrogen carbonate (Analar grade, B.D.H. Chemicals).

Potassium dihydrogen phosphate (Analar grade, B.D.H. Chemicals).

Sulphuric acid (Analar grade, B.D.H. Chemicals).

Gum tragacanth (Sigma Chemical Co.)

Farrants medium (Searle Diagnostic, High Wycombe, England).

Absolute Alcohol (Alchemist brand, Heynes Mathew, Cape Town).

n-Hexane (Merck, Darmstadt, Germany.)

Iron Sulphide (Merck).

Toluidine blue (Searle Diagnostic).

Synthetic Human gastrin (SHG-17-I) (Produced by I.C.I. London, England and distributed with CEA/Sorin gastrin radio-immunoassay kits.)

Histamine dihydrochloride (Merck).

Carbamylcholine chloride (Merck).

Acetazolamide (Lederle Laboratories).

Atropine (Merck).

Cimetidine (Smith, Kline & French Laboratories, Herts. U.K.)

Hepes buffer was made up by titrating Hepes to the required pH with a concentrated solution of potassium hydroxide solution.

8:2 Laboratory Equipment:

The dispensing apparatus was built by the Department of Bio-engineering, Groote Schuur Hospital, according to specifications provided by us. A diagram of the apparatus is shown in Fig. 8.1.

A Kipps apparatus was used to generate hydrogen sulphide. Iron sulphide granules were allowed to react with 50% concentrated hydrochloric acid. The Kipps apparatus was kept in a laboratory fume cupboard.

A Slee cryostat (Vickers Ltd., Vickers Instruments, Croyden, England) was used to cut the frozen sections. The scanning and integrating microdensitometer (M85) was supplied by Vickers Ltd., Vickers Instruments, York, U.K.

Female Duncan Hartley outbred (DHP) guinea-pigs, weighing 400-500 gms. were supplied by the Animal Unit, University of Cape Town Medical School.

8: 3 Methods:

8: 3: 1: Removal of the Stomach:

A 450 - 500 gram female DHP guinea-pig was starved for 12 hours and then asphyxiated in nitrogen. The peritoneal cavity was immediately opened and the stomach excised, and rinsed in 0.025M Hepes buffer pH 7.0. A strip of fundus (about 3 - 4mm wide) was cut circumferentially from a constant portion just below the oesophago-gastric junction. This was rinsed in fresh buffer and undigested pieces of food particles were removed by gentle brushing with a fine camel hair paintbrush. The strip of fundus was then cut into cubes which were rinsed again in fresh buffer.

8. 3. 2: The Freezing Procedure:

Crushed solid CO_2 was added to a chilling bath filled with absolute alcohol until a saturated solution was obtained. This state became obvious when, (a) the alcohol CO_2 mixture became viscous; (b) the addition of more solid CO_2 did not cause bubbling and (c) the thermometer recorded about $-70^{\circ}C$. A pyrex beaker, containing 30 - 50 mls. of n-Hexane was put into the bath and covered with a glass lid. More solid CO_2 was added to the bath to maintain the temperature. The Hexane was allowed to cool to about $-65^{\circ}C$, when it was ready for use.

The cubes of tissue were then dropped individually into the cooled hexane. Care was taken to ensure that the tissue was plunged straight into the hexane, without touching the sides of the beaker.

The specimen was left in the hexane for 1 minute and then transferred by pre-cooled forceps to a dry tube, pre-cooled to -70°C. The tube was corked and stored, encased in dry ice in a Dewar flask. All specimens were used within 60 hours of freezing.

8: 3: 3: Mounting the Tissue:

During this procedure, care had to be taken to prevent the tissue from being warmed at any stage. All handling of the tissue was thus done with pre-cooled forceps. A piece of tissue was removed from the tube and placed in a cavity in a piece of CO_2 ice so that its orientation could be determined. To keep it at the required temperature it was covered by a second flat piece of CO_2 ice. Another alcohol-solid CO_2 bath was prepared at about -70°C. The metal chuck from the cryostat was placed in this bath with the tip clear of the alcohol and left to equilibrate temperatures with the bath. A drop of water was then placed onto the chuck. This froze rapidly until only a thin film of water of comparable size to that of the specimen remained. The tissue was then transferred to this film of water and orientated to lie in a transverse plane. As the water continued to freeze, the specimen was held fast in the ice. The chuck was then transferred to the cryostat chamber where it was stored until use.

8: 3: 4: Cutting the Sections:

Sections were cut in the cryostat chamber. The chamber was set at an ambient temperature of -20°C while the knife was cooled to -70°C by packing the hasp with CO_2 ice. Sections 18 µm thick were cut and were lifted from the knife by carefully bringing a glass microscope slide at laboratory temperature, up to and parallel to the section on the knife.

The temperature gradient between the knife and the slide causes the sections to "jump" off the knife onto the slides. The central position of the sections on the slides was ensured by using a prepared transparent template, which fitted over the reverse surface of each slide. The slides were then stored in the cryostat chamber for up to 8 hours until use.

8: 3: 5: The Reaction

8: 3: 5: 1: Preparation of Standard Reference Solutions:

The standard reference solutions were made up by diluting the test substance in 0.025M Hepes buffer pH 7.0 to the final concentrations desired. Each dilution of test substance was reacted in triplicate in each experiment, with a total of 24 sections being stained during the course of each assay.

8: 3: 5: 2: The Secretagogue Reaction:

The microscope slides were removed from the cryostat chamber, labelled, laid flat in the staining trough and allowed to dry for 10 minutes. The syringes of the dispensing apparatus were filled with 200 µl of the test substances. When the dispensing apparatus was placed in position over the staining trough, each syringe was orientated directly above a single section. The syringes were then emptied directly and simultaneously onto the sections. The reaction was allowed to proceed for 90 seconds before being stopped by the addition of the staining reagents.

8: 3: 5: 3: Preparation of the Staining Reagents:

Two solutions were made up from stock solutions and were added together just prior to use.

Solution A consisted of 750mg $NaHCO_3$ in 40 mls. of 0.1M Hepes buffer, pH 7.4 + 0.001% gum tragacanth.

Solution B consisted of 6 mls of 0.1M $CoSO_4$

6 mls 0.5M $H_2 SO_4$

4 mls distilled water

1 ml $KH_2 PO_4$

This was added together to give a final solution consisting of:

1.05×10^{-2} M Co SO_4

5.3×10^{-2} M $H_2 SO_4$

15.7×10^{-2} M Na HCO_3

1.17×10^{-3} M $KH_2 PO_4$ in the Hepes buffer with a final volume of 57ml.

The mixture was very effervescent and prior to use was de-bubbled for a few seconds on the vacuum attachment of a laboratory tap.

8: 3: 5: 4: The Staining Reaction:

At the end of the 90 second secretagogue treatment, the staining reaction mixture was poured carefully over the slides. The staining trough was agitated gently, to ensure the even spread of the reaction mixture. The volume of the reaction mixture was such that only a thin film of liquid covered the sections. The reaction was allowed to proceed for 2 minutes at room temperature, when the slides were washed twice in cold water. The slides were then placed in a solution of H_2S in water for 90 seconds.

This resulted in the formation of a dark brown precipitate of cobalt sulphide, which was visible in the sections. The slides were again washed in cold water and the sections were mounted in Farrants medium. Fig. 8.2 summarizes the method used.

8: 3: 7: Quantitation of the Reaction:

The sections were read on the Vickers M85 integrating and scanning microdensitometer. This is basically a spectrophotometer which works through a microscope. The tissue is inspected with normal white light, and the cell to be read is selected. The rest of the section is then cut off by means of a stop. A monochromatic system allows the cells to be illuminated with light which is absorbed maximally by the histochemical reaction product and this image is projected onto a photo-multiplier. To overcome the variation of stain which occurs within the cell, the image is transmitted point by point by means of an automatic scanning system. Each point in the selected image is measured separately and all readings of the selected field are integrated by the machine which registers the integrated absorption present in that field. The machine readings could then be converted to absolute units of extinction by measuring the absorption produced by a neutral density filter with a known density of 1.

In the case of the parietal cells, readings were taken at a wavelength of 550 nm and a spot size of 1. Parietal cells selected for readings were those which presented a full profile. These completely filled the A2 mask on the densitometer, which has a measured field diameter of 20mm when the 20 x objective lens is used.

Approximately 15 to 20 cells per section were selected for readings. Particular care was taken that the cells were in a uniformly stained area of the section, that the section was not folded or frayed, and that the cell borders did not overlap adjacent cells, as this would give spuriously elevated readings.

To eliminate the effects of non-specific attachment of cobalt to tissues and optical errors due to light scattering, the same number of measurements were made in the muscularis mucosae of each section. The mean of the muscle measurements were subtracted from the mean of the cell measurements for each section. To correct for instrument variation, the instrument was calibrated by taking readings of the standard filter with an optical density of 1 prior to, and after taking the readings in each section. These measurements were used to calculate the mean integrated extinction, (MIE), which for convenience, was multiplied by a hundred.

$$MIE = (\text{Mean reading of cells} - \text{mean reading of muscle}) \times \frac{100}{D_1}$$

where D_1 is the reading of the standard density filter, optical density of 1.

Standard dose response curves were constructed relating the percentage integrated extinction to the log $_e$ secretagogue dose. For the purposes of the cytochemical bioassay for carbonic anhydrase stimulating activity (CASA) of serum, SHG-17-I was used as the standard reference preparation. The MIE x 100 of the diluted unknown sera was similarly plotted on a semi-log scale and the CASA determined by extrapolation from the standard dose response curve or substitution in the equation of the straight line relationship between extinction and the log gastrin dose.

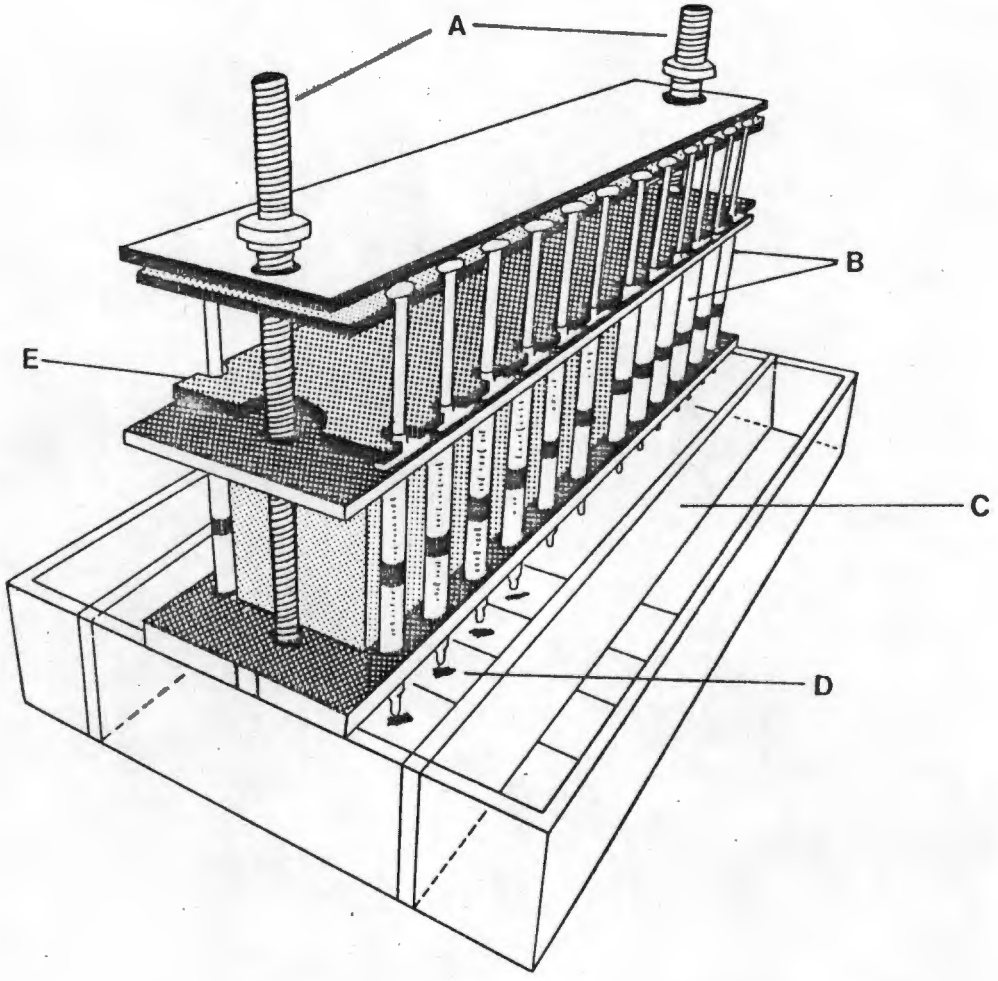


FIG.8:1 Dispensing Apparatus for Cytochemical Staining

The entire instrument was constructed from perspex. The staining trough (33 x 17cm) contained 24 microscope slides (D), held in place by the crossbeam (C). The upper part of the apparatus containing the 1 ml. disposable syringes (B) was detachable from the staining trough below. The syringes were held by the base-plate (E) and the plunger plate. The plunger plate was kept steady with the guide screws (A) and allowed all the syringes to be filled and emptied simultaneously.

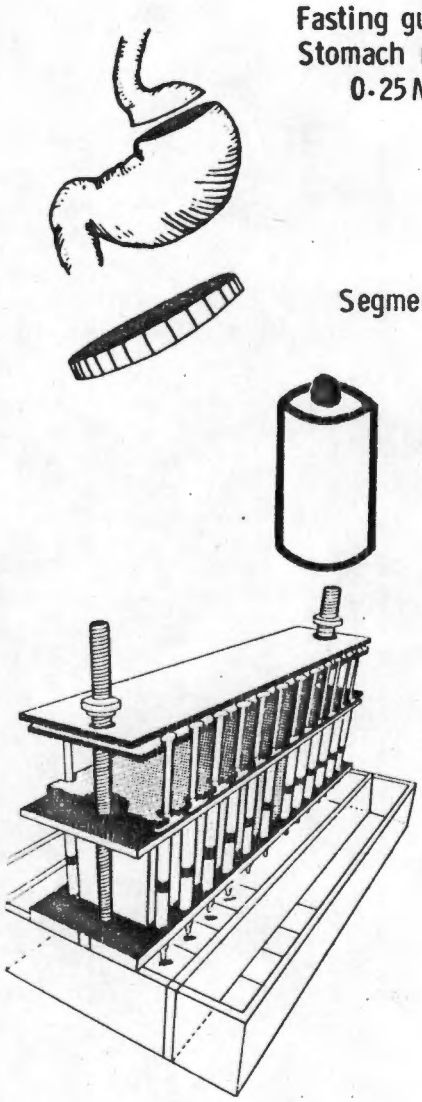


FIG. 8:2 Summary of Method for Cytochemical Staining

8:4: Results:

Fig. 8:3 and 8:4 shows the fundic mucosa stained for carbonic anhydrase activity by the method outlined. The cytoplasm of the parietal cells are stained dark brown by the cobalt sulphide precipitate. The nucleus of the cell remains pale.

A dose response curve of gastrin 2.5×10^{-16} to 2.5×10^{-10} mol/l is showm in Fig. 8:5. The response is linear from 2.5×10^{-15} to 2.5×10^{-12} mol/l.

The readings taken from a typical gastrin dose response curve are shown in Table 8:1. The coefficient of variation of the density measurements between cells of a given section ranged from 2.2 to 6.9% while the variation in the muscle measurements ranged from 3.7 to 10.3%. Thus in order to reduce the error between 10 and 15 cells and muscle fields were measured on each section. This reduces the percentage error to between 0.7 and 3.2%.

The coefficient of variation of readings taken by the instrument on a single cell was measured on several occasions and was always found to be less than 1%. This variation was sufficiently low for a single reading on each cell or muscle field to be acceptable.

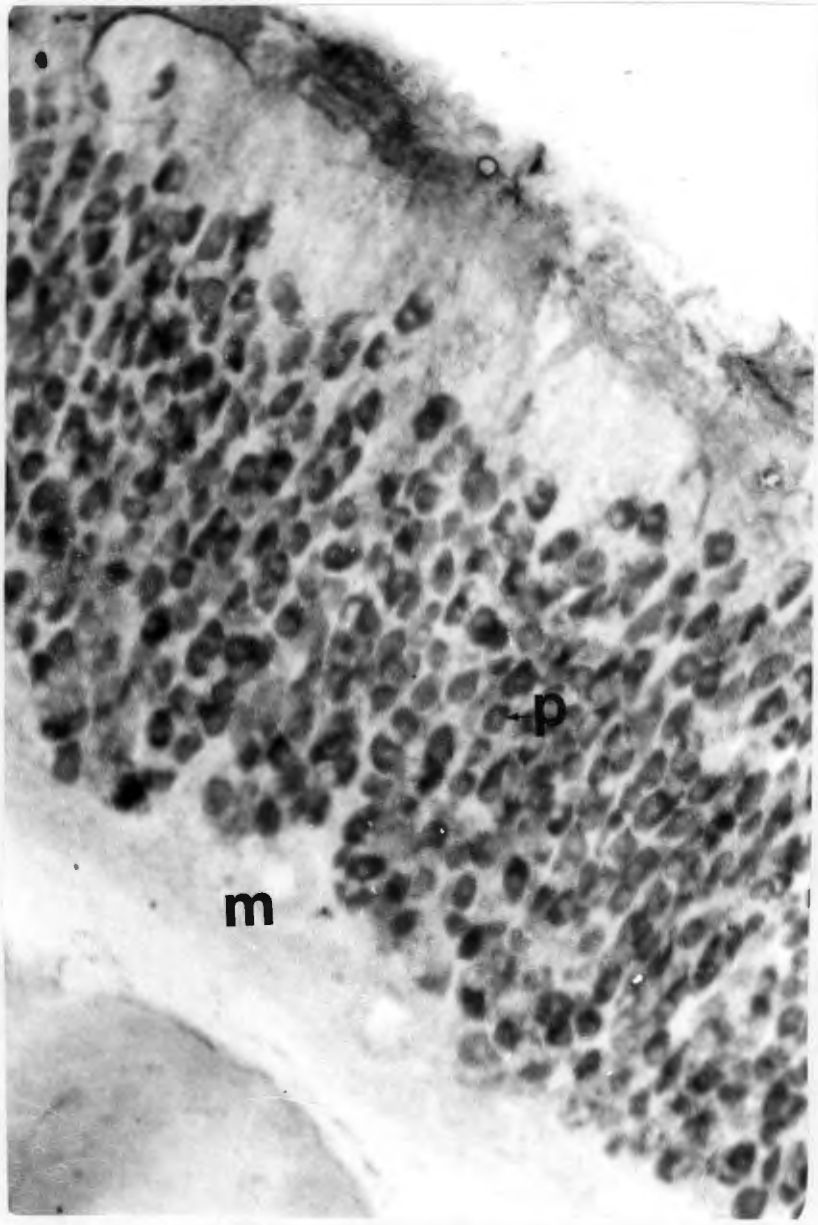


FIG 8:3 Guinea pig fundic mucosa stained for carbonic anhydrase activity. The cobalt sulphide precipitate stains darkly in the cytoplasm of the parietal cells, leaving the nucleus pale.

M Muscularis mucosa

P Parietal cell

X 600

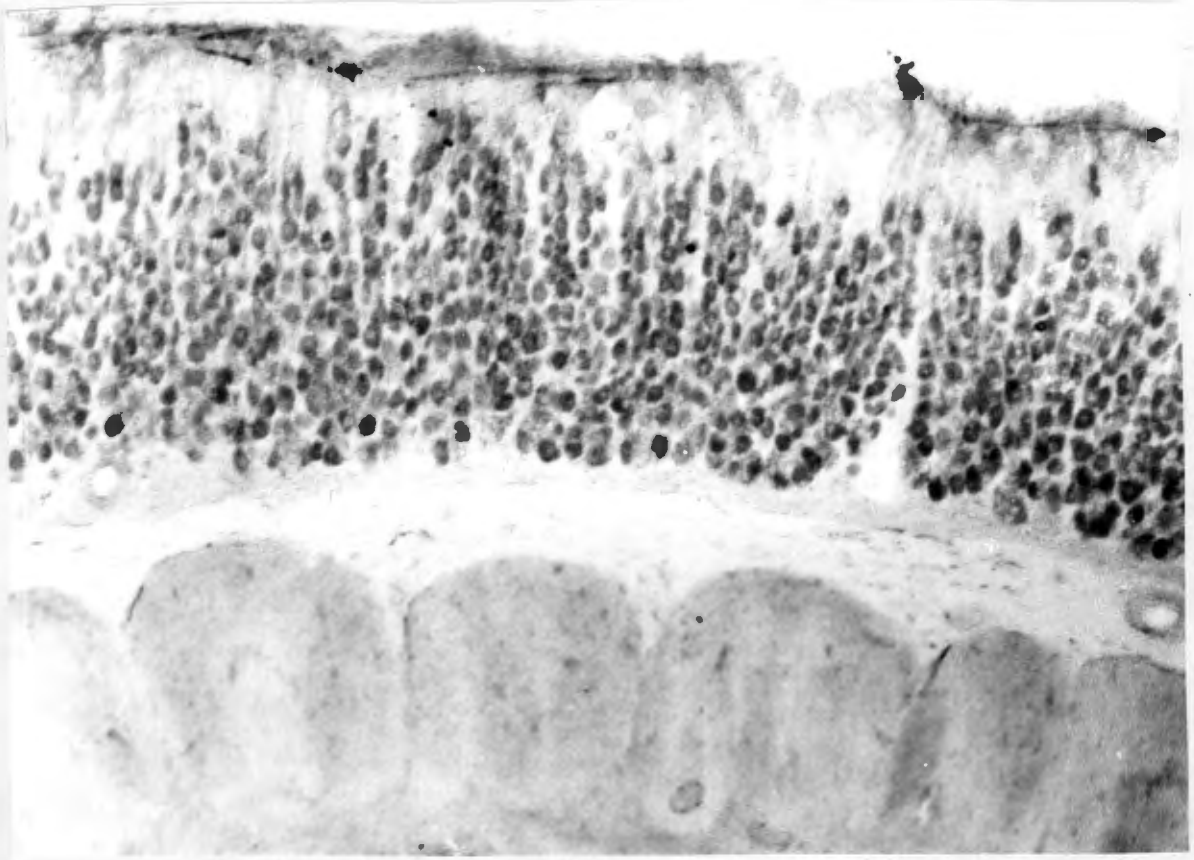


FIG. 8:4 Low power view of guinea pig fundic mucosa stained for CA activity.

X 150

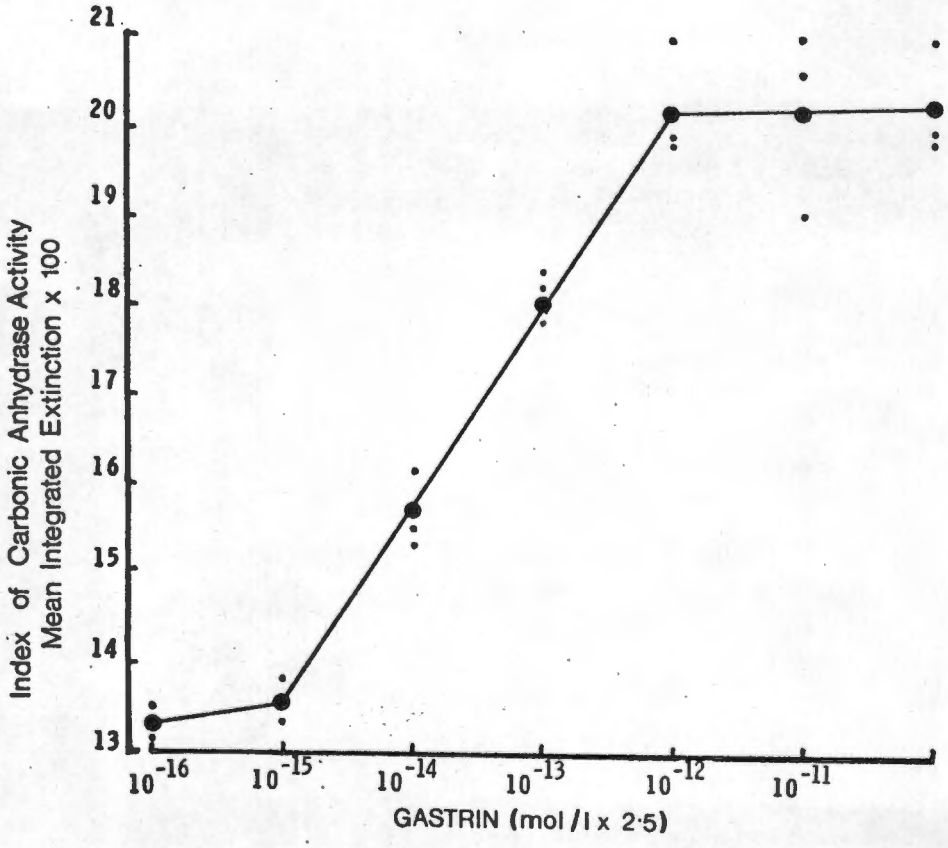


FIG: 8:5 Dose Response Curve of Gastrin 2.5×10^{-16} to 2.5×10^{-10} mol/l. The curve is linear from 2.5×10^{-15} to 2.5×10^{-12} mol/l.

TABLE 8: 1:

GASTRIN STANDARD DOSE RESPONSE CURVE — GUINEA PIG NO. 47

2.5×10^{-12} m/litre — 2.5×10^{-13} m/litre

SLIDE	a		b		c		a		b		c	
CELL NO.	CELL	MUSCLE	CELL	MUSCLE	CELL	MUSCLE	CELL	MUSCLE	CELL	MUSCLE	CELL	MUSCLE
1	32.92	17.31	37.36	21.3	32.73	17.85	29.67	16.61	33.70	20.96	30.61	16.64
2	34.65	18.94	32.17	18.92	34.62	19.23	29.63	16.57	35.04	22.93	30.97	17.29
3	32.93	21.47	32.80	18.22	33.77	18.33	31.25	17.45	30.86	17.81	31.32	18.02
4	35.94	18.64	36.92	21.00	34.84	19.55	29.64	16.21	32.83	19.20	32.19	19.14
5	32.25	21.13	34.99	19.79	35.23	19.70	32.65	20.46	34.13	21.84	31.89	1[illegible].10
6	35.79	23.03	33.03	18.37	35.55	20.93	29.10	16.43	36.01	22.78	32.03	19.54
7	38.67	21.82	32.89	18.07	33.59	18.50	32.39	19.17	29.77	20.18	28.61	16.57
8	37.01	19.16	34.08	17.87	32.54	18.10	31.31	18.47	32.87	17.58	30.64	17.12
9	33.13	19.87	30.21	17.81	35.88	19.46	30.35	17.56	30.13	17.80	30.65	17.36
10	34.14	19.04	31.11	17.35	31.84	17.70	33.80	20.66	32.94	18.86	28.59	16.54
Mean	34.74	20.04	33.56	18.87	34.06	18.94	30.98	17.96	32.83	20.00	30.77	17.73
SD	2.079	1.758	2.32	1.375	1.377	1.014	1.570	1.661	2.054	2.058	1.280	1.149
Coeff. variation (%)	6	8.8	6.9	7.3	4	5.4	5.1	9.2	6.3	10.3	4.2	6.5
SEM	0.657	0.556	0.734	0.435	0.321	0.321	0.496	0.525	0.650	0.651	0.404	0.363
% Error	1.9	1.8	2.2	2.3	1.3	1.7	1.6	2.9	2.0	3.2	1.3	2.0
Density of 1 (D_1)	69.9		70.6		71.3		71.9		71.3		72.2	
Mean Integrated Extinction x 100	21.0		20.8		21.2		18.1		18.0		18.1	

2.5×10^{-14} m/litre — 2.5×10^{-15} m/litre

	a		b		c		a		b		c	
	CELL	MUSCLE	CELL	MUSCLE	CELL	MUSCLE	CELL	MUSCLE	CELL	MUSCLE	CELL	MUSCLE
1	28.24	15.05	33.0	21.74	31.18	20.91	30.07	21.35	28.95	18.41	29.14	18.28
2	27.49	16.34	32.15	21.04	32.20	21.48	30.49	20.74	27.68	17.93	30.47	21.23
3	28.95	17.29	32.90	21.15	31.97	20.40	30.72	18.95	27.23	17.73	31.04	21.37
4	29.40	18.35	34.84	22.29	33.94	21.65	30.95	21.24	27.17	17.14	29.83	20.53
5	27.16	16.23	33.76	21.85	29.87	18.64	30.15	21.44	27.09	17.98	29.50	20.78
6	27.52	16.80	31.78	20.74	30.24	18.66	31.38	21.07	29.25	21.20	29.58	20.43
7	30.96	19.06	33.12	20.59	30.53	18.73	29.26	21.48	28.50	19.01	29.10	17.61
8	27.12	15.75	31.98	21.07	30.62	19.00	29.40	21.07	29.53	19.81	30.06	18.80
9	28.86	17.74	34.22	24.16	32.40	21.05	30.71	21.35	30.13	20.40	30.52	18.71
10	26.48	15.32	34.12	22.46	31.81	21.56	30.48	20.31	20.10	19.47	28.64	21.16
Mean	28.22	16.79	33.19	21.71	31.48	20.21	30.36	20.9	28.46	18.91	29.79	19.89
SD	1.343	1.313	1.035	1.069	1.23	1.302	0.661	0.773	1.099	1.300	0.744	1.393
Coeff. variation (%)	4.8	7.8	3.1	4.9	3.9	6.4	2.2	3.7	3.9	6.9	2.5	7
SEM	0.425	0.415	0.327	0.338	0.389	0.412	0.209	0.245	0.348	0.411	0.235	0.440
% Error	1.5	2.5	1.0	1.6	1.2	2.0	0.7	1.2	1.2	2.2	0.8	2.2
Density of 1 (D_1)	71.9		72.5		72.6		72.9		71.8		74.2	
Mean Integrated Extinction x 100	15.9		15.8		15.5		13.0		13.3		13.3	

Mean Integrated Extinction = (cell - muscle) x $\frac{100}{D_1}$

8:5 Discussion:

The cytochemical section bioassay for gastrin (Loveridge, Bloom et al, 1974), whose method has been modified here, entailed pre-incubation of segments of gastric fundic mucosa in maintenance culture for 4 to 5 hours prior to their treatment with gastrin. The tissue was then treated with gastrin, snap-frozen and stained for carbonic anhydrase (CA) activity. When their method was modified to the section bioassay (Hoile and Loveridge, 1976), this pre-incubation stage was excluded. However these workers found that the basal CA activity tended to vary between animals and suggested that this may have been due to differences in the basal background gastrin level of the animal (Loveridge, Hoile, et al 1978). They therefore re-introduced the pre-incubation of the segments in Trowell's T8 medium, followed by the pre-treatment of segments with a sub-threshold dose of gastrin (Loveridge, 1978). These segments are then frozen, sections cut and treated with gastrin in much the same method as outlined above. Loveridge, Hoile et al (1978) stated that this pre-incubation step decreased and standardised the blank activity without altering the sensitivity of the response. An attempt was made to confirm these findings in this study. The author found that the problems associated with the pre-incubation of tissue did not warrant the addition of this step. These problems included:

1) Bacterial contamination of the incubation medium which was unavoidable without added antibiotics.

2) pH changes in the Trowell's medium due to gas leaks and possible acid secretion by the segments.

Although it may be true that the pre-incubation phase may reduce the background gastrin effect, many of the amino-acids in Trowell's medium are potent stimulators of acid secretion themselves, some independent and others dependent on histamine (Konturek, Tasler et al, 1977). There is thus no reason why the pre-incubation phase may not produce its own background effect. The author found that in practice, unstimulated sections gave very constant readings of background carbonic anhydrase activity. The coefficient of variation for these readings was 3.2% which was highly acceptable. This low variability appeared to depend on two factors: firstly the animal had to be starved for a minimum of 12 hours prior to sacrifice and secondly, the time from sacrifice to freezing of the tissue had to be kept to a minimum with as little trauma to the tissues as possible. Furthermore, the omission of the preincubation phase allowed up to four staining 'runs' to be done per day, whereas the preincubation phase limited this to one or possibly two per day. It was thus felt that the disadvantages of the preincubation phase outweighed the advantages and this step was omitted.

The reason for freezing the tissue segments in hexane rather than in liquid nitrogen is discussed by Chayen, Bitensky and Butcher (1973). The rate of cooling of the tissue in hexane is twice as fast as when chilling in liquid nitrogen. As such, fewer temperature gradients are set up within the tissue and ice formation, which may distort or damage the tissue, does not occur. This has been shown by Lynch, Bitensky and Chayen (1966), using a thermocouple in the tissue; by Silcox, Poulter et al (1965)

following microscopic examination for the ice crystals or damage produced by them; using dark ground illumination to investigate denaturation-aggregation of the protoplasma and lipid protein associations (Chayen, 1968a); or studies of the permeability of subcellular membranes (Chayen, 1968b).

Mounting of the tissue before sectioning was done in ice, as the use of synthetic mounting materials prevented the secretagogue reaction from occurring. This possibly occurred by interfering with the receptor-binding sites on the cells.

The sections were cut in the cryostat to a thickness of 18 µm. This thickness was chosen so as to contain not more than one parietal cell. A parietal cell varies in diameter from 12 to 17 µm. (Davenport, 1977). In addition Loveridge (1978), showed that the extinction increased linearly with thickness over the range 10 to 22 µm.

The removal of the sections from the cryostat knife is the method described by Chayen, Bitensky and Butcher (1973). Using this method the section moves across a temperature gradient from the knife (-70°C) to the slides (room temperature). There is no need to press the slides against the section and the section remains frozen in the low ambient temperature of the cryostat chamber (-20°C.) and can be stored there until use.

The pH of the buffer in which the gastrin was diluted was 7.0. Loveridge, Bloom et al, (1974) found an increased basal level of CA activity when the segments were maintained at a higher pH.

This was confirmed by the author (Fig. 8:6), although differences noted at higher pH were far less than those reported by Loveridge, Bloom et al (1974). Thus it appeared that the pH 7.0 gave the lowest and most consistent basal staining.

Initially there were two major problems encountered during the development of the staining method.

(a) Staining was faint and non-specific.

(b) The sections washed off the slides and were lost during the staining procedure.

(a): For some time during the early development of the method it was found that the sections stained lightly and that the staining was non-specific. In many cases the muscle layer stained darker than the mucosa. Lonnerholm (1975) found in staining for CA that the tissue had to float on top of the reaction medium. This appeared to be critical as sections which dipped below the surface of the medium remained unstained. He proposed that the presence of staining reagent above the section prevented the free loss of CO_2 which is a rate-limiting factor in this reaction. As it was impossible with the cytochemical bioassay to float the sections on the reaction medium it was decided to reduce the volume of the reaction medium so that the tissues would be covered by a film of liquid of approximately 1mm in depth. Using this method specific staining was obtained. Thus the total volume of 57mls of staining reagent was critical for the staining trough that was used. This finding was subsequently confirmed by Loveridge (1978), who showed decreased staining with increased depth of reaction medium.

(b): The second major problem was that the sections did not stick to the slides and were washed off during the staining procedure. It was noticed that during the staining reaction small bubbles appeared on the sections in the effervescent medium. It was suggested that these bubbles of effervescence may have had the effect of lifting the sections off the slides. De-bubbling the reaction medium by vacuum pump for a few seconds prior to use appeared to solve this problem.

The reaction medium was modified by Loveridge (1978) from that of Hansson (1967) and Lonnerholm (1975). Both these latter workers used a non-buffered reaction medium. However the evolution of CO_2 in the reaction medium (due to the reaction between sulphuric acid and bicarbonate) causes a change in pH in the medium. Loveridge (1978) found that the pH rose rapidly to over 8 over the first two minutes after mixing solutions A and B. In an attempt to slow this excessive change in pH, the reaction medium was buffered by Hepes pH 7.4. This was chosen because it had been shown that it did not affect the biochemical characteristics of two of the carbonic anhydrase isoenzymes (Christiansen and Magid, 1970). Loveridge showed that the buffered reaction medium markedly slowed the pH change and the pH remained between 7.0 and 7.4 for the duration of the staining run.

Loveridge (1978) demonstrated a linear CA reaction for up to 4 to 6 minutes. Shortening the reaction time to two minutes minimized the time related pH change in the medium but allowed sufficient discrimination between levels of enzyme activity at each gastrin concentration. Other modifications to the constituents of the reaction medium proposed by (Loveridge (1978) and followed

by the author, included the use of phosphate at concentration 1.2 mmol/l, which appeared to aid in the activation of the enzymes.

Phosphates at higher concentrations caused an increased non-specific staining, possibly due to a decrease in the solubility of the reaction product. In addition, the concentration of cobalt in the medium was increased to 10mmol/l, as when the buffer was included in the reaction medium there was competition with the cobalt for the hydroxyl ions. Furthermore, gum tragacanth was included in solution B as it was found to retard the precipitation of uncatalysed reaction products. (Loveridge, 1978).

For several cytochemical staining techniques the solubility of the enzyme makes it obligatory for a colloid stabilizer such as polyvinyl alcohol or a collagen preparation designated polypeptide 5115 to be used in the reaction medium (Chayen, Daly et al, 1976). Loveridge has shown that CA is tissue-bound under the conditions of the assay and thus the reaction medium does not require these additives. This is in spite of the report by Narumi and Kanno (1973), who showed that CA activity occurred mainly in the cytosol fractions of gastric mucosal homogenates.

The density of the staining was quantitated by the Vickers micro-densitometer. As the density of stain is an indication of reaction products and as the reaction was in each case allowed to proceed under similar conditions of temperature, time and pH, the density of the stain reflected CA activity.

While it is beyond the scope of this work to discuss the theory of microdensitometry, the microdensitometer is basically an instrument in which a microscopically enlarged image of a cell is scanned by a slit and the light from the slit is passed sequentially on to a photomultiplier. The size of the slit is sufficiently small that no optical inhomogeneity occurs within it; the photomultiplier is thereby confronted with a large number of single optically homogeneous fields, and the responses for each slit field are integrated to give the mean integrated extinction. This makes it possible to measure precisely the density of reaction products within the cells, even though the product is not distributed homogeneously. The scanning and integration occurs within about 5 seconds. The wavelength of 550nM was used for readings, as at this wavelength there was maximal discrimination between the cells and the background readings. This is shown in Fig. 8:7 where the readings of a single cell and muscle field are plotted at varying wavelengths. This wavelength was used for all studies.

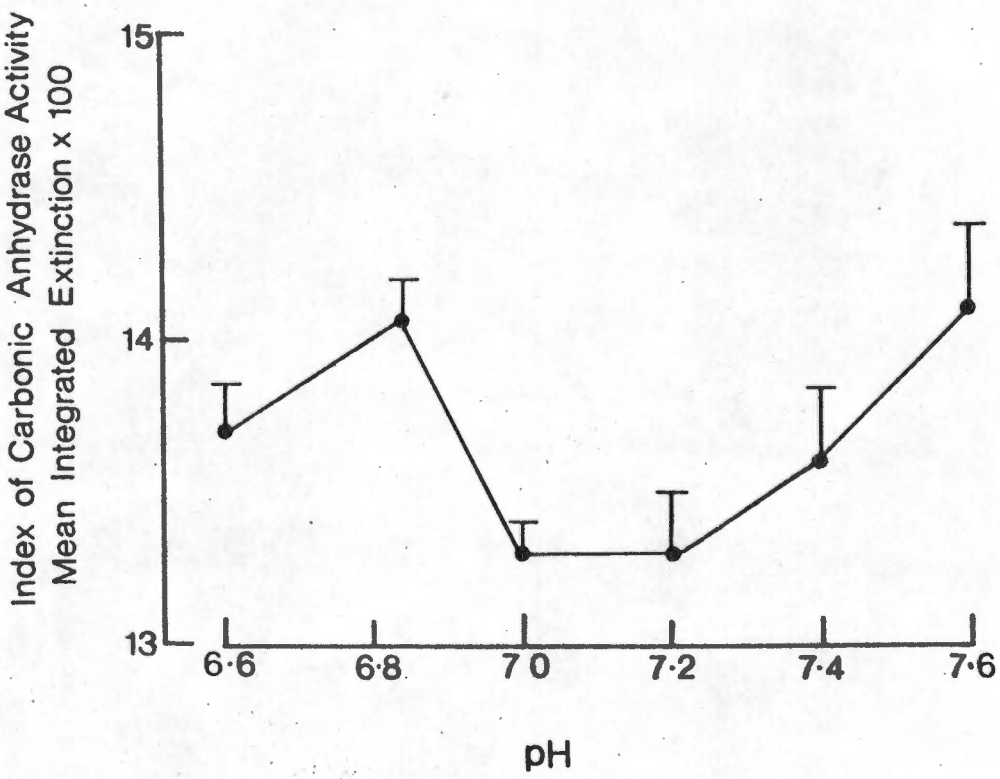


FIG 8:6: Basal Carbonic Anhydrase Activity with a Variation in the pH of the Hepes Buffer. Each point indicates the mean $\pm$ SEM.

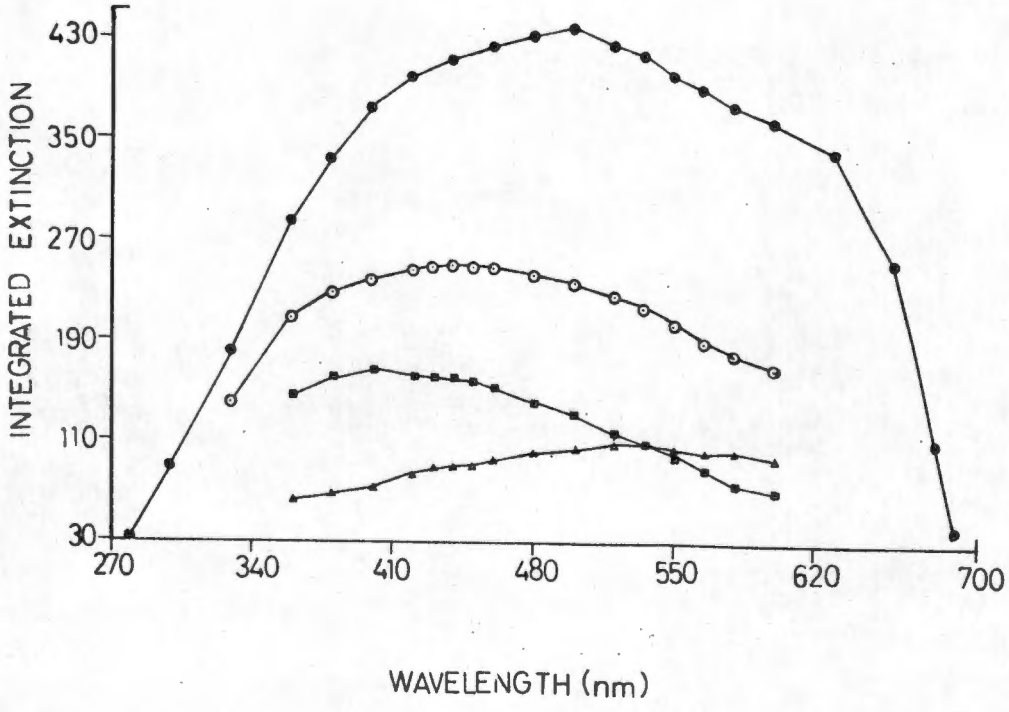


FIG. 8:7: Readings at varying wavelengths of a single cell (open circles) and a single muscle field (squares) stained for C.A. activity. The cell/muscle difference (triangles), is maximal at about 550mm. The closed circles designate readings taken of the standard filter.

8:6: Validation of the Reaction:

Having described the method, certain of the technical problems associated with development and the steps taken to ensure optimum conditions for the technique, we now turn to the validation of the technique. As has been mentioned, the mean integrated extinction is being regarded as an index of CA activity. The question arises whether this is in fact so. Perhaps the best method of answering this question may have been to establish a parallel system to measure CA by an alternative chemical method. Unfortunately this was not possible without altering the basic model to such an extent that the two methods would no longer be considered strictly comparable. Therefore, for the purposes of validating the enzyme reaction, the tissue was boiled to denature the enzyme and the specific CA inhibitor, acetazolamide, was used. Lastly, as acid secretion is inhibited by anoxia (Hersey and High, 1972), the effect of anoxia on CA activation was examined.

8:6:1: Enzyme Denaturation:

In the first study, sections of fundus were placed in boiling water for 5 minutes. This will denature most large proteins and enzyme activity is usually destroyed by this procedure. The segments were then snap-frozen, sectioned and treated with gastrin in the usual way. The results are shown in Fig. 8.8. After boiling the tissue, gastrin no longer caused an increase in the integrated extinction. It is of note that staining of the tissue still occurred with a basal mean integrated extinction $\times$ 100 of 12.5. Thus, in spite of the absence of enzyme activity, there is a basal non-specific reaction occurring, which accounts for more than 50% of the maximum integrated extinction achieved after full gastrin stimulation.

8: 6: 2: Carbonic Anhydrase Inhibition:

In the second study, acetazolamide (10^{-5}M) was added to the sections with each dose of gastrin. Again there was complete inhibition of the gastrin response (Fig. 8:8). The basal, uncatalysed reaction again accounted for more than 50% of the peak percentage integrated extinction induced by the maximum dose of gastrin.

8:6:3: The Effect of Anoxia on Carbonic Anhydrase Activation

Acid secretion is associated with an increase in oxygen utilization by the parietal cell. Furthermore, in the presence of anoxia, acid secretion in isolated amphibian mucosa is inhibited (Hersey and High, 1972).

In order to examine the effect of anoxia in the system, tissue was treated with gastrin in the usual manner, while under a nitrogen atmosphere. Secondly, the tissue was kept in a nitrogen atmosphere for ten minutes and then treated with gastrin five minutes after removal of the nitrogen. The gastrin effect was almost entirely inhibited by the presence of anoxia. (Fig. 8:8). In the tissue that was allowed to recover after ten minutes of anoxia, there was partial but incomplete recovery of the gastrin effect.

8:6:4: Discussion:

It thus appears that the staining reaction consists of two, approximately equal parts. Firstly a basal uncatalysed reaction which accounts for 50-60% of the maximum reaction achieved and secondly, a reaction which is inhibited by boiling and by a CA inhibitor. Thus, by implication, the second part of this reaction is CA mediated.

The question arises why the uncatalysed reaction seems to predominate. The answer probably lies in the staining method. In Hansson's method for cytochemical localization of CA, the reagents were made up in distilled water and were not buffered. The use of buffer made it necessary to increase the concentration of cobalt in the reaction medium six-fold, as the buffer competes with the cobalt to trap the hydroxyl ions produced in the reaction (Loveridge, 1978). This increase in cobalt, coupled with the de-bubbling of the reaction of the reaction medium prior to use which accelerates the uncatalysed reaction, results in a high non-specific staining. However, as the secretagogue-induced increase in CA activity could always be clearly detected over and above the non-specific staining this was acceptable. In addition the non-specific staining remained approximately constant between experiments and did not materially alter sensitivity or reproducibility of the method.

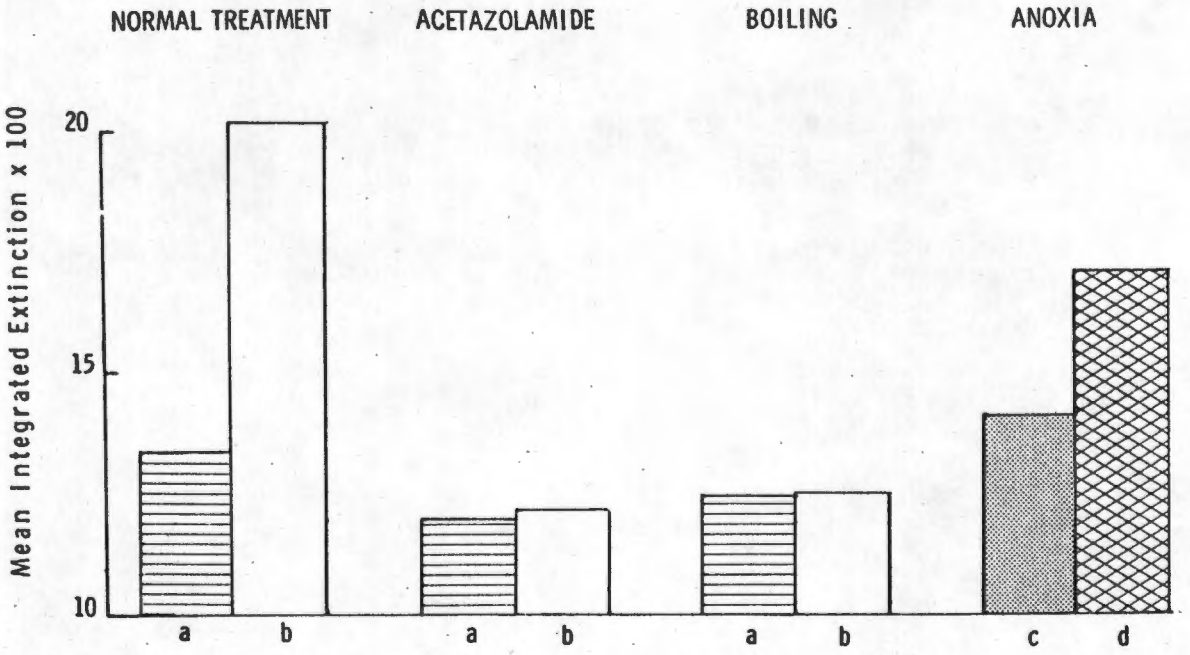


FIG. 8:8: Effect of acetazolamide (10^{-5}M), boiling the tissue and anoxia on gastrin-stimulated CA activity.

a= basal

b= Gastrin (2.5×10^{-12}mol/l)

c= Gastrin (2.5×10^{-12}mol/l) in nitrogen atmosphere

d= Gastrin (2.5×10^{-12}mol/l) after removal from nitrogen.

CHAPTER 9:

THE EFFECT OF THE ACID SECRETAGOGUES ON CARBONIC ANHYDRASE ACTIVITY

9: 1: INTRODUCTION:

Under physiological conditions carbonic anhydrase (CA) appears to be necessary for the maintenance of neutral intracellular pH in the parietal mucosa of the stomach (O'Brien, Rosen et al, 1977, Davenport, 1977). Through the action of this enzyme system, CO_2 is hydroxylated to HCO_3^- and H^+ (Maren, 1967). The bicarbonate ions are exchanged for Cl^- at the nutrient membrane of the cell, accounting for the 'alkaline tide' of acid secretion (Davenport 1977), while the H^+ ions replenish the intracellular H^+ pool (Fig. 6:1). Thus under physiological conditions CA activity may be regarded as an index of parietal cell function.

Acid secretion is mediated by hormonal, paracrine, neurocrine and luminal factors which include the three major acid secretagogues histamine, acetylcholine and gastrin. (Soll and Grossman, 1978). The aim of this section of the study was to demonstrate the effects of these secretagogues on CA activity in guinea pig parietal mucosal sections quantitated by the method described above.

9:2: Gastrin:

9:2:1: Time Course of Gastrin-stimulated CA Activation:

9:2:1:1: Method:

To demonstrate the optimal time exposure of gastrin to stimulate CA activity, the sections were treated with three concentrations of gastrin (2.5×10^{-14}, 2.5×10^{-13} and 2.5×10^{-12} mol/l) for 30, 60, 90, 120, 150, 180 and 240 seconds. At the end of these time periods the reaction was terminated by the addition of the staining reagents.

9:2:1:2: Results:

Gastrin caused a mean peak activation of CA 90 seconds after treatment at each concentration tested. (Fig. 9:1). Following the peak at 90 seconds there was a rapid fall in CA activity. With gastrin concentrations 2.5×10^{-12} mol/l this fall was significant at 120 seconds ($p < 0.01$) while with gastrin concentrations 2.5×10^{-13} mol/l, the fall was significant at 150 seconds ($p < 0.001$). The activity then returned to basal levels.

In view of the finding of peak CA activity occurring at 90 seconds, this stimulation time was used for subsequent studies.

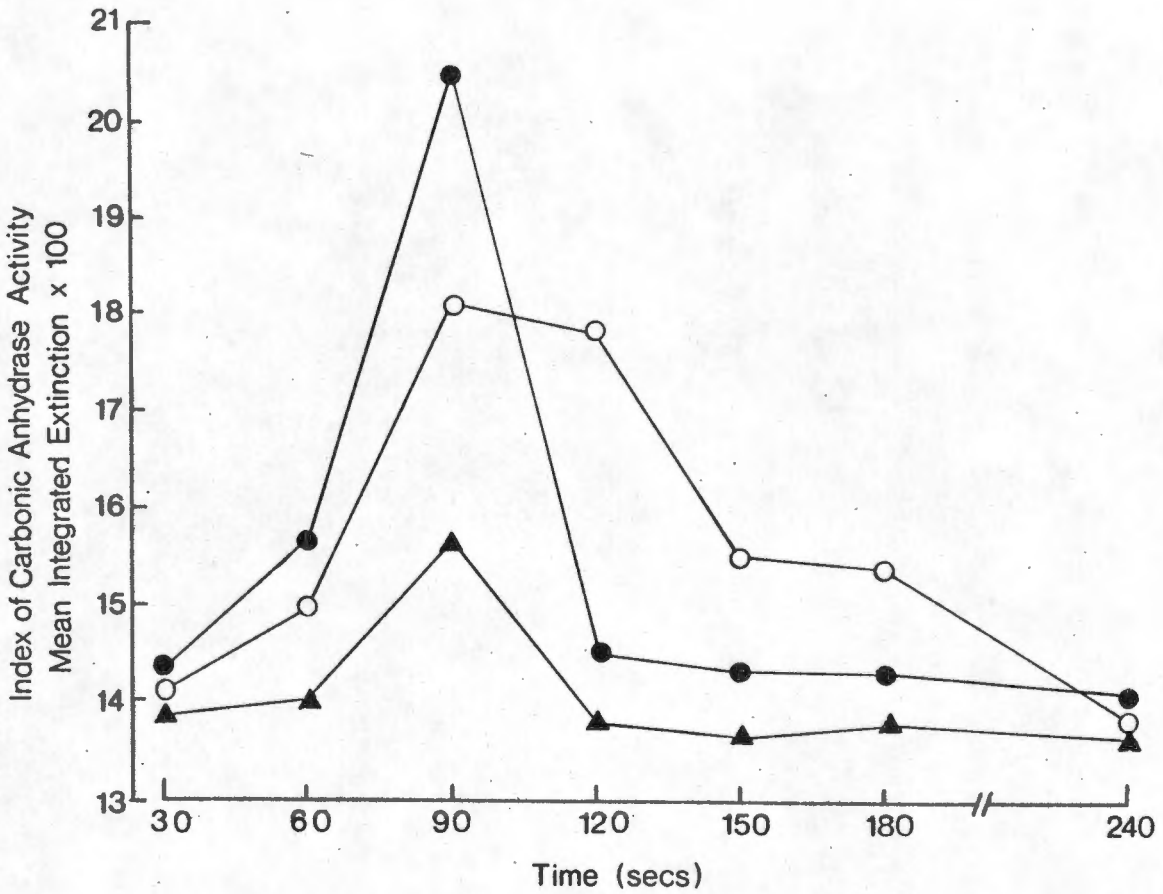


Fig. 9:1: Time Course of Activation Following Exposure of Parietal Mucosa to SHG-17-I .

▲ 2.5×10^{-14}M

o 2.5×10^{-13}M

● 2.5×10^{-12}M

9:2:2: The Dose Response Effect of Gastrin on CA Activity.

9:2:2:1: Method:

The effects of gastrin 2.5×10^{-16} to 2.5×10^{-10} mol/l on CA activity was tested after a gastrin exposure time of 90 seconds.

The results were plotted relating the mean integrated extinction to the log dose of gastrin. Gastrin was tested in 10 experiments (9 animals) and a regression line, calculated by the least mean squares methods (Appendix 2), was fitted to the data on the linear portion of each dose response curve.

The mean basal activity was calculated by measuring the mean extinction of control sections treated with buffer alone during the same experiments. The point of intersection of the linear regression lines with the mean basal CA activity was designated the minimum effective dose (D_{min}). Furthermore, the D_{50} (the dose causing 50% of the maximal effect) was calculated from each regression line.

9:2:2:2: Results:

Fig. 9:2 shows the mean dose response effects of gastrin (2.5×10^{-16} to 2.5×10^{-10} mol/l) on CA activity in 10 consecutive experiments. The linear portion of the dose response curve was between 2.5×10^{-15} and 2.5×10^{-12} mol/l. The calculated D_{min} was $2.3 \pm 0.2 \times 10^{-15}$ mol/l (mean $\pm$ sem) while the D_{50} was $78 \pm 3.7 \times 10^{-15}$ mol/l. The mean slope of the 10 curves was 2.35 ± 0.06 and the coefficient of variation of the slopes was 8.7%. The data of the regression equations of the indivisual experiments is shown in Table 9:1.

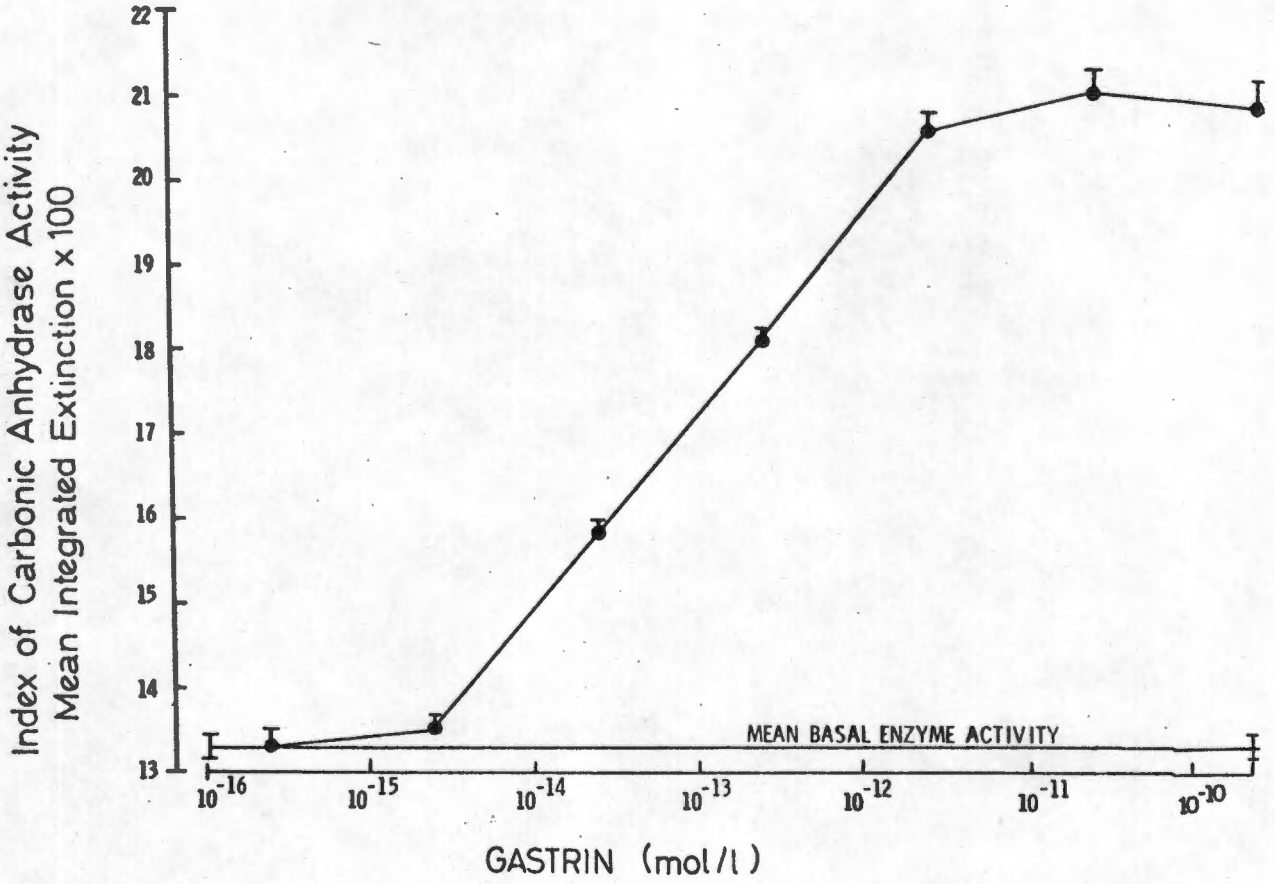


Fig. 9:2: Mean Dose Response Curve of Gastrin in 10 Consecutive Experiments. Points represent mean ± SEM

TABLE 9:1

THE REGRESSION EQUATIONS OF THE LINEAR SECTIONS OF THE INDIVIDUAL GASTRIN DOSE RESPONSE CURVES.

EXPERIMENT	NO	n	EQUATION (f(y))	CORR.COEFFICIENT (r)	CALCULATED D_{50}	CALCULATED D_{min}
Gastrin:					moles/l x 10^{-15}	moles/l x 10^{-15}
(2.5×10^{-15} - 2.5×10^{-12}M	1	10	2.36 x +13.0	0.9864	85	3.4
	2	8	2.24 x +13.2	0.9825	85	2.7
	3	12	2.37 x +13.3	0.9925	80	2.5
	4	9	2.25 x +13.4	0.9926	85	2.3
	(5)	8	2.37 x +13.2	0.9849	95	3.0
	(6)	10	2.17 x +13.6	0.9839	70	1.9
	7	11	2.72 x +13.4	0.9868	80	2.3
	8	10	2.41 x +13.7	0.9799	65	1.7
	9	11	1.98 x +14.1	0.9839	55	1.0
	10	10	2.58 x +13.5	0.9949	80	2.1
Mean			2.35		78	
Sem			0.06		37	±0.2

All r values were significant $p < 0.01$
n = no. of points in the regression equation
D_{50} = dose causing 50% of maximal effect
D_{min} = calculated minimum dose

The numbers bracketed are those in which two experiments were done on the tissues of a single animal. For the purpose of calculating the regression equations 2.5×10^{-15}M was deemed to be on the Y axis and equal 0; 2.5×10^{-14}M = 1, etc.

9:3 The Time Course and Dose Response of Histamine-Stimulated CA Activity.

9:3:1: Method:

The method of evaluating the time course of histamine-stimulated CA activity was similar to that described for gastrin. Histamine time course was studied in the concentrations 1.0×10^{-14} and 1.0×10^{-15} mol/l, whilst the histamine dose response was tested over the concentrations 1.0×10^{-17} to 1.0×10^{-12} mol/l in 12 consecutive experiments (9 animals). The methods of calculating the D_{min} and D_{50} are described above.

9:3:2: Results:

The time course of CA activation following exposure to histamine is shown in Fig. 9:3. In contrast to gastrin, the increase in CA activity following exposure to histamine 1.0×10^{-14} mol/l was rapid and was significantly higher than the basal values at 30 seconds ($p = 0.001$). The activity then rose slightly but insignificantly, to a peak at 90 seconds and was still significantly above basal values at 150 seconds ($p < 0.001$). By 180 seconds the CA activity had fallen significantly from the peak ($p < 0.001$) to near basal levels. Histamine 1.0×10^{-15} mol/l had a similarly patterned time course, but the maximal activation was lower.

The dose response curve for histamine (1.0×10^{-17} to 1.0×10^{-12} mol/l) is shown in Fig. 9:4. The maximal effects above basal of histamine on CA activity is 62% that of gastrin. The doses 1.0×10^{-16} to 1.0×10^{-14} mmol/l comprised the linear portion of the dose response curve.

The mean D_{min} was $5.8 \pm 0.9 \times 10^{-17}$ mol/l (mean $\pm$ sem), while the mean D_{50} was $76.8 \pm 7.6 \times 10^{-17}$ mol/l. The mean slope of the 12 regression lines was 1.84 ± 0.07 with a coefficient of variation of 13.6%. This differed significantly from the mean slope of the gastrin curves ($p < 0.01$). The regression equations from the individual experiments is shown in Table 9.2.

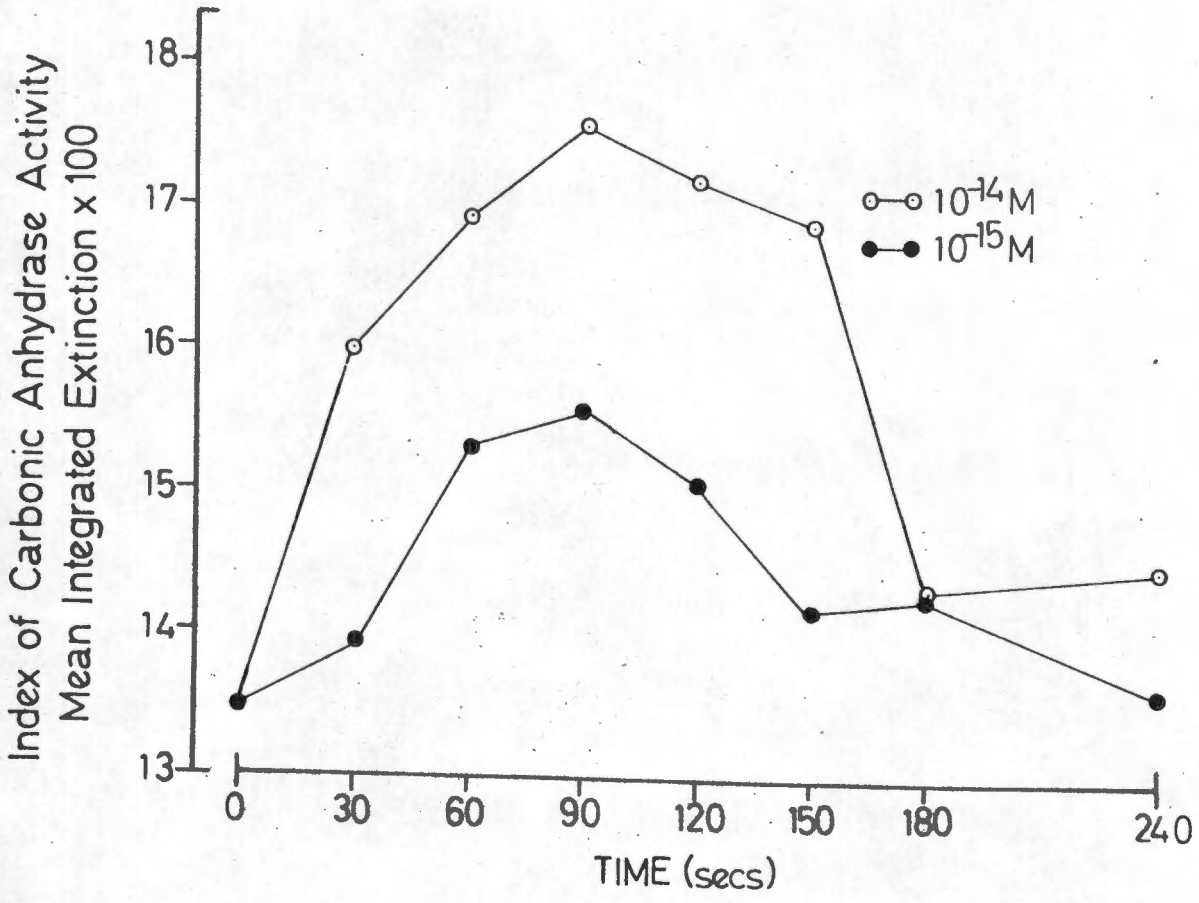


FIG. 9:3: Time Course of CA Activation following Exposure of Parietal Mucosal Sections to Histamine.

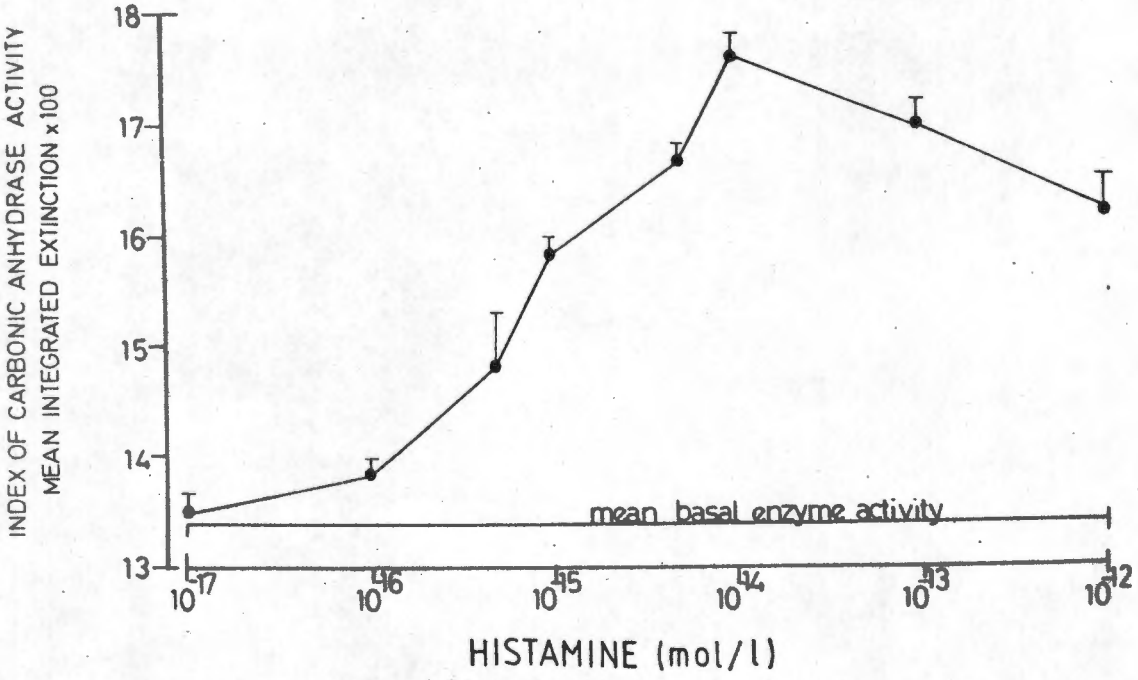


FIG. 9:4: The Effect of Histamine on Carbonic Anhydrase Activity. Each point indicates the mean $\pm$ sem of 12 pooled experiments.

TABLE 9:2

THE REGRESSION EQUATION OF THE LINEAR SECTIONS OF OF THE INDIVIDUAL HISTAMINE DOSE RESPONSE CURVES.

EXPERIMENT	NO	n	EQUATION (f(y))	CORR.COEFFICIENT (r)	CALCULATED D_{50}	CALCULATED D_{min}
Histamine ($1.0x10^{-16}$ -$1.0x10^{-14}$M)					moles/$1x10^{-17}$	moles/$1x10^{-17}$
	1	6	1.95x +13.6	0.8969 *	80	7
	2	8	1.46x +14.9	0.9337 +	27	0.8
	3	6	2.21x +13.8	0.9665 +	80	6
	(4)	8	2.07x +13.7	0.9651 +	80	6.5
	(5)	12	2.08x +14.0	0.8969 +	130	4
	6	14	2.03x +13.1	0.9561 +	95	13
	7	7	2.09x +14.9	0.8140 *	44	2.1
	8	8	1.68x +14.2	0.9527 +	62	3.7
	(9)	7	1.58x +13.7	0.9658 +	68	5
	(10)	7	1.67x +13.5	0.8247 *	100	9
	(11)	11	1.72x +13.7	0.8351 +	78	6.4
	12	11	1.57x +13.9	0.7770 +	78	6.0
Mean			1.84		76.8	5.88
Sem			±0.07		±7.6	±0.9

n = no. of points in each regression line

D_{50} = Dose causing half maximal effect.

D_{min} = Calculated minimal effective dose

The bracketed experiments were conducted on tissue from the same animal

For the purpose of calculating the regression equations 1.0 x 10^{-16}M was deemed to lie on the Y axis and equal 0; 1.0 x 10^{-15}M = 1, etc.

* $p < 0.05$

\+ $p < 0.01$

9:4: The Time Course and Dose Response of Carbamylcholine-Stimulated CA Activity.

9:4:1: Introduction:

The third major physiological stimulant of acid secretion is acetylcholine. It was noted at the onset of these experiments however, that although acetylcholine stimulated CA activity, the response was extremely variable. This was not found to the same extent with the analogue carbamylcholine and it was decided to use the latter in the following experiments. The reason for the variable response to acetylcholine is not known but it is thought to be possibly due to rapid tissue degradation of the substance.

9:4:2: Methods:

The methods are similar to those described for gastrin and histamine. The time course of carbamylcholine-stimulated CA activity was tested at the concentrations 1.0×10^{-9} and 1.0×10^{10} mol/l, whilst the dose response effect was tested at concentrations 1.0×10^{-13} to 1.0×10^{-8} mol/l in 12 experiments (8 animals). D_{min} and D_{50} were calculated as above from the regression equations.

9:4:3: Results:

The time course of CA activity following exposure to carbamylcholine is shown in Fig. 9:5. Like histamine, carbamylcholine caused a significant rise in CA activity by 30 seconds ($p < 0.05$), peaked at 90 seconds and then fell. Carbamylcholine-(1.0×10^{-9} mol/l) stimulated activity was still significantly ($p < 0.01$) above basal at 240 seconds after stimulation.

In contrast carbamylcholine-(1.0×10^{-10} mol/l) stimulated activity had returned to basal at 180 seconds. The dose response curve for carbamylcholine(1.0×10^{-13} to 1.0×10^{-8} mmol/l)is shown in Fig. 9:6. The maximal effect of carbamylcholine above basal is approximately similar to that of histamine and about 60% that of gastrin. The doses 1.0×10^{-12} to 1.0×10^{-9} mol/l comprised the linear portion of the dose response curve. The mean D_{min} was $1.2 \pm 0.3 \times 10^{-12}$ mol/l (Mean $\pm$ sem) and the mean D_{50} was $31.3 \pm 4.3 \times 10^{-12}$ mol/l. The mean slope of the 12 regression lines was 1.33 ± 0.05 with a coefficient of variation of 13%. This differed significantly ($p < 0.001$) from both the gastrin and histamine slopes. The data for the individual experiments is shown in Table 9.3.

Fig. 9:7 compares the dose response curves of histamine, G17 and carbamylcholine. If the potencies of the secretagogues are compared by comparing the D_{50s} histamine is approximately 100 times more potent than gastrin, while carbamylcholine is approximately 400 times less potent than gastrin.

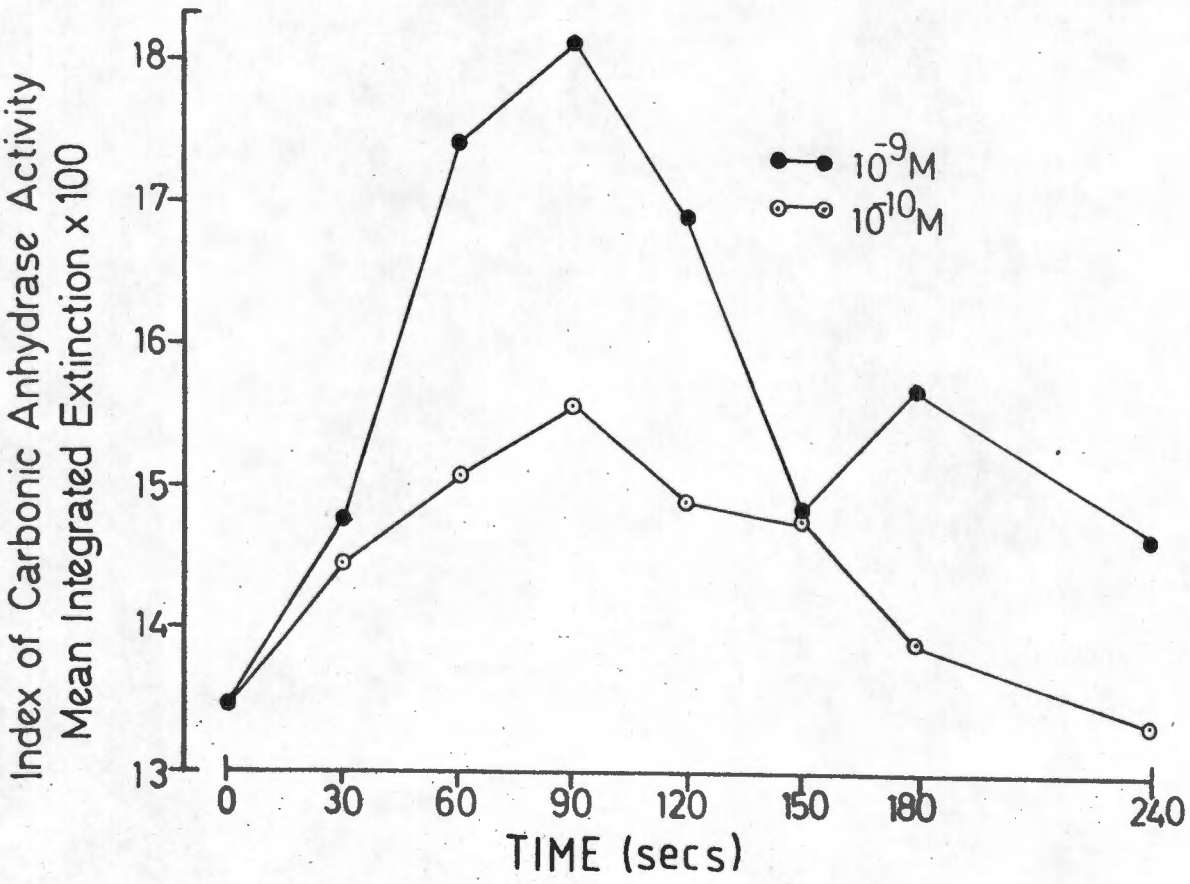


Fig. 9:5: Time Course of CA Activation in Parietal Mucosal Sections following Exposure to Carbamylcholine.

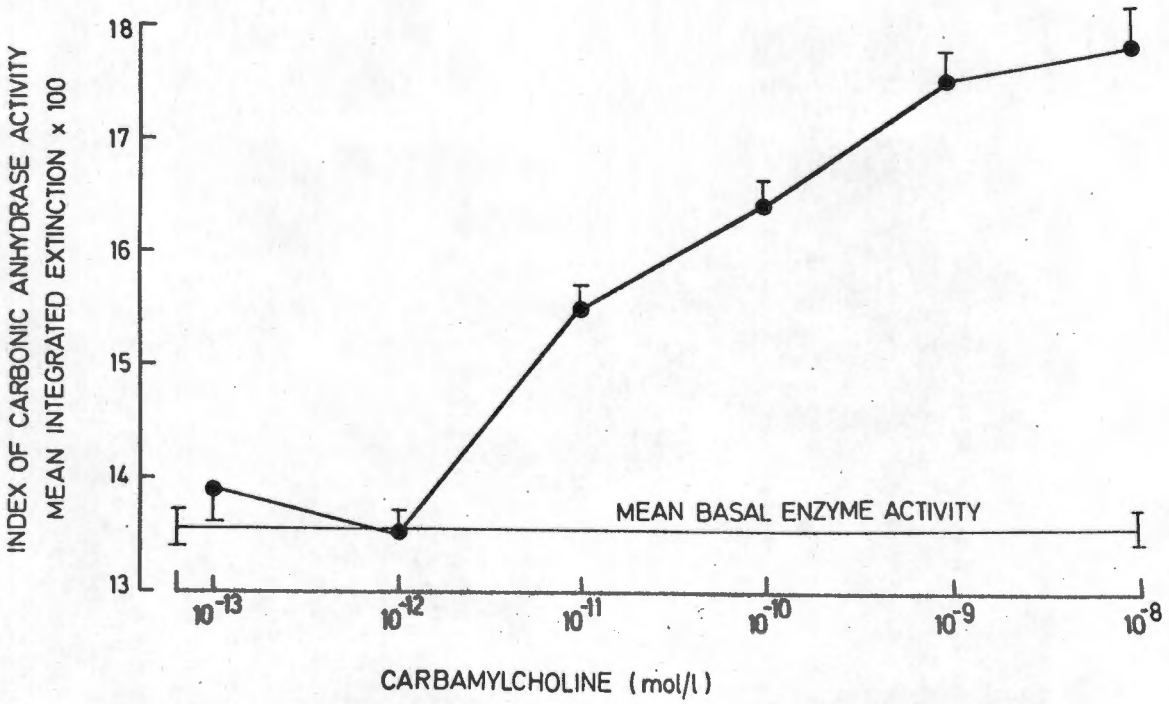


Fig. 9:6: Mean Dose Response Curve of Carbamylcholine-stimulated CA Activity in 12 Consecutive Experiments. Each point represents the mean ± SEM of the pooled data.

TABLE 9:3: THE REGRESSION EQUATIONS OF THE LINEAR SECTIONS OF THE INDIVIDUAL CARBAMYLCHOLINE DOSE RESPONSE CURVES

EXPERIMENT	NO	n	EQUATION (f(y))	CORR. COEFFICIENT (r)	CALCULATED D_{50}	CALCULATED D_{min}
Carbamylcholine					moles/$1x10^{-12}$	moles/$1x10^{-12}$
($1.0x10^{-12}$ - $1.0x10^{-9}$M)	(1)	11	1.19x +14.4	0.9132	13	0.2
	(2)	8	1.12x +13.7	0.8541	18	0.75
	3	12	1.27x +13.3	0.8617	42	1.7
	4	8	1.31x +13.5	0.9777	32	1.0
	5	9	1.06x +14.3	0.8153	15	0.25
	(6)	10	1.51x +12.8	0.9264	56	3.2
	(7)	9	1.32x +13.0	0.8684	54	2.7
	(8)	9	1.46x +13.4	0.8649	25	0.65
	(9)	9	1.60x +13.2	0.8394	38	1.5
	10	10	1.34x +14.3	0.8765	17	0.28
	11	9	1.35x +13.3	0.8394	42	1.7
	12	7	1.48x +13.4	0.9734	23	0.56
Mean			1.33		31.3	1.2
± Sem			±0.05		4.3	0.3

n = No of points in each regression line

D_{50} = Dose causing half maximal effect

D_{min} = Calculated minimal effective dose

The bracketed experiments were calculated on tissue from the same animal

All r values $p < 0.01$

For the purpose of calculating the regression equations, 1.0×10^{-12}M was deemed to lie on the Y axis and equal 0; 1.0×10^{-11}M = 1, etc.

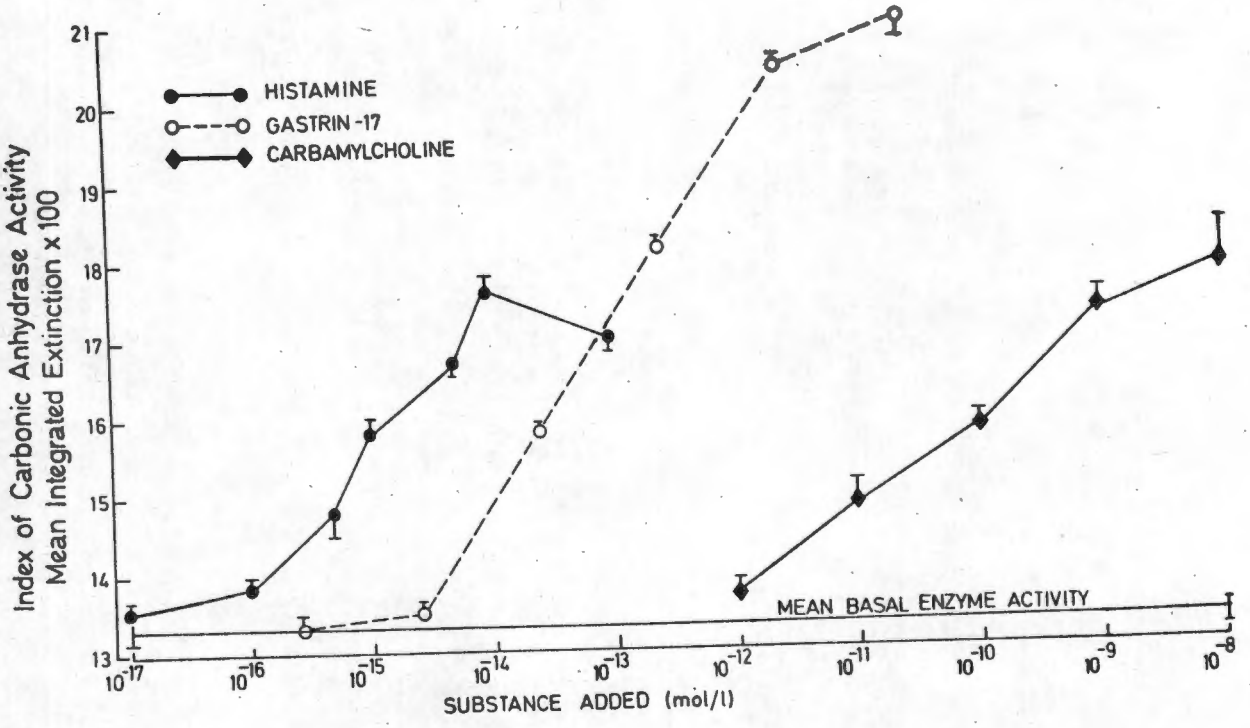


FIG. 9:7: Comparison of the Dose Response Curves of Histamine, G17 and Carbamylcholine.

Histamine is 100 times more potent than gastrin which is 400 times more potent than carbamylcholine.

Each point represents the mean $\pm$ SEM

9:5: Discussion:

In this section activation of CA has been demonstrated in response to all three of the major acid secretagogues, gastrin, histamine and carbamylcholine (acetylcholine) in a dose-dependent manner.

As has been previously discussed, CA may, under physiological conditions, be regarded as an index of parietal cell function. Acid secretion in the presence of CA inhibition continues at a low rate (Maren, 1967), which is equivalent to the rate of formation of H^+ by the non-catalysed hydroxylation of CO_2. This cannot be increased by further histamine stimulation. (Maren, 1967).

All three agents in this system have an effect on CA activity in surprisingly low concentrations. Gastrin affects CA activity in the dose range 2.3×10^{-15} to 2.5×10^{-12} mol/l which is about 10-fold lower than the level of circulating gastrins in normal fasting serum. Thus it could be argued that the carbonic anhydrase activation in this system does not reflect physiological acid secretion as if that were so, the parietal cell would always be under maximal gastrin stimulation. This may be so but on the other hand there is little data on the concentrations of these substances at the cellular receptor level. As peptide degradation occurs in the tissues, the concentration within the serum may not reflect the concentration reaching and activating the cells. In this particular system, the active substances are delivered directly onto the cell sections and this may account for the sensitivity achieved.

The finding that histamine caused a maximal effect which was only 62% that of gastin, may be comparable to the findings of Adashek

and Grossman (1963), who found that maximal acid secretion stimulated by histamine was only 58% that of gastrin in rats. In dogs (Johnson and Grossman, 1969) however, and in man (Brooks, Johnson and Grossman, 1970), histamine and gastrin have a similar maximal effect on acid secretion. Carbamylcholine has approximately the same efficacy as histamine. However the most potent activator of CA is histamine, followed by G17 and lastly, carbamycholine.

The optimum time course of the secretagogue reactions, i.e. 90 seconds, is very short compared to other experimental models. In the cytochemical method for gastrin described by Loveridge, Bloom et al 1974, the optimal time course for CA activation was 5 minutes, while the maximal binding of (^{125}I)-gastrin (Del Mazo and McGuigan, 1976) and (^{3}H)-gastrin to isolated parietal cells (Soumarmon, Cheret and Lewin, 1977) occurs about 30 to 40 minutes after exposure. Once again the short activation time in the present study may be partly due to placing the secretagogue directly onto the surface of these sections. With histamine and carbamylcholine the activation time was even shorter, although maximal activation also occurred at 90 seconds. Forte, Machen and Forte (1977), showed that 30 seconds after the addition of histamine to isolated piglet mucosa, there was a change in the intracellular potential difference. This is thus comparable with the present study. They found that the earliest morphological changes attributable to acid secretion appeared at 3 minutes, while acid secretion per se was detectable at 5 - 10 minutes. This is about similar to the delay in acid secretion in vivo after the intravenous injection of histamine (Brooks , Johnson and Grossman 1970; Grossman and Konturek, 1974).

Furthermore, Domschke, Domschke et al (1973), found that cyclic AMP in the isolated perfused rat stomach doubled within 60 seconds of histamine administration. Acid secretion occurred only after a lag period of 4 minutes. CA may therefore be activated early after stimulation of the cell and the short time course noted may correspond to the onset of intracellular acid production rather than to the intraluminal acid secretion. Although there are no data similar to that of Forte, Machen and Forte (1977) regarding gastrin-stimulated acid secretion, heptadecapeptide gastrin, when injected intravenously into humans also caused acid secretion within about 10 minutes (Walsh, Isenberg et al (1976). Thus it appears that histamine and gastrin have approximately similar time courses, both in acid secretion in vivo and in the maximal activation of CA in vitro.

The fall in CA activation after 90 seconds noted with all three agonists cannot be easily explained. It is possible that this reflects an auto-regulatory phenomenon, either within the cell or at the respective receptors. An inhibitory action of large doses of gastrin (Prugh, Schorr et al, 1975) or gastrin extract (Gillespie and Grossman, 1964) has been previously described. Prugh, Schorr et al, postulated two orders of receptors for gastrin on the parietal cell - a high affinity stimulatory receptor and a low affinity inhibitory receptor. Furthermore, an autoregulatory mechanism for gastrin has been proposed in another experimental model. Morgan, Schmalz et al, (1978) have shown that heptadecapeptide gastrin increases the amplitude and frequency of spontaneous action potentials in canine smooth muscle.

With increasing doses of gastrin there was apparent fall in the amplitude from the peak achieved at approximately 10^{-9}M. These workers suggested that the bell-shaped dose response curve may indicate that gastrin and pentagastrin were autoregulatory. However these conclusions were purely speculative.

Likewise the total acid secretory response to very large doses of histamine may be less than the total acid output obtained with lower doses, (Marks, Komarov and Shay, 1960). Furthermore when histamine is infused into dogs for a prolonged period, acid secretion tends to fall (Johnson and Grossman, 1969). This may be due to a desensitization effect of the histamine receptor in the presence of large doses of histamine, but could also reflect an autoregulatory mechanism. However, whether this is related to the fall in CA activity which occurred after peak histamine stimulation is speculative.

Much of this discussion has thus proved to be speculative and perhaps it can be rightly argued that at present the physiological relationship of CA activation in this model and quantitative acid secretion by the parietal cell is at best, obscure. However, in view of the known physiological role of CA, in view of the time course of activation which closely reflects the time course for acid stimulation and in view of the activation of the enzyme system by specific acid secretagogues acting via independent mechanisms, which will be discussed in the following chapter, it is probable that this relationship does exist.

It is possible for example, that the model reflects an early event occurring at the initiation of activation of the parietal cell mechanisms which eventually leads to acid secretion. Whatever this relationship is, however, this technique appears to provide a valid model for secretagogue-parietal cell interaction to study secretagogue actions, interactions and biological secretagogue potencies.

CHAPTER 10

THE INTERACTION BETWEEN THE ACID SECRETAGOGUES

10:1: Introduction:

In 1938 MacIntosh proposed that the liberation of histamine in gastric mucosa might mediate the secretagogue action of vagal stimulation while in 1956, Code contended that histamine was the final common chemostimulator of acid secretion. According to this hypothesis, histamine is the agent which stimulates gastric acid secretion. Gastrin and cholinergic agents stimulate gastric acid secretion solely by increasing the availability of histamine. This hypothesis was supported by Black, Duncan et al (1972), who synthesized the first histamine H_2-receptor blocker, burimamide and its successors metiamide and cimetidine (Brimblecombe, Duncan et al, 1978). These agents caused a reduction in both basal (by about 50-90%) (Sewing, Hagie et al, 1978; Richardson, 1978) and stimulated (80-95%) acid secretion (Sewing, Hagie et al, 1978 ; Richardson, 1978; Grossman and Konturek, 1974; Richardson, Bailey et al, 1975).

Grossman (1975) suggested that there are at least three stimulatory receptors in the parietal cell membrane (histamine, gastrin and acetylcholine); and that even in the resting state some of these receptors are occupied by their respective agonists; and that each agent permissively allows the full action of the others while conversely, blockade of the receptors for any one of these agents will depress the response to the other stimulants.

Soll (1978a, b, 1980a) found that histamine, carbamylcholine and gastrin could independently cause an increase in oxygen uptake and (^{14}C)-aminopyrine accumlation in isolated canine parietal cells.

Histamine potentiated the effect of the other two agents, while gastrin alone had a small effect only in large concentrations (10^{-7}M) and only in the presence of a background of the phosphodiesterase inhibitor isomethylbutylxanthine.

As has been demonstrated above, carbonic anhydrase (CA) activity in guinea pig parietal cells increases with exposure to all three of these stimulants. In this section experiments will be described aimed at demonstrating the interrelationships between these agents in the stimulation of carbonic anhydrase activity. In addition the effects of the histamine H_2-receptor blocker cimetidine and the anti-cholinergic agent atropine on secretagogue-stimulated CA activity will be shown.

Lastly, since Soll (1979) has postulated that histamine acts via a cyclic-AMP mechanism, whereas acetylcholine acts via calcium transfer, the effects of lanthanum chloride on secretagogue action will be shown, to confirm the independent modes of action of these agents. Lanthanum chloride displaces calcium from cell membranes and stops calcium fluxes across membranes (Hellman, Sehlin and Taljedal, 1976) and thus effectively blocks activation dependent upon extracellular calcium.

10:2 Methods:

10:2:1: Secretagogue Interaction

In order to investigate whether a potentiating relationship existed between the secretagogues, a dose of each agent which was below the calculated minimum dose (Chapter 8) and which thus had no significant effect on carbonic anhydrase activity itself was added to each dose in the dose response curves of the other agents.

The sub-threshold doses were: gastrin 2.5×10^{-16} mol/l, histamine 1.0×10^{-17} mol/l and carbamylcholine 1.0×10^{-13} mol/l. The time exposure was 90 seconds for all experiments as this coincided with the peak activation induced by all three substances. Dose response curves were plotted as previously described and the mean D_{min}, D_{50} and slopes were compared to those of the agonist alone.

10:2:2: Secretagogue Inhibition

To test the effects of an histamine H_2-receptor blocker, cimetidine 10^{-5}M was added to gastrin (2.5×10^{-15} to 2.5×10^{-12} mol/l), to histamine (1.0×10^{-16} to 1.0×10^{-14} mol/l) and to carbamylcholine (1.0×10^{-9} to 1.0×10^{-12} mol/l). This large concentration of cimetidine was chosen with the aim of blocking all H_2-receptors in the tissue. To ensure that cimetidine did not alter CA activity independently of gastrin and histamine, cimetidine (10^{-5}M) was also added to the tissues alone and CA activity measured. The anti-cholinergic agent atropine (10^{-5}M) was similarly added to the tissues in the absence and presence of each concentration of gastrin, histamine and carbamylcholine and the tissue processed in the routine manner. Lastly, lanthanum chloride (10^{-6}M) was added to the tissues in the presence of each concentration of secretagogue,

The maximal rise above basal enzyme activity stimulated by the agent alone was considered 100% and inhibition was calculated from the reduction of this rise caused by the inhibitory agent.

Differences were compared by the Student's T test for unpaired values. (Appendix 2).

10:3 Results

10:3:1: The Interaction Between the Secretagogues:

The effects on CA activity of adding a subthreshold dose of histamine (1.0×10^{-17}M) to gastrin (2.5×10^{-15} to 2.5×10^{-11}) (four experiments) compared with the effects of gastrin alone are shown in Fig. 10.1. The mean integrated extinction of the two curves differ significantly at gastrin concentrations 2.5×10^{-15} and 2.5×10^{-14} mol/l ($p < 0.01$) and at 2.5×10^{-13} and 2.5×10^{-12} mol/l ($p < 0.05$). There was no significant difference at the highest concentration of gastrin (2.5×10^{-11} mol/l).

The regression equations of the individual dose curves of gastrin with subthreshold histamine are shown in Table 10.1. The mean ($\pm$ SEM) slope is 2.12 ± 0.06 and the coefficient of variation of the slope is 6%. The added histamine caused a significant reduction in both the D_{min} and the D_{50} of the gastrin dose responses and a significant alteration in the slopes of the regression lines. (Table 10.2).

Similarly, the addition of gastrin (2.5×10^{-16} mol/l to the histamine dose response curve (1.0×10^{-17} to 1.0×10^{-13} mol/l) (four experiments) caused a significant reduction in the D_{50} and D_{min} for histamine (Table 10.2). Fig. 10.2 shows that the subthreshold dose of gastrin significantly increased the mean integrated extinction, at all concentrations of added histamine. The data of the individual dose response curves of histamine with subthreshold gastrin is shown in Table 10.1. The mean slope is 1.12 ± 0.13 with a coefficient of variant of 22%. This differed significantly from the mean of the histamine slopes (Table 10.2).

In contrast neither the addition of subthreshold concentrations of gastrin nor subthreshold histamine to the carbamylcholine dose response curves significantly altered the D_{50} or D_{min} of carbamylcholine (Fig. 10.3). (Table 10.2). Likewise, carbamylcholine did not significantly alter the histamine dose response curve (Fig. 10.4) but did slightly reduce the maximal effect achieved by gastrin alone (Fig. 10.5). This occurred without significantly altering the D_{min} or D_{50} of the gastrin response (Table 10.2). The data from the individual experiments is again shown in Table 10.1.

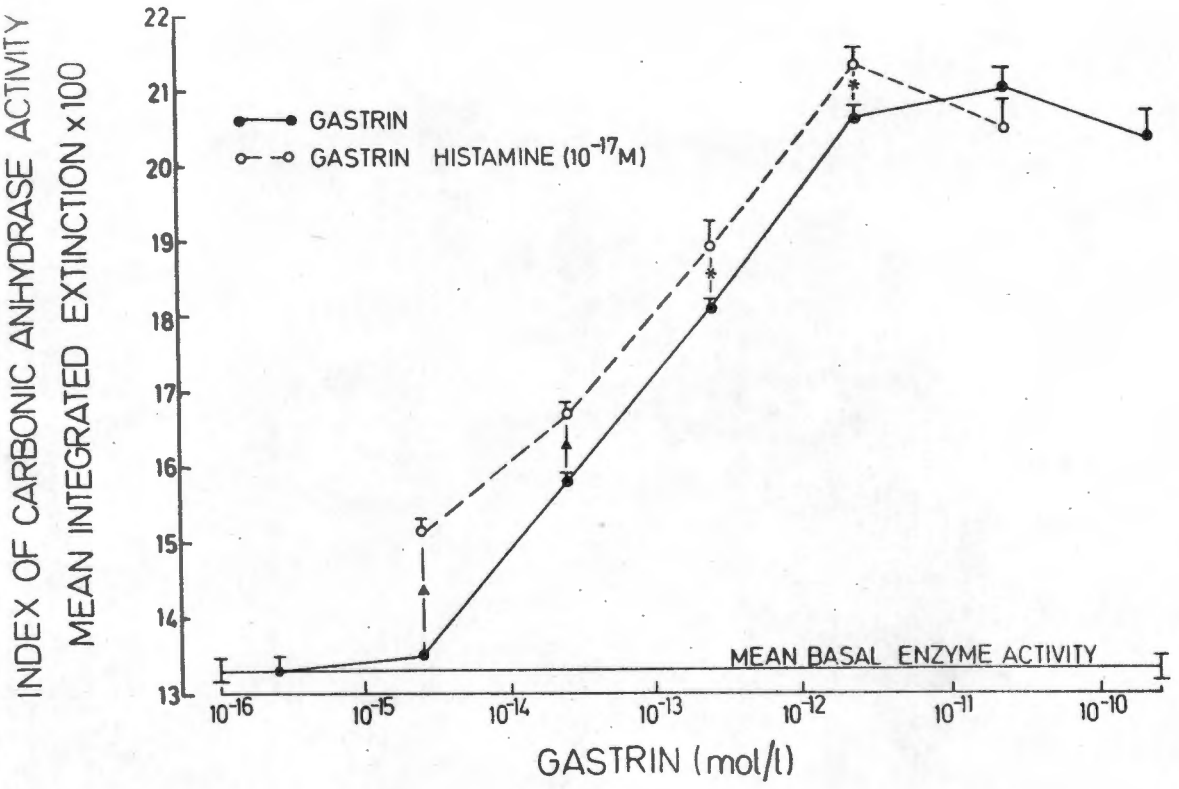


FIG. 10.1.

The addition of a subthreshold dose of histamine (10^{-17}M) to the gastrin dose response curve causes a significant shift to the left of the curve. Each point represents the mean ± SEM of the pooled data.

* $p < 0.05$

▲ $p < 0.01$

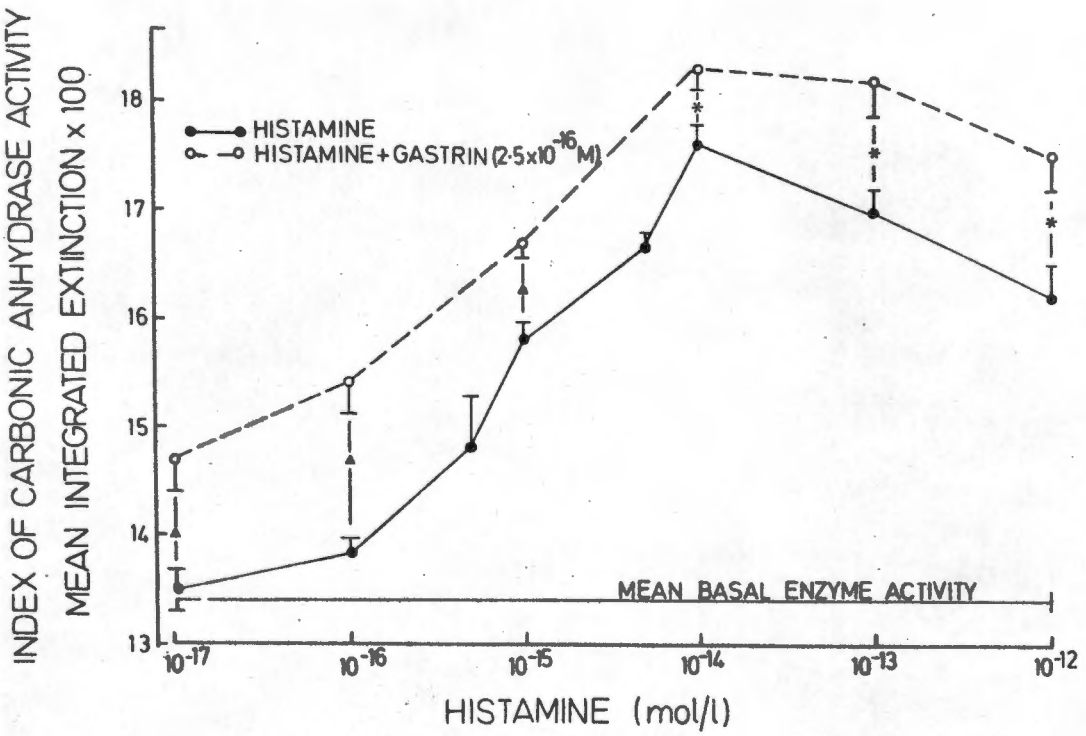


FIG. 10.2.

The addition of a subthreshold dose of gastrin (2.5×10^{-16}M) significantly increased both efficacy and potency of the histamine dose response. Each point represents the mean ± SEM of the pooled data.

* $p < 0.01$

▲ $p < 0.001$

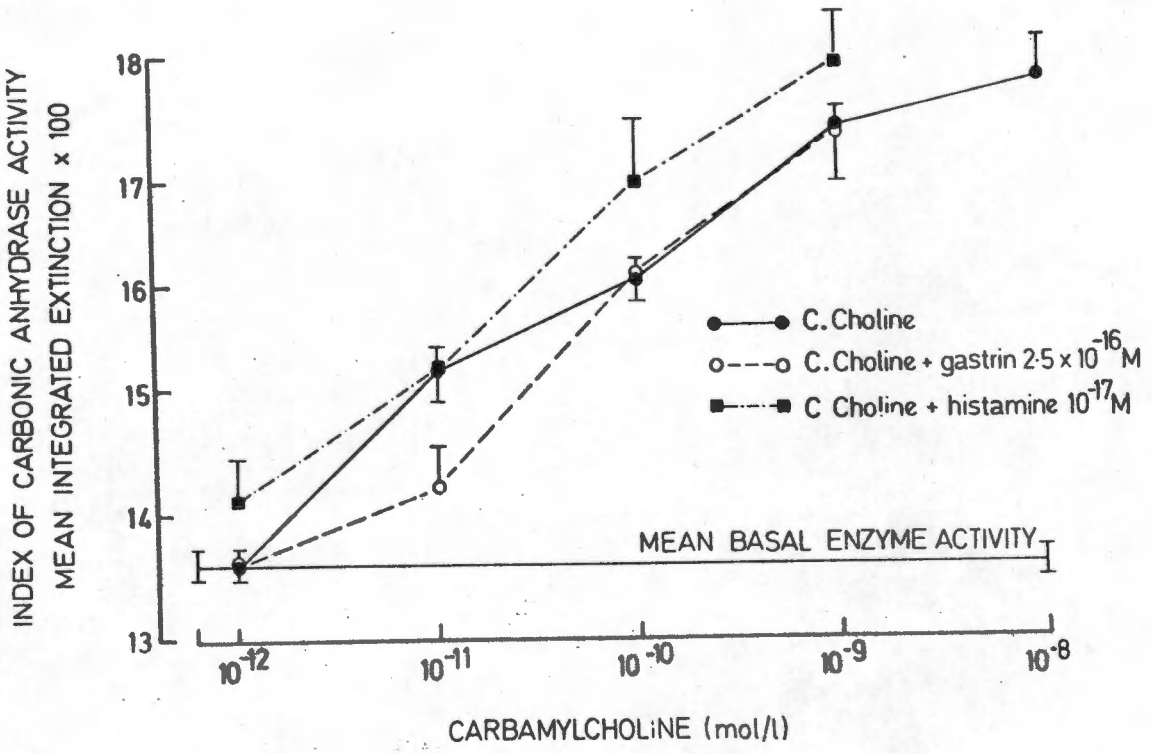


FIG. 10.3.

The addition of subthreshold doses of gastrin and histamine did not significantly alter the carbamylcholine (C.Choline) dose response curve.

Each point represents the mean $\pm$ SEM

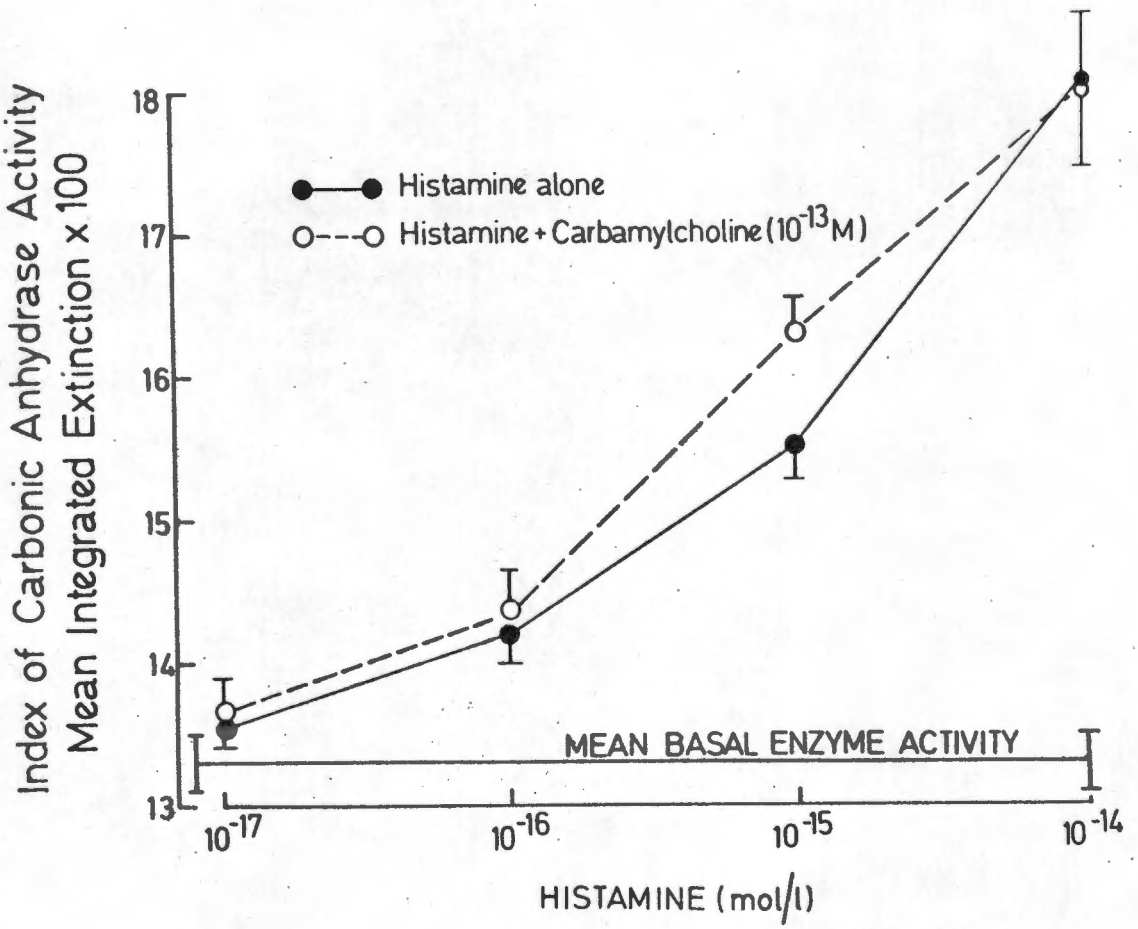


FIG. 10:4.

The addition of a subthreshold dose of carbamylcholine (10^{-13}M) did not significantly alter the histamine dose response curve. Each point represents the mean $\pm$ SEM.

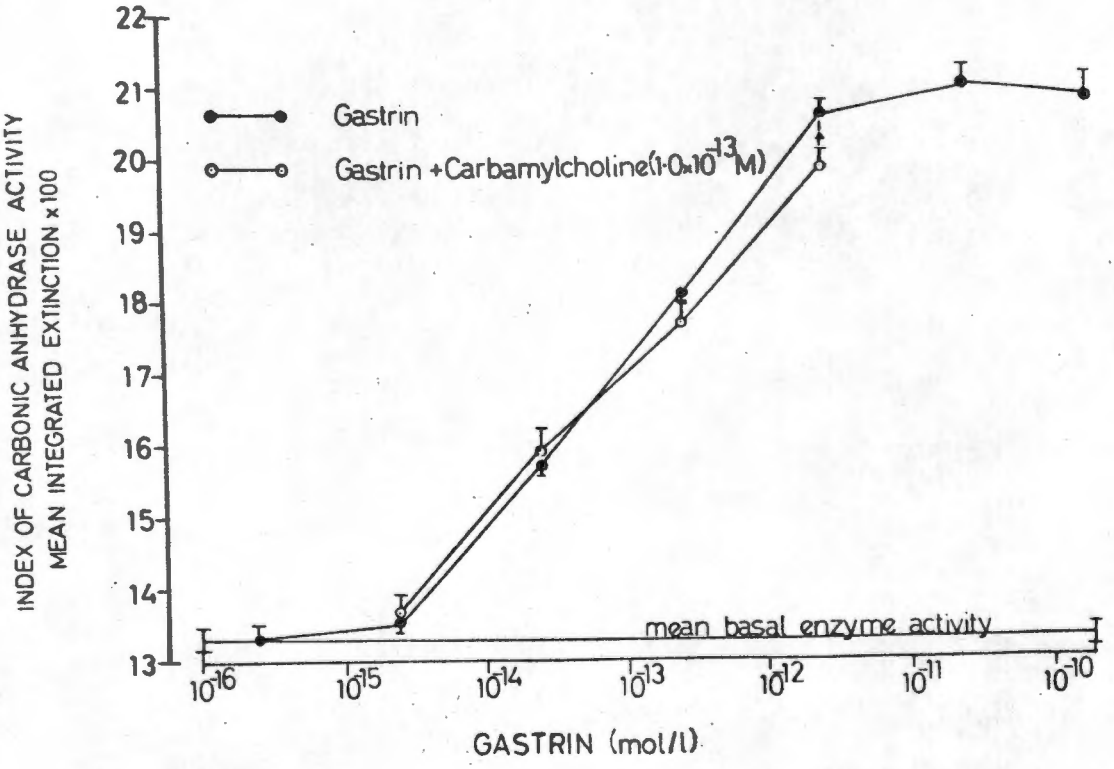


FIG. 10.5:

The addition of a subthreshold dose of carbamylcholine did not significantly alter the D_{min} or D_{50} of the gastrin dose response curve but did slightly decrease the gastrin effect at concentration 2.5×10^{-12} mol/l.

Each point represents the mean $\pm$ SEM.

▲ $p < 0.01$

TABLE 10.1. THE REGRESSION EQUATIONS OF THE INTERACTING SECRETAGOGUE DOSE RESPONSE CURVES

EXPERIMENT	NO	n	EQUATION f(y)	CORR COEFF (r)	CALCULATED D_{50}	CALCULATED D_{min}
					(mol/l x 10^{-15})	(mol/l x 10^{-15})
C (2.5 x 10^{-15} - 2.5 x 10^{-12}M)	1	6	2.08 x + 15.0	0.9875	31	0.36
	2	9	2.15 x + 14.3	0.9533	48	0.80
+ H (10^{-17}M)	3	6	1.99 x + 15.0	0.9719	36	0.38
	4	7	2.29 x + 15.1	0.9667	29	0.27
G (2.5 x 10^{-15} - 2.5 x 10^{-12}M)	1	10	2.03 x + 13.9	0.9733	68	1.8
	2	9	1.84 x + 13.9	0.9215	68	1.7
+ C (10^{-13}M)	3	8	1.76 x + 14	0.9712	66	1.6
	4	10	2.42 x + 13.2	0.9538	87	4.2
H (1.0 x 10^{-17} - 1.0 x 10^{-14}M)					(mol/l x 10^{-17})	(mol/l x 10^{-17})
+ G (2.5 x 10^{-16}M)	1	10	1.23 x + 14.0	0.9287	17	0.3
	2	9	0.98 x + 14.8	0.9303	5.4	0.03
	3	9	1.42 x + 14.3	0.8495	14	0.2
	4	7	0.86 x + 15.6	0.9094	1.4	0.02
H (1.0 x 10^{-17} - 1.0 x 10^{-14}M)	1	12	1.93 x + 15.2	0.8969	31	1.0
	2	13	1.33 x + 14.2	0.7741	41	1.8
+ C (10^{-13}M)	3	7	2.69 x + 13.3	0.9920	105	10
	4	8	1.80 x + 14.3	0.8359	56	2.8
C (1.0 x 10^{-12} - 1.0 x 10^{-9}M)					(mol/l x 10^{-12})	(mol/l x 10^{-12})
	1	11	1.65 x + 13.5	0.8550	32	1.1
+ H (10^{-17}M)	2	10	1.45 x + 13.6	0.8530	32	1.1
	3	9	1.00 x + 14.8	0.7689*	7	0.05
	4	8	1.03 x + 14.7	0.9357	9	0.09
C (1.0 x 10^{-12} - 1.0 x 10^{-9}M)	1	12	1.20 x + 13.4	0.8800	42	1.3
	2	10	1.32 x + 13.5	0.8704	37	1.3
+ G (2.5 x 10^{-16}M)	3	10	1.20 x + 13.4	0.8386	38	1.5

All r values $p < 0.01$ except where indicated by asterisk

H = Histamine

G = Gastrin

C = Carbamylcholine

n = no of points in regression equation

D_{50} = Dose causing half maximal response

D_{min} = Calculated minimum effective dose

TABLE 10:2

INTERACTIONS OF THE SECRETAGOGUES

Added Subthreshold Dose		SECRETAGOGUE DOSE RESPONSE CURVES								
		GASTRIN (mol/l x 10^{-15})			HISTAMINE (mol/l x 10^{-17})			CARBAMYLCHOLINE (mol/l x 10^{-12})		
		D_{50}	D_{min}	Slope	D_{50}	D_{min}	Slope	D_{50}	D_{min}	Slope
Nil	n	10	10	10	12	12	12	12	12	12
	Mean	7.8	2.3	2.35	76.8	5.8	1.84	31.3	1.2	1.33
	SEM	3.7	0.2	0.06	7.6	0.9	0.07	4.3	0.3	0.05
Histamine (1.0×10^{-17}M)	n	4	4	4	-	-	-	4	4	4
	Mean	36**	0.45**	2.12*	-	-	-	20.0	0.6	1.28
	SEM	4	0.12	0.06	-	-	-	7.0	0.3	0.16
Gastrin (2.5×10^{-16}M)	n	-	-	-	4	4	4	3	3	3
	Mean	-	-	-	9.5**	0.14**	1.12**	39	1.4	1.24
	SEM	-	-	-	3.6	0.07	0.13	1.5	0.07	0.04
Carbamylcholine 1.0×10^{-13}M	n	4	4	4	4	4	4	-	-	-
	Mean	72.3	2.3	2.01	58	3.9	1.93	-	-	-
	SEM	5	0.6	0.15	16	2.1	0.29	-	-	-

* < 0.05 compared with agent alone

10.3.2. Secretagogue Inhibition

Cimetidine (10^{-5}M) alone had no effect on the basal CA activity of the parietal cell sections. The effect of cimetidine on histamine-stimulated CA activity is shown in Fig. 10.6. Cimetidine caused 76$\pm$ 7.8% inhibition of histamine-stimulated CA activity at the maximal effective dose of histamine (1.0×10^{-14}mol/l).

Gastrin-stimulated CA activity was only slightly affected by cimetidine (Fig. 10.7). The D_{min} of gastrin and cimetidine was $1.7 \pm 0.3 \times 10^{-15}$ mol/l and was not significantly different from that of gastrin alone. However the slope of the gastrin dose response curve was altered (1.68 ± 0.2) (< 0.01). At the maximal effective dose of gastrin (2.5×10^{-12} mol/l), cimetidine inhibited gastrin-stimulated CA activity by $23 \pm 4\%$ ($p < 0.01$).

Carbamylcholine-stimulated CA activity was not significantly altered by cimetidine (Fig. 10.8).

Atropine did not alter basal, histamine, or gastrin-stimulated CA activity (Fig. 10.9). Atropine however markedly inhibited carbamylcholine-stimulated CA activity with total inhibition at concentrations 1.0×10^{-11} and 1.0×10^{-10} mol/l and $79 \pm 9\%$ inhibition at the maximal effective dose of carbamylcholine (1.0×10^{-9} mol/l) ($p < 0.001$). (Fig. 10.10).

The effects of atropine and cimetidine on gastrin-, histamine-, and carbamylcholine-stimulated CA activation are compared in Fig. 10.11.

Lastly, the effects of lanthanum chloride (10^{-6}M) on gastrin (2.5×10^{-12}mol/l) histamine- (1.0×10^{-14}mol/l) and carbamylcholine (1.0×10^{-9}mol/l) stimulated CA activation is shown in Fig. 10.12.

Carbamylcholine- (1.0×10^{-9} mol/l) stimulated CA activity was inhibited by $77 \pm 8\%$ ($p < 0.001$) gastrin (2.5×10^{-12} mol/l) by $45 \pm 10\%$ ($p < 0.01$) and histamine (1.0×10^{-14} mol/l) by $2 \pm 9\%$ ($p > 0.05$).

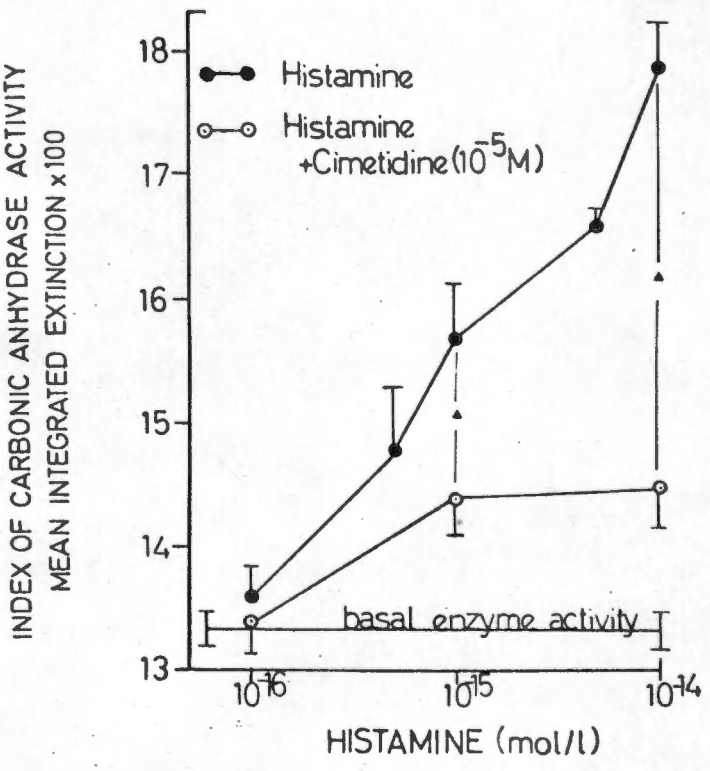


FIG. 10.6

The effect of cimetidine (10^{-5}M) on histamine-stimulated CA activity. Each point represents the mean $\pm$ SEM

▲ $p < 0.01$

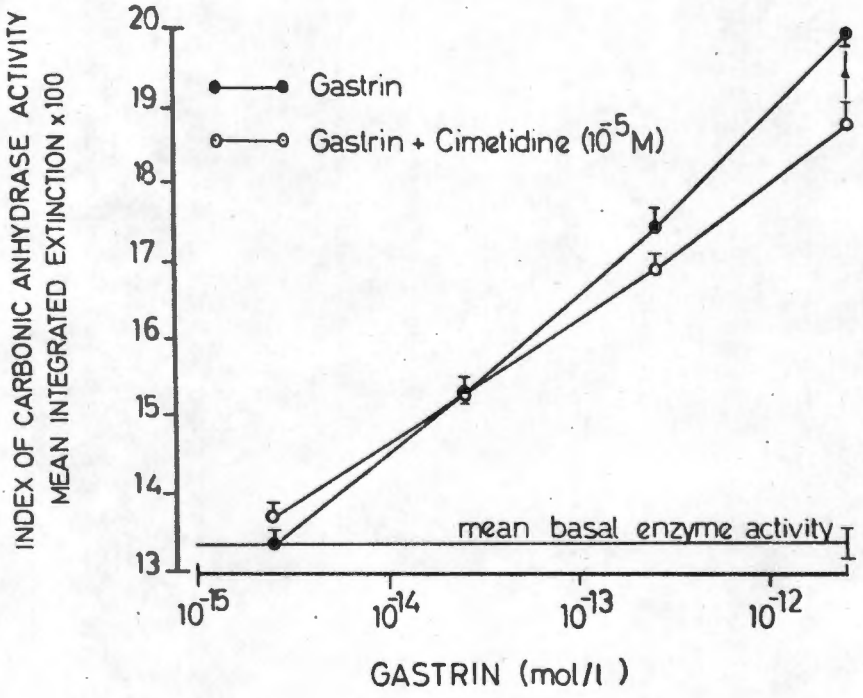


FIG. 10.7

The effect of cimetidine (10^{-5}M) on gastrin-stimulated CA activity. Each point represents the mean ± SEM

▲ p <0.01

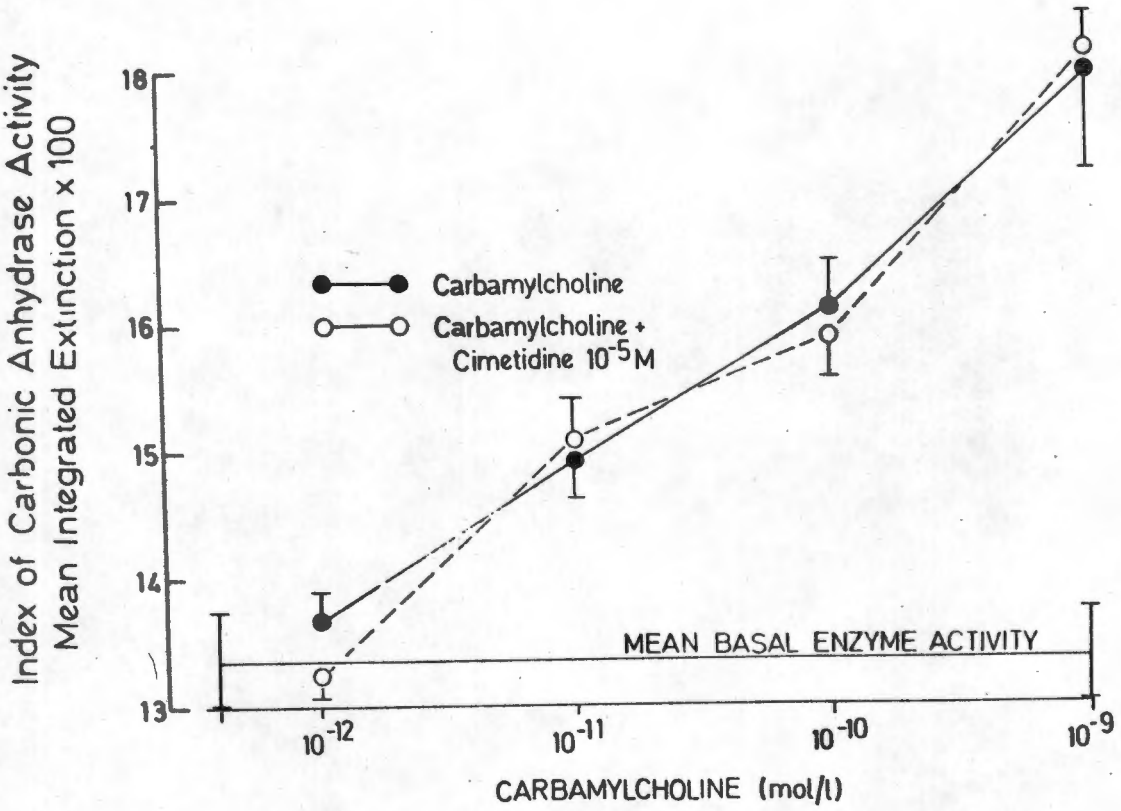


FIG. 10:8.

The effects of cimetidine 10^{-5}M on carbamylcholine-stimulated CA activity. Each point represents the mean $\pm$ SEM.

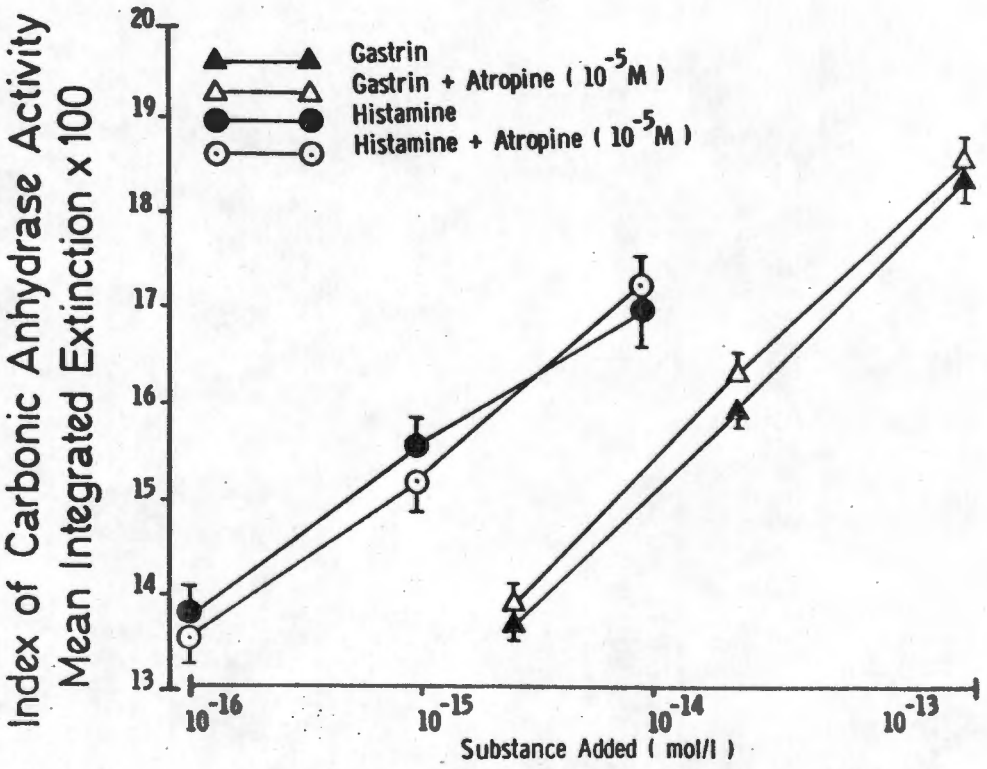


FIG. 10.9

The effect of atropine (10^{-5}M) on gastrin- and histamine-stimulated CA activity.

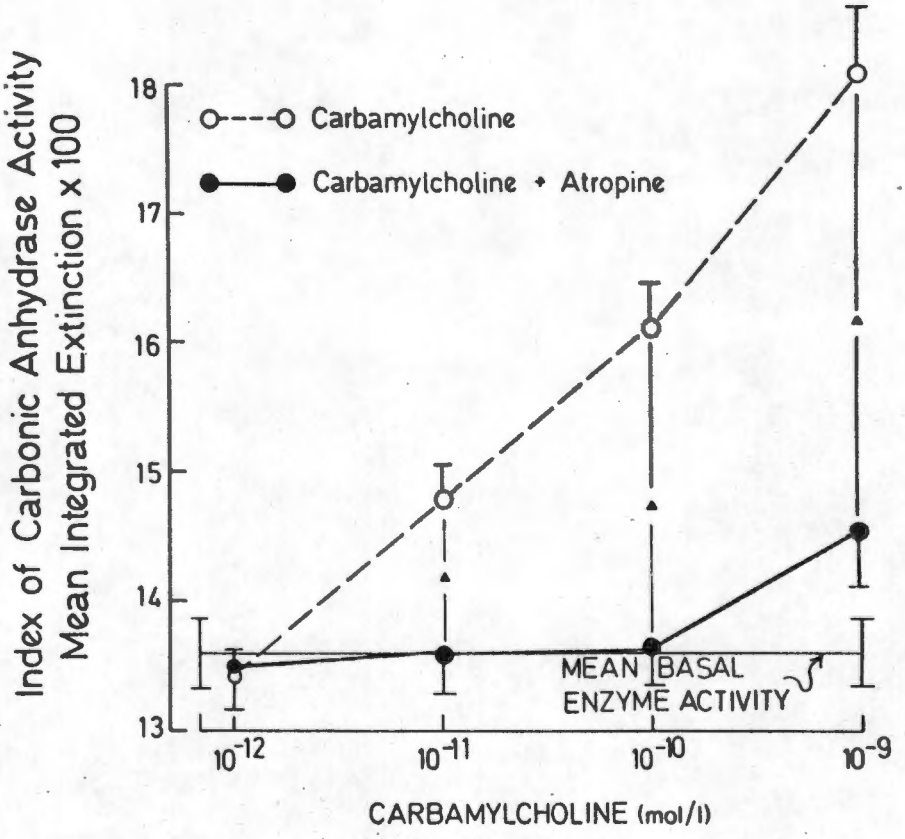


FIG. 10.10

The effect of atropine (10^{-5}M) on carbamylcholine-stimulated CA activity.

▲ $p < 0.01$.

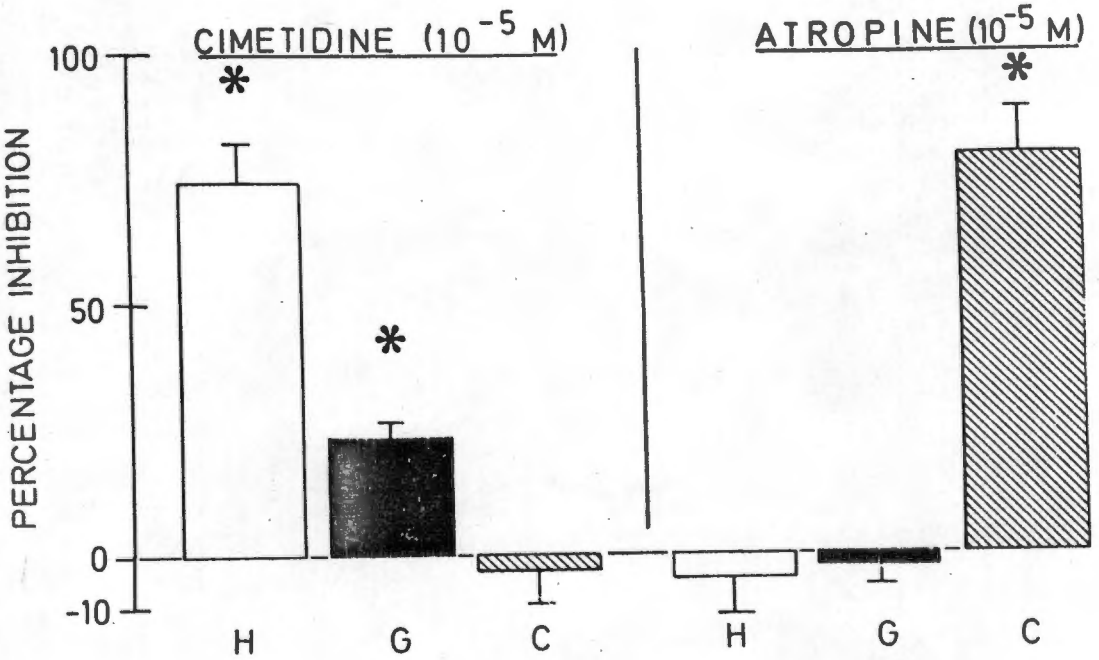


FIG. 10:11

Percentage inhibition ($\pm$ SEM) of carbamylcholine (10^{-5}M) and atropine (10^{-5}M) on histamine- (H) (1.0×10^{-14}M), gastrin- (G) (2.5×10^{-12}M) and carbamylcholine- (C) (1.0×10^{-9}M) stimulated CA activity.

*$p < 0.01$

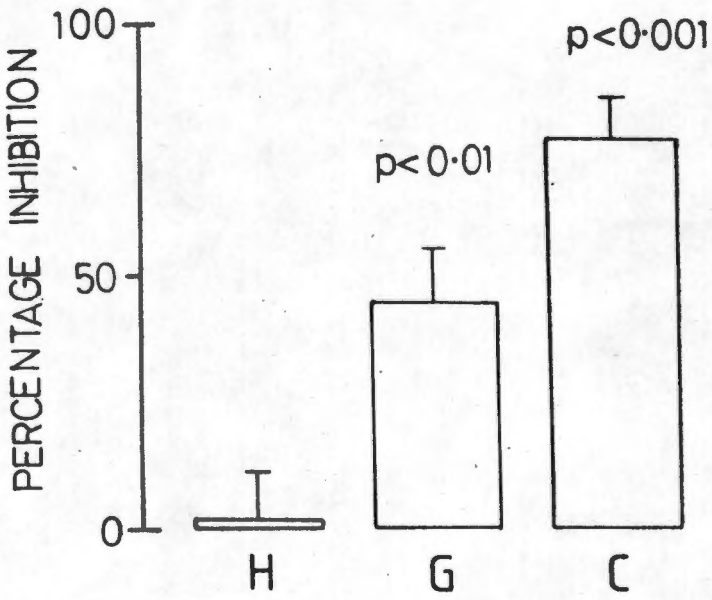


FIG. 10:12

Percentage inhibition (± SEM) of histamine- (H) (1.0×10^{-14} mol/l) gastrin- (G) (2.5×10^{-12} mol/l) and carbamylcholine- (C) stimulated CA activity caused by lanthanum (10^{-6} M).

10:4 Discussion

Potentiation refers to the phenomenon in which the effect of two agents in combination is significantly greater than the sum of the effect of each agent alone (Gardner, Jackson et al, 1978). This can occur in two ways, by an increment in efficacy or an increase in potency. "Efficacy" refers to the magnitude of a response caused by a maximally effective concentration of a particular agent. "Potency" refers to the concentration of a particular agent required to produce an effect of given magnitude, e.g. the concentration required to produce a half maximal response (Gardner, Jackson et al, 1978). Thus the increase in the maximal effect and the shift to the left of the histamine response caused by a dose of gastrin, which itself had no significant effect, is suggestive of potentiation due to both an increase in efficacy and potency, while the shift to the left of the dose response of gastrin caused by a subthreshold dose of histamine may be interpreted as a potentiating effect due to an increment in potency.

These results are therefore not wholly comparable to those of Soll (1978a,b, 1980a) who showed by oxygen and (^{14}C)-aminopyrine uptake in isolated canine parietal cells that histamine increased the efficacy of the response to gastrin. However, Soll's data indicated that increased potency had also occurred in his model as the lowest dose of gastrin alone to cause an effect was 10^{-7} M whilst gastrin 10^{-10}M enhanced the effect of histamine.

In further contrast to Soll's findings (1978a,b 1980a) this present work did not demonstrate significant potentiating interactions between histamine and carbamylcholine.

Although both the carbamylcholine and histamine dose response curves tended to be shifted to the left by subthreshold histamine and carbamylcholine respectively, this did not achieve statistical significance. Subthreshold gastrin furthermore did not alter the carbamylcholine curve but it was interesting to note that the subthreshold carbamylcholine caused a small decrease in the magnitude of the maximal gastrin response, thus apparently decreasing the efficacy without a significant alteration in the potency. The significance of this is unknown and it may be spurious since it was demonstrated at only a single point.

Soll (1978ab, 1980a) found no potentiating interaction between gastrin and carbamylcholine in his isolated cells. Gastrin and histamine have been demonstrated to potentiate each other in vivo, (Brooks, Johnson and Grossman, 1970; Johnson and Grossman, 1969) whilst acid secretion following antrectomy, which removes the major source of gastrin, is decreased in response to histamine (Bergegardh, Broman et al, 1976). Further evidence for secretagogue interaction is the finding that both H_2-receptor antagonists (Grossman and Konturek, 1974; Black, Duncan et al, 1972; Brimblecombe, Duncan et al, 1974; Sewing, Hagie et al, 1978; Richardson, Bailey et al, 1978) and anticholinergics such as atropine (Konturek, Oleksy and Wysocki, 1968; Hirschowitz and Sachs, 1969) block the effects of secretagogues other than their specific agonists. These reports contrast with the present findings that atropine, which inhibited carbamylcholine-stimulated CA activity, had no effect on either the gastrin or histamine-stimulated CA activity, while cimetidine inhibited only 23% of the gastrin effect, 7% of the carbamylcholine effect and 76% that of histamine.

In addition, in vivo potentiating interactions between gastrin and cholinergic agents (Grossman, 1974) and between cholinergic agents and histamine (Gillespie and Grossman, 1964b; Johnson and Grossman, 1969) have been demonstrated. The reason why this interaction is not apparent in-vitro systems is not known. It is possible that the interactions may be three-way interactions (Soll, 1978c), a possibility which has not been tested in this study or there may be species differences. Grossman (1975) suggested that all secretagogues act on their own receptors in the parietal cell, but the action is diminished if the other receptors are not occupied simultaneously. This could explain the slight reduction noted in the maximal effect of gastrin caused by cimetidine which may have been due to blockade of endogenous tissue histamine acting together with the gastrin to produce the gastrin effect. However this data indicates relative independence of the cholinergic pathways from that of gastrin and histamine in guinea pig mucosa.

The finding that lanthanum chloride inhibits carbamylcholine-stimulated but not histamine-stimulated CA activation is very similar to the findings of Soll (1979). Thus Soll has suggested that carbamylcholine acts via a calcium pathway, whereas histamine acts via cyclic AMP (Wollin, Soll and Samloff, 1979), This again implies a separation of the pathways of secretagogue action.

Lastly, in spite of the inhibition of both gastrin and acetyl-choline stimulated acid secretion by the H_2-receptor antagonists there has also been recent suggestions that anticholinergic agents augment the acid inhibition achieved by cimetidine in duodenal ulceration (Feldman, Richardson et al, 1977), whilst

vagotomy reduces the cimetidine inhibition of acid in the Zollinger Ellison syndrome. (Richardson, Feldman et al, 1979). This is thus a further clinical demonstration of the secretagogues acting through individual but interdependent pathways.

Thus it is clear that neither the isolated cell system of Soll, nor the quantitative cytochemical technique, is able to answer all the questions regarding secretagogue interaction. However this work and that of Soll's, both demonstrate an independent action by all three secretagogues, a potentiating interaction between gastrin and histamine and distinctly separate modes of action, although details of the latter are still not clear. Furthermore this independence of action and the potentiation between gastrin and histamine is not compatible with the final common pathway theory of Code (1956) and suggests that H_2-receptor blockers inhibit acid secretion by inhibition of the potentiation effect of histamine rather than by blockade of gastrin-or cholinergically mediated histamine release.

CHAPTER 11:

COMPARATIVE POTENCY OF THE GASTRINS

11:1 Introduction:

Thus far the effects of synthetic non-sulphated heptadecapeptide gastrin (SHG-17-I), histamine and carbamylcholine on carbonic anhydrase (CA) activity in the parietal cell sections have been examined. Histamine is the most potent activator of CA with D_{50} approximately 100 times lower than G17 and carbamylcholine, the least potent agonist, with a D_{50} approximately 400 times higher than G17.

The gastrins are composed of several molecular forms including "big gastrin" comprising 34 amino acids (G34), Big-big gastrin (BBG) and component I of undetermined molecular structure,"little gastrin" (G17) and "mini-gastrin" (G14) (Walsh and Grossman, 1975). In addition the carboxyterminal tetrapeptide common to gastrin and CCK (CCK-4) has been found to be present in the nerves of the gastro-intestinal tract (Larsson and Rehfeld, 1979). These different forms have different potency in acid secretion (Strunz, Thompson et al, 1978). As the gastrins are present in the circulation, the potency of these molecules is dependent not only upon their actions at the cellular level but also on their fate within the circulation. In the cytochemical bioassay the latter variable is not a factor and the technique therefore may be used to measure potency at a cellular level. In this chapter the potency of several gastrin peptides on the activation of CA are compared.

11:2 Methods

11:2:1: Laboratory Methods

The method for quantitative cytochemical estimation of CA activity is as previously outlined.

The standard reference preparation for all these studies was SHG-17-I in doses ranging from 2.5×10^{-15} to 2.5×10^{-12} mol/l and the potency of the following substances in the activation of CA was compared:

1. Natural human gastrin 17-I (NHG 17-I)*
2. Natural human gastrin 17-II (NHG 17-II)*
3. Natural human gastrin 34-I (NHG 34-I)*
4. Synthetic amino-terminal gastrin fragment 1-13 (G 1-13) +
5. Synthetic carboxy-terminal fragment 8-17 (G 8-17) +
6. Synthetic cholecystokinin octapeptide (CCK-8) (SQ 19844) (donated by Dr. Ondetti, Squibb Laboratories, U.S.A.)
7. Synthetic pentagastrin (PG) (Imperial Chemical Industries (ICI) U.K.)

11:2:2: Statistical Analysis

In bioassay systems the presence of parallelism between the standard dose response curve and several dilutions of the test substance implies that the test substance contains the same material or another material acting in an identical manner to that present in the standard preparations (Cornfeld, 1964). In contrast, the absence of parallelism implies that the response of the test substance is being affected by another substance which may either inhibit or enhance the effect of the material being measured or that the test material is dissimilar to the standard preparation.

* These peptides kindly donated by Dr. M. Grossman of CURE Los Angeles, were isolated from tumour extracts prepared by Drs. Gregory and Tracy.

+ These peptides were kindly donated by Dr. J. Morley of ICI.

However the presence of parallelism does not necessarily exclude the presence of a co-existing inhibitor which may be diluting out in the same manner as the effector substance over the range of dilutions tested.

In this study, because the different peptides did not in all cases cause identical maximal or parallel dose responses, the relative potencies were calculated from the D_{50} of the substance relative to the D_{50} of SHG 17-I expressed as a percentage ($\pm$ 95% confidence limits) (Appendix 2). The mean basal enzyme activity was calculated by measuring the mean integrated extinction of control sections treated with buffer alone. The slopes of the dose response curves were determined by regression of the (MIE x 100) against the $\log_{10}$ peptide concentration by least mean squares analysis (Appendix 2). Parallelism of regression lines was assessed by analysis of covariance (Appendix 2).

11:3: Results:

The potencies of the gastrin-like peptides are compared in Table 11.1. The dose response effect of SHG 17-I was parallel to that of NHG 17-I, NHG 17-II, G 8-17, PG and CCK-8 but differed significantly from that of NHG 34-I ($p < 0.01$). The standard mean dose response curve of NHG 34-I is compared to that of SHG 17-I in Fig. 11.1.

The N-terminal peptide G 1-13 had no biological activity whereas the C-terminal decapeptide G 8-17 had similar potency to SHG 17-I. The dose curves of G 1-13 and G 8-17 are compared in figure 11.2. Pentagastrin and CCK8 had 35% and 7% respectively the potency of SHG 17-I.

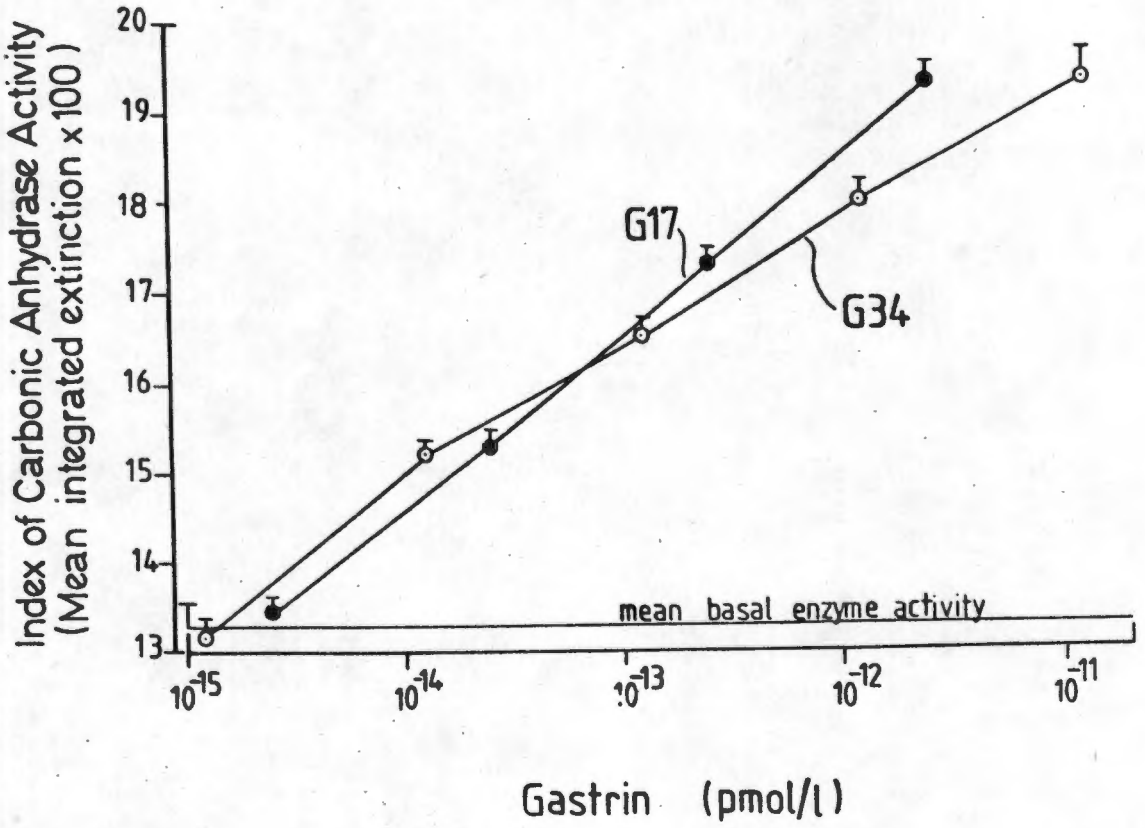


FIG. 11:1

Comparison of the dose response curves of the SHG-17-I (G17) and NHG-34-I (G34).

Each point indicates the mean and SEM of 6 pooled experiments.

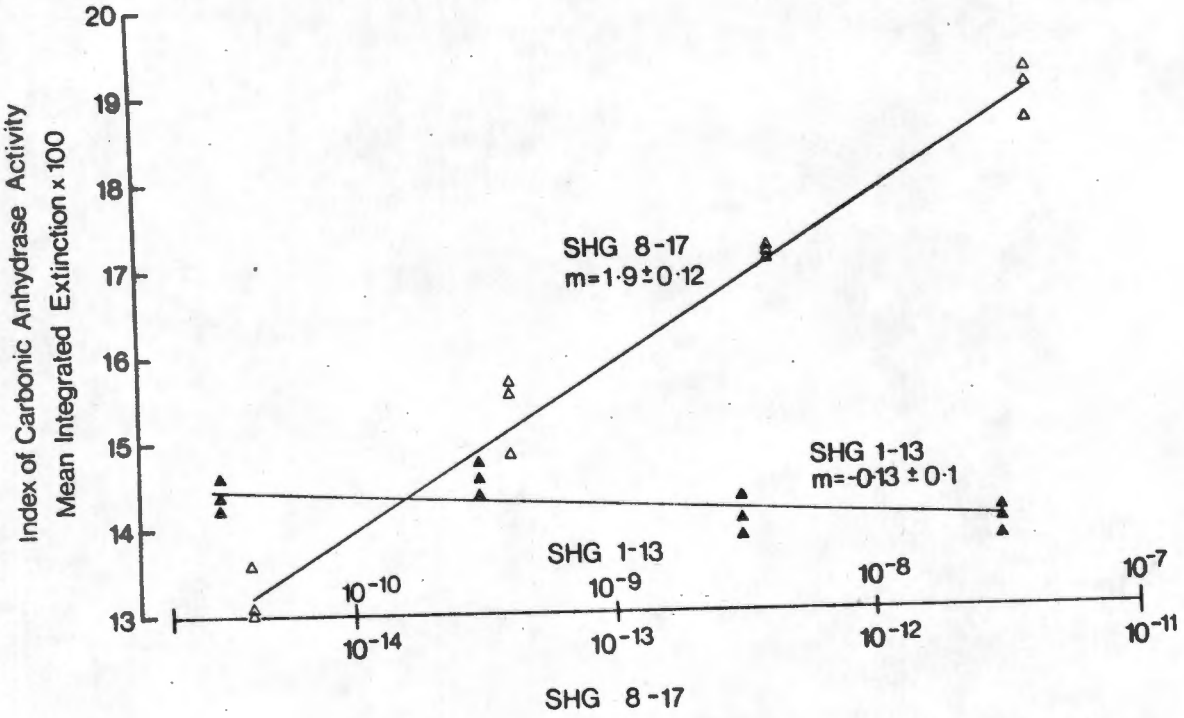


FIG. 11:2

The effects of synthetic gastrin carboxyterminal fragment (SHG 8-17) and aminoterminal fragment (SHG 1-13) on CA activity.

The biological activity of gastrin resides in the carboxyterminal

m = slope $\pm$ SD

TABLE 11:1

COMPARISON OF POTENCIES OF GASTRIN-LIKE PEPTIDES

Peptide	Potency % D_{50} of SHG-17-I				Slope Mean ± SD	
	NO	%	Confidence limits (95%)	Significance[x]		Significance[x]
SHG-17-I	14	100	-	-	2.08 ± 0.10	-
NHG-17-I	3	120	80 - 150	ns	2.21 ± 0.12	ns
NHG-17-II	2	83	57 - 128	ns	2.16 ± 0.16	ns
NHG-34-I	6	70	53 - 100	ns	1.60 ± 0.07	$p < 0.01$
C-terminal 8-17	2	100	70 - 133	ns	2.01 ± 0.13	ns
N-terminal 1-13	2	0	-	$p < 0.01$	-	-
Pentagastrin	3	32	25-42	$p < 0.01$	1.92 ± 0.10	ns
CCK-octapeptide	2	4	3-6	$p < 0.01$	1.95 ± 0.12	ns

x compared to SHG-17-I
ns = not significant
no = number of experiments (results pooled)

11.4 Discussion:

The potency of acid secretagogues as agonists of parietal cell function is dependent upon events at the cellular level and in the instance of circulating substances, on the fate of the substance within the circulation. At the cellular level the potency of agonists is dependent upon:-

(a) The nature of interaction with the receptor upon which the substance acts, including, the affinity of the substance for the receptor.

(b) The degradation and removal of the substance from the system.

(c) Post-receptor cellular events.

The potency of systemically administered agonist is dependent upon events at the cellular level and in addition the route and manner of administration into the system. Thus G34 and G17 when infused in equimolar concentrations are equipotent in causing acid secretions (Walsch, Isenberg et al, 1976), which is supported by these findings of similar potencies at the cellular level. However, as G34 has a half-life disappearance time six times longer than G17 (Walsch, Isenberg et al, 1976) which results in a higher circulating blood level, these workers calculated that G34 was six times less potent than G17 at the cellular level. This calculation may be erroneous as a later study by the same group (Feldman, Walsch et al, 1978), shows that all gastrin-stimulated acid secretion after a peptone meal is attributable entirely to G17 in spite of the high circulating G34 levels. This suggests that the gastrins in circulation may not have the expected biological effects on acid secretion, thus rendering potency calculations from blood levels and half life disappearance times rather unreliable.

In this study it was found that G34 and G17 had similar potencies in activating CA. However, their actions, as determined by non-parallelism of the dose response curve, were not similar. The reason for this is not clear. In view of their molecular similarities, it is unlikely that these molecules are acting at different receptor sites. It is possible that G34 interacts with endogenous substances such as histamine in a different manner to G17 or that the amino-terminal elongation alters the nature of the interaction with the receptors or hinders degradation at the receptor site and thus alters the dose response curves. However, this is speculative. Strunz, Thompson et al, (1978) have shown that gastrins of various chain lengths have different potencies depending on whether the peptide is given via the portal or systemic route. This is due to different rates of hepatic inactivation of the peptides. Carboxyterminal peptides of 8 amino-acids or less were more than 90% inactivated by the liver. Thus in vivo the carboxy-terminal decapeptide of gastrin (G10) has 53%, and the shorter pentagastrin molecule 12% the potency of G17. For contrast in the cytochemical bioassay, G10 has a 100% and pentagastrin 33% the activity of G17, a discrepancy possibly due to inactivation in the circulation. This data also confirms that the aminoterminal of gastrin is biologically inactive at the cellular level as well as when given systemically.

CCK-8 caused activation of CA which was parallel to but less potent than that of gastrin. This finding would thus be compatible with the proposal of Johnson and Grossman (1971) that CCK is a partial agonist of acid secretion in certain species.

In conclusion this work confirms that differences in potency of gastrin molecules vary with molecular configuration and that the cytochemical bioassay is a method for measuring secretagogue potency at the cellular level. In the next chapter the secretagogue potency of the serum of normal subjects will be measured by this technique.

CHAPTER 12

THE MEASUREMENT OF CARBONIC ANHYDRASE STIMULATING ACTIVITY IN HUMAN SERUM.

12:1 Introduction:

The acid secretory response to a mixed meal is influenced by numerous interacting factors. These may be both stimulatory or inhibitory and act via neurocrine, paracrine, hormonal and luminal factors (Soll and Grossman, 1978). There is poor correlation between the levels of circulating gastrins and the amounts of acid released with a meal. (Richardson, Walsch et al, 1976). One of the reasons for this may be that gastrins circulate as several peptides. (Walsch and Grossman, 1975). This heterogeneity will have two effects. Firstly, radioimmunoassay measurements of gastrin will vary according to the specificity of different antisera for different gastrin peptides and secondly the biological activity of the various gastrin-like peptides differ.

After a mixed meal 75% of the gastrin released is G34. The remaining 25% is accounted for by G17 and to a lesser extent G14. (Walsch, Isenberg et al, 1976). These workers calculated that G34 probably contributes less than half of the acid stimulating activity of the gastrins released in response to a meal. Taylor, Dockray and Walker (1979) however reported that 50% of the gastrin released after a meal is G17, whilst Lamers, Harrison et al (1979) reported that the levels of G34 in the resting state were twice times that of G17. Following a meal, G17 increased four times and G34 twice times to give an approximate 1:1 ratio.

Feldman, Walsch et al, (1978) showed that the G17 released in response to a peptone meal could account for all gastrin-stimulated acid secretion. Thus it appears that the gastrins released after a meal do not display their expected biological effects and this together with the heterogeneity of the gastrins may account for the dissociation noted between acid secretion and serum gastrin concentrations measured by radioimmunoassay.

The aim of this section of the study was to examine the secretagogue potency in serum before and after a mixed meal in normal people and in known hypergastrinaemic patients.

As has been previously demonstrated gastrin, histamine and acetylcholine are all activators of CA in the cytochemical bioassay, and may be present in blood. In addition, other acid secretagogues may circulate in blood. For this reason the assay may not be specific for gastrin and will be considered as an assay for the CA stimulating activity (CASA) of serum.

12:2: Methods;

12:2:1: Cytochemical Bioassay

The method is as previously described. The CA stimulating activity (CASA) of the serum samples diluted 1:100 and 1:1000 in 0.025 Hepes buffer pH 7.0 were compared to standard reference solutions of synthetic non-sulphated heptadecapeptide gastrin (SHG-17-I) in doses ranging from 2.5×10^{-15} to 2.5×10^{-12} mol/l. Treatment time for both was 90 seconds. Both standard preparations and unknown serum samples were assayed in triplicate within a single assay. Thus two unknown samples could be assayed with each standard dose response curve.

12:2:2: Assay Variation and Precision

To assess the variation in measurementof CASA between identically tested sections within a single assay (intra-assay variation) the CASA of the four standard gastrin concentrations were tested on six sections each during one experiment.

To assess inter-assay variation a serum sample was divided into aliquots, frozen and a single aliquot measured at 2 dilutions (1:100 and 1:1000) in six consecutive assays. Lastly, a further estimation of inter-assay variation was calculated from the variation of the slopes of the standard dose response curves (Appendix 2).

The index of precision λ (Gaddum, 1933) for each individual dose response curve was calculated as follows:

$$\lambda = Sxy/M$$

where Sxy is the standard deviation of the individual values about the straight regression line and M is the slope of the line. By Lorraine's criteria (1958), assays with λ of 0.2 or less are very precise and well-suited for chemical studies, while assays with a λ of 0.2 to 0.3 are less precise but still regarded as acceptable. Any dose response curve with a λ of more than 0.27 was regarded as unacceptable for the purpose of this study and the results were discarded and assays repeated.

In addition the fiducial limits (95% confidence limits) were calculated for each standard curve. (Appendix 2). Parallelism of the dose response curve and the serial serum dilutions were calculated as above. (Chapter 11:2:2) (Appendix 2).

If the slope of the dilution curve of the unknown samples did not parallel that of the standards on the first experiment, the samples were repeated in a second assay and at different dilutions. In addition, samples reading beyond the maximum capacity of the assay were repeated in greater dilutions so that they could be read on the sensitive portion of the curve.

Thus in summary the following calculations were made on each dose response curve:

(a) Correlation coefficient by the least mean squares method (r).

(b) Slope and standard deviation of the slope of the regression line. ($M \pm SD$)

(c) Index of precision (λ)

(d) Confidence limits of the curve (95%).

The assay was repeated on the following criteria:

(a) If the slope of the standard curve fell outside the 95% confidence limits of the mean curve.

(b) If the index of precision of the standard dose response curve or the serial serum dilution curve was greater than 0.27.

(c) Non-parallelism with standard dose response curve.

(d) If the unknown sample read in the insensitive portion of the dose response curve. In the case of (c) and (d) the sample would be diluted to a greater number of dilutions.

12:2:3 Recovery of Gastrin from Serum:

One of the tests of the efficiency of an assay system is to add known amounts of the substance measured to the tissue fluid being assayed and to measure the substance in both the untreated and treated samples.

In a perfectly efficient system, the amount added should be exactly equal to the difference in the measurements between the treated and untreated samples, i.e. 100% recovery. If these amounts do not tally there are several possible reasons. Firstly the substance may be unstable in the medium to which it has been added. This may result in an activity which is lower than expected. Secondly, in a bioassay system, the biological activity may be altered by interfering substances. Lastly, it may reflect an inefficiency in the assay system. The recovery of SHG-17-I in the cytochemical bioassay was measured by adding SHG-17-I to a fasting serum sample in concentrations anticipated to cover the range of the assay. These samples were also measured by radio-immunoassay using the G2 and 2604/7 antiserum. (Appendix 1).

12:3: Specificity of the Cytochemical Bioassay

Prior to measuring the CASA in the serum of normal and hypergastrinaemic subjects, an attempt was made to characterise this activity in serum. This was done by the removal of gastrin from serum in two ways.

(1) Charcoal Adsorption

(2) Adsorption on a specific affinity chromatography column.

Both these techniques were performed on pooled fasting serum as described in Appendix I.

12:3:1: Activated Charcoal Adsorption

Activated charcoal added to serum adsorbs peptides and small molecules. In a 2% suspension the charcoal will remove the majority of gastrin 17 and gastrin 34 from the serum. However big-big gastrin (BBG) which elutes in the void volume of a sephadex G50 column and has a molecular weight of 20,000 is not affected by charcoal treatment.

(Yalow and Wu, 1973). This differential removal by charcoal of small small from large peptides is used in the gastrin radio-immunoassay to separate free (^{125}I)-gastrin from antibody bound (^{125}I)-gastrin (Appendix 1).

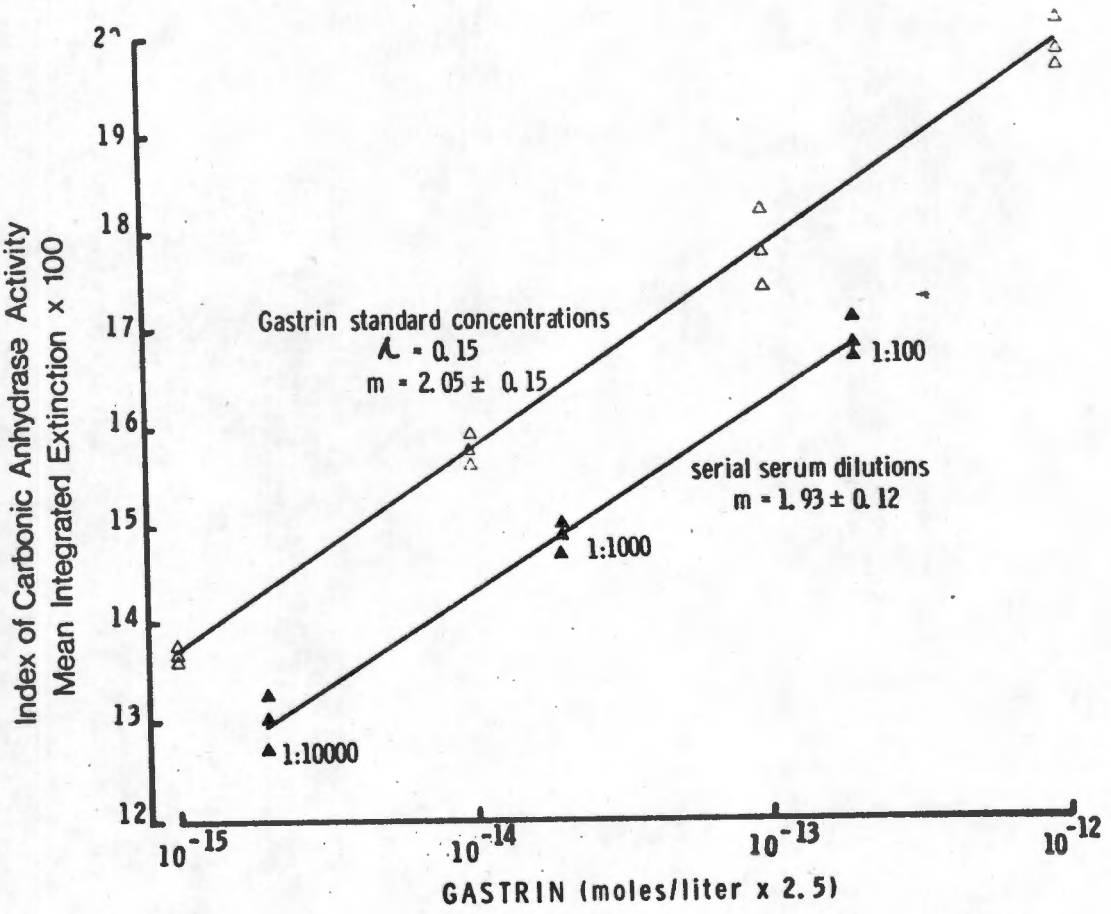


FIG. 12:1.

Serial dilutions of serum are parallel to the gastrin dose-response curve.

λ = Index of precision

m = Slope $\pm$ SD

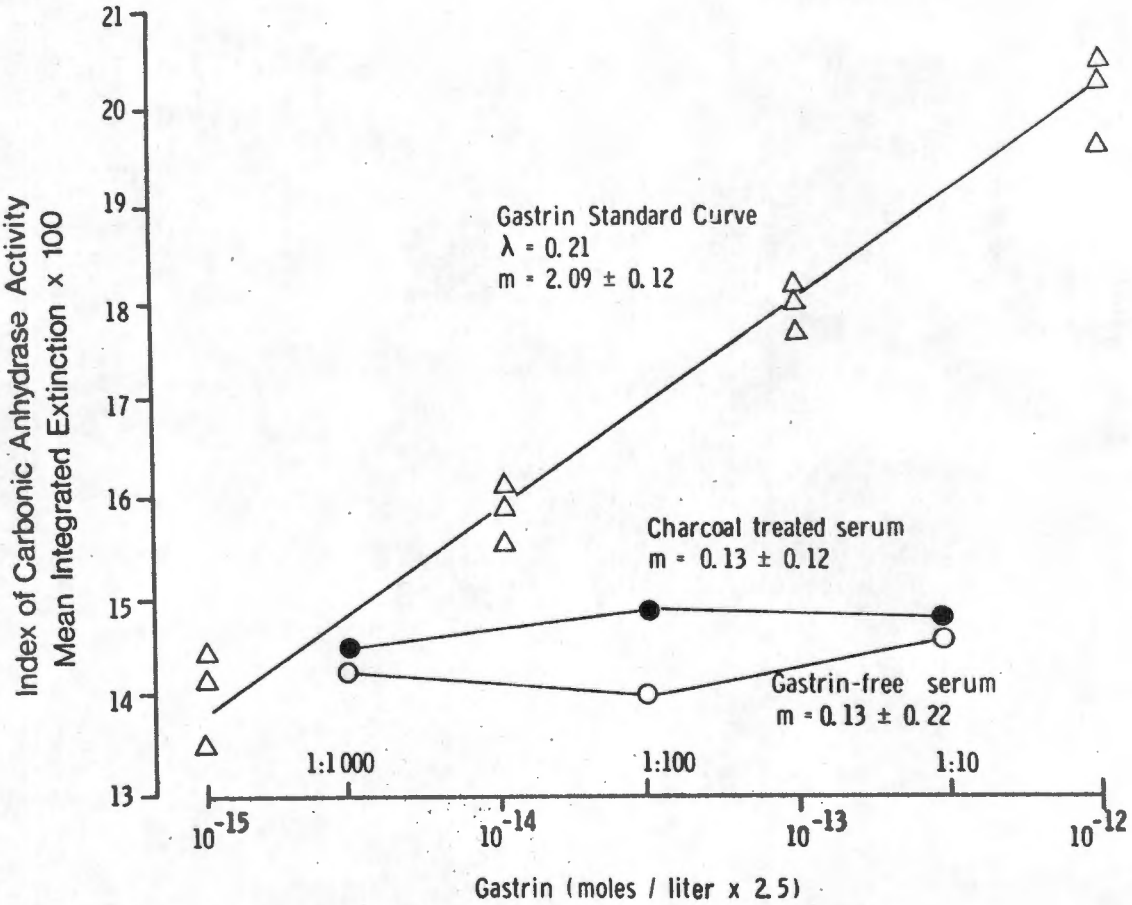


FIG. 12:2

Effect of serial dilutions of charcoal treated serum and serum purged of gastrins by affinity chromatography on CA activity as compared to the gastrin dose response curve.

12:3:2 Affinity Chromatography

Affinity chromatography is a method of adsorbing a specific substance from a solution or suspension. In order to do this a ligand is required which is specific for that substance. A convenient ligand for the adsorption of a peptide is an antiserum directed against that peptide. For this purpose an anti-gastrin antiserum G9 prepared by the inoculation of a rabbit with SHG 17-I coupled to whelk haemocyanin was used (Napier, 1978). This antiserum which cross-reacted with both natural and synthetic human gastrins G17 and G34 was coupled to AH Sepharose (R) 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) using carbadiimide as the coupling agent by the method of Cuatrecasas(1970). (Appendix 1).

12:3:3: Results:

The effects of serial dilutions of untreated serum is shown in Fig 12.1. This is compared to serum treated by charcoal adsorption and affinity chromatography in Fig. 12.2. CASA was removed by both forms of treatment and parallelism with the standard dose response curve was lost. In contrast the radioimmunoassay gastrin was reduced from 17.5 pmol/l in the untreated serum to 12.5 pmol/l in the charcoal treated serum and to undetectable levels in the affinity chromatography serum.

12:3:4. Discussion

The finding that the removal of gastrin from fasting serum coincided with the removal of most of the CASA of serum suggested that gastrin plays a major role in this activity.

Nevertheless it should be stated that neither experiment confirmed absolutely the specificity of serum gastrins in accounting for the CASA of serum. Firstly charcoal adsorption is not a specific gastrin adsorbent and other molecules may be removed by this treatment. Secondly, whereas affinity chromatography may be a specific technique for adsorbing gastrins, the possibility of the degradation of other small molecules such as histamine and acetylcholine occurring during the affinity chromatography treatment cannot be excluded. Both these substances are inactivated very rapidly in blood (Code, 1977) via the histaminase and diamine oxidase enzymes (Baylin, Beaven et al, 1970) and acetylcholinesterase respectively. No specific measures to prevent this occurring during the affinity chromatography treatment were taken and as no methods sufficiently sensitive for measuring these substances were available it is not possible to speculate as to their fate during this treatment.

The finding that charcoal treatment of serum removed only 33% of the immunoreactive gastrins but 100% of the CASA is consistent with the finding of Yalow and Wu (1973). They suggested that the predominant circulating form of gastrin in the fasting state is BBG which is not adsorbed by charcoal. Although further studies would be required to prove that the remaining immunoreactivity is due to BBG, this data suggests that the material non-adsorbed to charcoal is biologically inactive. Whether this BBG represents a circulating large molecular weight prohormone form, or is non-specific protein interference, as suggested by Rehfeld, Schwartz and Stadil (1977), is still unclear.

12:4 Subjects:

12:4:1: Normal Subjects:

Ten normal volunteers consisting of 7 males and 3 females were studied. Their ages varied from 18 to 35 years. After an overnight fast a 19 gauge butterfly needle was inserted into an anticubital vein. This was maintained patent by flushing with a few mls. of heparinised saline after drawing blood samples. Venous blood was sampled at 15 minute intervals during the 30-minute basal period. The subjects then ate a standard breakfast (Table 12:1) over a period of 10 minutes. Further blood samples were taken at 5, 15, 30, 60, 120 and 180 minutes after the meal. These were separated immediately by centrifugation and the serum stored at-20^{o}C. All subjects gave written informed consent. All samples were assayed in at least two dilutions by the cytochemical bioassay for CASA and by radioimmunoassay using two antisera, G2 and 2604/7 (Appendix 1).

12:4:2: Hypergastinaemic Patients:

Ten patients with hypergastrinaemia had fasting serum samples analysed for CASA and immunoreactive gastrins. These patients included 6 patients with the Zollinger-Ellison syndrome (gastrin-producing tumours), three of which had Multiple Endocrine Adenomatosis Type I. Five of these patients had tumours removed, whereas one patient became normogastrinaemic following parathyroidectomy. Four of these patients, including the last mentioned, had positive secretin tests i.e., the serum immunoreactive gastrin increased more than 45pmol/l following the intravenous injection of 2 ^{u}/kg Boots secretin. In addition four patients with atrophic gastritis were tested, including three with pernicious anaemia and one with dyskeratosis congenita.

12:4:3: Statistical Methods: (Snedecor and Cochran, 1967).

Values were compared using the Wilcoxon Rank test for paired samples. Correlation coefficients were calculated by the least mean squares method (Appendix 2). Significance was accepted at the 5% level.

TABLE 12:1

STANDARD BREAKFAST

	PROTEIN (gm)	FAT (gm)	CARBOHYDRATE (gm)	KJOULES (gm)
2 boiled eggs	14	14	-	756
250 ml milk	8	8	12	714
30 ml skim milk	10	-	15	420
2 slices bread	4.4	1	30	605
20gm. butter	-	20	-	756
20gm. honey	-	-	15	252
TOTAL	36.4	43	72	3 503

12:5 Results:

12:5:1 Statistical Variations of the Assay

A dose response curve of gastrin with the calculated 95% confidence limits is shown in Fig. 12:3. The data utilized is that in Table 8:1.

The mean index of precision of 44 consecutive standard dose response curves was 0.15 with a range from 0.05 to 0.27. The slope of the dose response curve in these assays varied from 1.975 to 2.8889 with a mean of 2.38 and a coefficient variation of 8%. The 95%confidence limits were therefore calculated to range from 1.998 to 2.768 and assays in which the slope of the standard dose response curve fell outside these limits were repeated.

The interassay coefficient of variation of 6 consecutive assays was 30% (Table 12:2), whilst the intra-assay coefficient of variation at concentrations of gastrin 2.5×10^{-15}, 2.5×10^{-14}, and 2.5×10^{-13} mol/l was 15%, 9% and 19% respectively (Table 12:3). At concentration 2.5×10^{-12}mol/l, although the variation in readings was small (5.6%), this variation read off the x axis (log concentration) was unacceptably high (100%). This loss of sensitivity at the upper region of the curve was overcome by repeating test samples reading in this region in a greater number of dilutions. The lowest concentration of gastrin which could be differentiated from zero with 95% confidence was 3×10^{-15} mol/l.

The recovery of gastrin by the cytochemical bioassay is about 50% whereas the recovery measured by the radioimmunoassay was 55% for 2604/7 and 85% for G2 (Table 12:4).

TABLE 12:2

INTERASSAY VARIATION : GASTRIN (pmoles/litre)

ASSAY NO.	1	2	3	4	5	6
SERUM DILUTION 1/100	16	8	10.5	19	18	10
SERUM DILUTION 1/1000	15	9	14.5	18	11.5	9

MEAN 13.2 pmoles/litre

SD $\pm$ 3.99

Coeff. variation 30%

-14	10^{-15}				
.5	13.6				
.38	0.54				
.3	4				
	15				

in a single assay.

ings as well as the

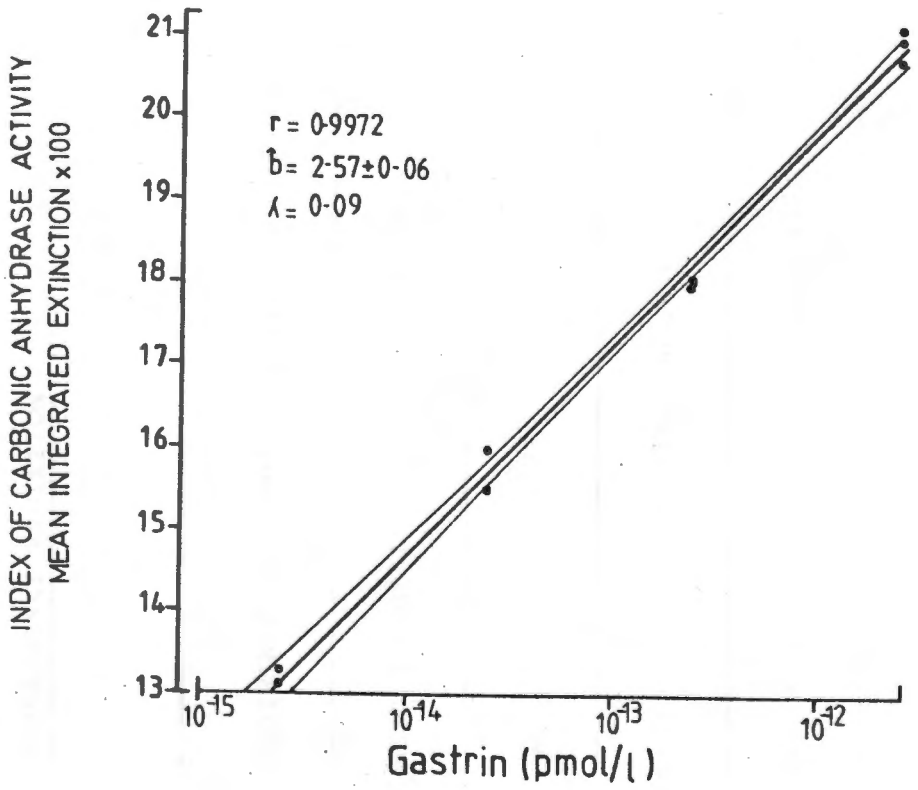


TABLE 12.4

RECOVERY OF GASTRIN

	Cytochemical Bioassay			Radioimmunoassay			
	Dilution	pmol/l gastrin	% Recovery	Antiserum 2604/7 pmol/l	% Recovery	Antiserum G2 pmol/l	% Recovery
Fasting Serum	1/100	14		16		15	
	1/1000	13					
Fasting Serum + 40pmol/l NHG-17-I	1/500	31	49	39	55	49	85
	1/5000	35					
Fasting serum + 80pmol/l NHG-17-I	1/1000	55	52	60	55	82	84
	1/10000	55					

NHG-17-I = Natural human heptadecapeptide gastrin

Each sample in the cytochemical bioassay was assayed in two dilutions and parallelism with the standard dose response curve was present in each set of dilutions (Table 12.5).

12:5:2 Normal Subjects

Table 12.5 shows the fasting CASA levels in the serum of the normal subjects as measured by cytochemical bioassay. The mean fasting CASA level was 15.8 $\pm$ 1.6 pmol/l (SEM) whilst the gastrins measured with 2604/7 was 50.4 pmol/l and with G2 25.3 $\pm$ 2.8 pmol/l (Fig. 12.4)

The table shows that all fasting sera diluted out in parallel with the SHG-17-I dose response curve. This is evident by the similarity of the measurements at the two dilutions tested.

There was a significant correlation between the RIA gastrins measured with the two antisera in the fasting state ($y = 1.15 x + 41.5$, $r = 0.7837$ $p < 0.01$) (Fig. 12.5). However, there was no significant correlation between the RIA values and CASA values in the fasting sera (Fig. 12.5).

Following the meal there was an immediate rise of immunoreactive gastrins. The mean gastrin concentrations measured by the G2 antiserum were significantly raised above basal levels, 5 minutes after the meal ($p < 0.01$) whilst the values measured by 2604/7 were significanty raised above basal 15 minutes after the meal.

The immunoreactive gastrins remained high, reached a peak at 120 minutes and at 180 minutes, were still significantly raised above the basal values (Fig. 12.4).

In contrast CASA levels rose much more slowly after the meal. CASA was significantly raised above basal at 30 minutes and reached a peak 15.1 $\pm$ 4.4pmol/l above basal at 60 minutes. CASA then fell rapidly and by 180 minutes had returned to basal values.

There was no correlation between the RIA and CASA levels post-prandially (G2 vs. CASA, $r = 0.1056$, 2604/7 vs. CASA, $r = 0.0709$) (Fig. 12.5).

The RIA values measured by the two antisera however, showed significant correlation (2604 vs. G2; $y = 1.23x + 35.5$; $r = 0.8500$, $p < 0.001$).

All samples of the serum from the post-prandial period diluted out parallel to the standard gastrin dose response curve.

12:5:3 Hypergastrinaemic Patients

The CASA levels in the serum of the hypergastrinaemic patients are shown in Table 12:6. All diluted out in parallel with the standard G17-I gastrin curves. The fiducial limits for each measurement are shown in the Table. These varied from 63% to 164% (mean 74.5% to 135%). The CASA of these patients is compared to the CASA of the 10 fasting normal subjects in Fig. 12:6. There is a significantly raised CASA in the hypergastrinaemic group. However, CASA did not correlate significantly with the immunoreactive gastrins in the same patients, measured with the G2 antiserum (Fig. 12.6) ($r = 0.5010$, $p > 0.05$).

TABLE 12:5

FASTING CASA VALUES MEASURED BY CYTOCHEMICAL BIOASSAY

	SUBJECT	Cytochemical Bioassay (pmoles/ gastrin) 1:100	1:1 000	AVERAGE	FIDUCIAL LIMITS
1.	Ka	10	15.5	12.8	9 - 18
2.	Bo	15	17	16	11.5 - 22.5
3.	Ga	16	25	20.5	12.5 - 30
4.	Bre	25.5	29.5	27.5	22 - 37
5.	Go	17	14	15.5	11.5 - 20
6.	Ta	16	15	15.5	13.5 - 17.5
7.	Bri	10.5	11.5	11	9.5 - 13.5
8.	Ge	12	11	11.5	6.5 - 19
9.	Bra	10	14	12	7.5 - 17
10.	Kn	15	17	16	11 - 21
MEAN ± SEM				15.8 ± 1.6	

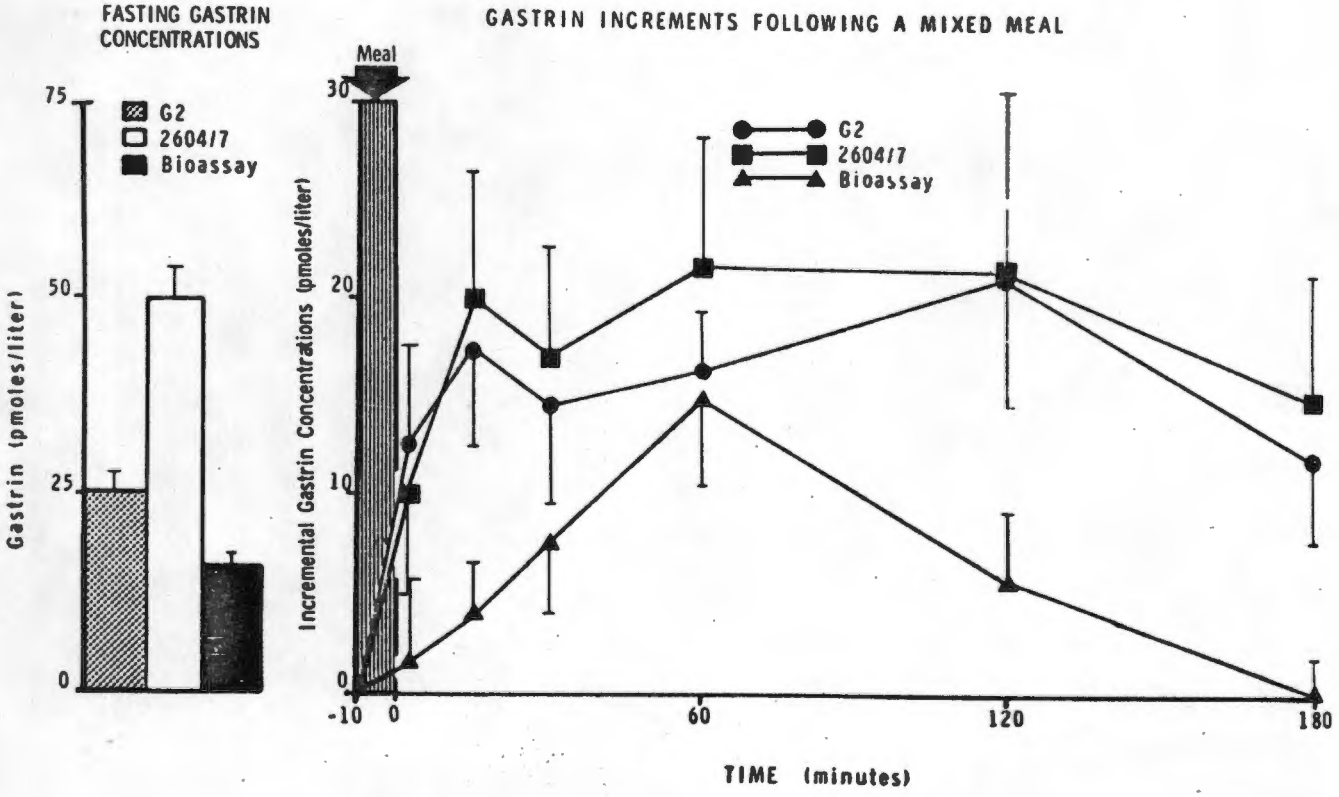


FIG. 12:4

The mean immunoreactive gastrin measured by the 2 antisera (G2 and 2604/7) and the CASA measured by the cytochemical bioassay in the fasting state and the incremental rise following the mixed meal. The points indicated are the mean ($\pm$ SEM) of 10 normal people, except at 5 minutes, where only 7 of the people were tested.

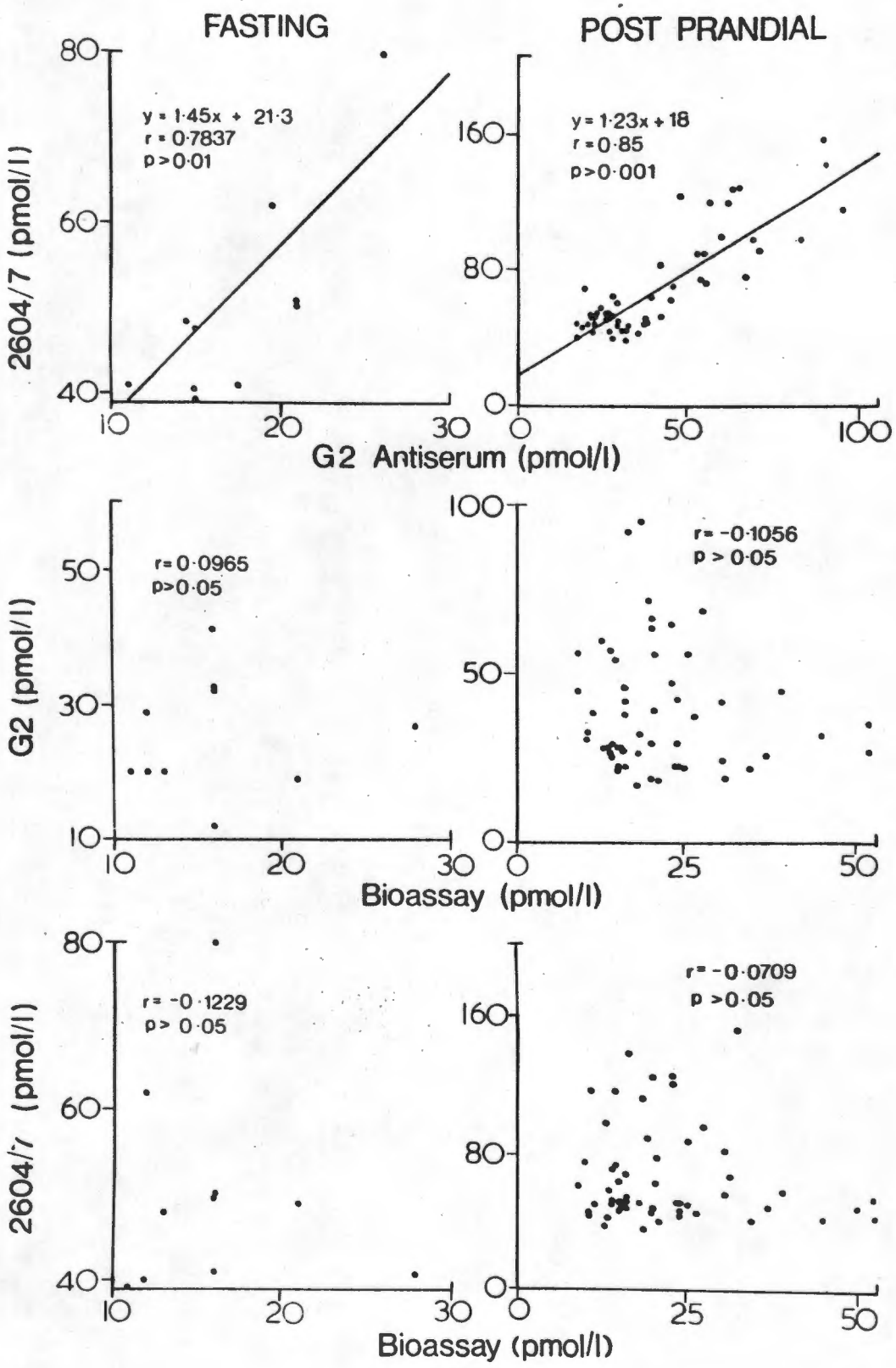


FIG. 12:5

Correlations between immunoreactive gastrins and CASA in fasting and post-prandial serum.

TABLE 12.7

CYTOCHEMICAL BIOASSAY MEASUREMENTS OF GASTRIN-LIKE ACTIVITY IN HYPERGASTRINAEMIC STATES DETERMINED BY RADIOIMMUNOASSAY

Subject	Clinical Presentation	Cytochemical Bioassay (pmol/l gastrin)				Radioimmunoassay (pmol/l)		Secretin Test	Comments
		1:1 000	1:10 000	Average	Fiducial Limits (95%)	Antisera 2604/7	G2		
BK	Multiple Ulcers	725	675	700	590-800	617	466	Not done	No tumour found -?occult
JA	Hypercalcaemia and multiple ulcers	125	140	133	100-165	-	123	Not done	MEA Type I with pancreatic tumour and hyperparathyroidism treated by total gastrectomy
RP	Multiple ulcers and hypercalcaemia	150	150	150	125-190	450	242	Positive	Hyperparathyroid tumour and gastrin-secreting tumour in tail of pancreas (MEA-I)
AU	Weight loss, gastric ulceration & diarrhoea	230	195	213	135-350	220	63	Not Done	Malignant Z.E. with liver metastases. Tumour secreting mainly sulphated gastrins.
GM	Steatorrhoea	250	240	245	190-310	-	645	Positive	Malignant Z.E. Primary tumour in pancreas (MEA Type-I)
VN	Hypercalcaemia and recurrent peptic ulcers	230	210	220	160-280	-	217	Positive	Gastrin normal after parathyroidectomy.
DG	Pernicious anaemia	90	120	105	70-150	434	245	Not done	On Vit B_{12} replacement
CD	Pernicious anaemia	350	350	350	275-400	795	342	Not Done	On Vit B_{12} replacement
CR	Dyskeratosis Conjenita	150	165	158	125-200	136	81	Not done	Fanconi-like anaemia. typical skin lesions, family history
AM	Pernicious anaemia	150	175	163	115-235	324	242	Not done	On Vit B_{12} replacement

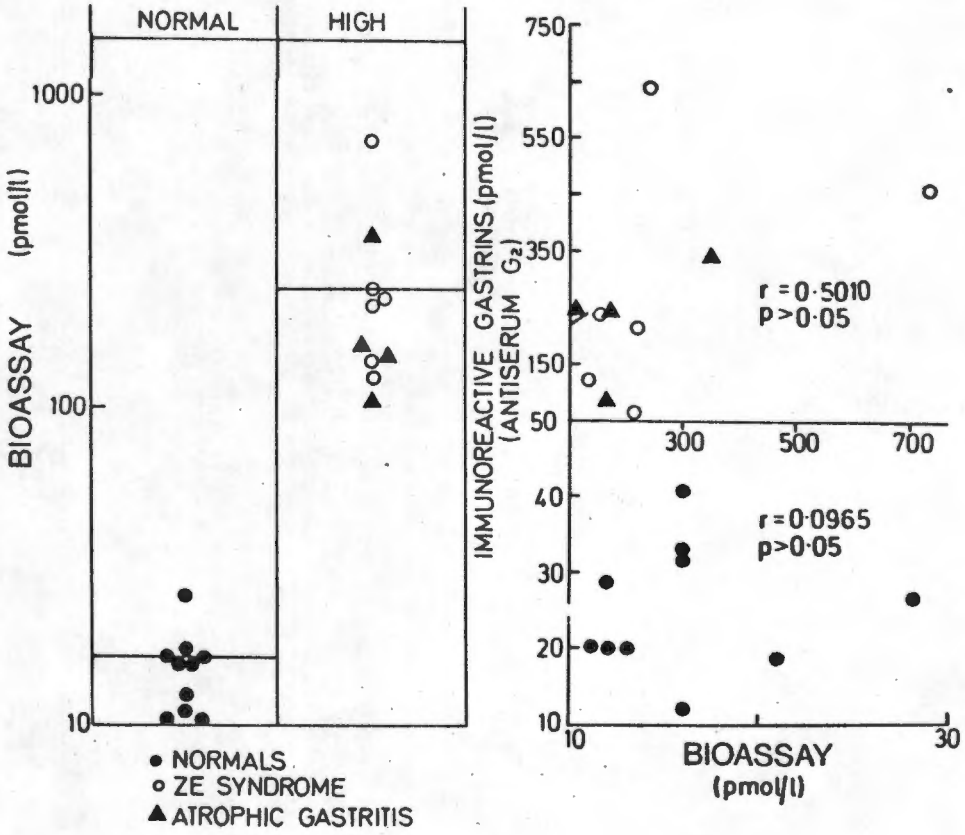


FIG. 12:6

Comparison between the serum CASA of normal fasting subjects and hypergastrinaemic subjects and the correlation between the immunoreactive gastrins and serum CASA in these subjects.

12:6 Discussion

The cytochemical section bioassay has streamlined the original segment bioassay described by Loveridge, Bloom et al, (1974). This was a tedious procedure in which segments of gastric mucosa were pre-incubated in tissue culture medium for 5 hours prior to treatment with different concentrations of gastrin. In contrast the section assay has the advantage of using a single segment of gastric mucosa for the entire assay, thus eliminating excessive variations between segments. In addition, starving the guinea pigs for 24 hours prior to killing eliminates significant variation in basal carbonic anhydrase (CA) activity and the response to gastrin stimulation is very reproducible. Thus the slopes of the standard curves varied less than 10% between assays whereas the interassay variation of a sample assayed on 6 occasions was 30% which is acceptable for a bioassay. The major problem however with the cytochemical bioassay in serum is that one is unsure of its specificity. Although it is unlikely that acetylcholine affects the assay as circulating levels are probably too low, histamine which activates CA in extremely low concentrations (Chapter 8), may well be present in serum, in concentrations high enough to have an effect on the CA activation. The finding of parallelism of the sample dilutions with the standard gastrin dose response does not exclude this possibility, as the mean slope of the histamine dose curve (Chapter 8) is not very dissimilar to the slope of the gastrin curves. In addition the use of affinity chromatography for gastrin may not have been totally specific for the reasons outlined.

In addition there is a possibility that the CA activity is affected by acid inhibitors present in serum. Indeed, Loveridge, Hoile et al, (1978) have shown that secretin competitively antagonizes the action of gastrin in the assay. Other possible enterogastrones have not been studied. For these reasons it was decided that rather than measuring biologically active gastrins in serum, this assay was measuring the carbonic anhydrase stimulatory activity (CASA) of serum.

The presence of the numerous substances in serum affecting acid secretion is almost certainly one of the reasons for the finding of a poor correlation between the immunoreactive gastrins released after a meal and acid secretion (Richardson, Walsch et al, 1976). Another possible reason is that secretagogues stimulate acid secretion via luminal, paracrine and neurocrine routes (Soll and Grossman, 1978), whilst a third reason may be altered sensitivity of the parietal cell mass to gastrins, as occurs in duodenal ulcer patients (Lam, Isenberg et al, 1980).

The findings in this study, of a lack of correlation between immunoreactive gastrins and CASA levels in serum are therefore not surprising, although they are at variance with those of Loveridge, Bloom et al, 1974.

Richardson, Walsch et al, (1977) reported that the acid response to modified sham feeding with gastric distension was similar to that occurring in the first 30 minutes after a meal, and was independent of a change in immunoreactive gastrins. The gastrin effect on acid secretion only became evident after 60 minutes.

These workers suggested that food may release inhibitors which counteract the acid secretory effect of gastrins. The findings of a slow rise in CASA after a meal which did not parallel the immediate response of immunoreactive gastrins would be in accord with this suggestion. Although the presence of inhibitors was not suggested by a loss in parallelism between the serial serum dilutions and the standard curve, this does not necessarily exclude the parallel dilution of an inhibitor, particularly as only two dilutions of serum were, in general, tested.

The more rapid fall in CASA levels compared with the RIA may be explained by changes in circulating forms of gastrins due to differences in half-life times. Following a meal, the concentration of G17 decreases more rapidly than G34. This was demonstrated by Dockray and Taylor (1976), who showed that by 120 minutes after a meal, G17 levels in the circulation were low, when the predominant remaining immunoreactive peptide was G34. As G17 is the predominant gastrin secreted after a meal (Lamers, Harrison et al, 1979), the fall noted in CASA levels may reflect the disappearance of G17 from the circulation.

Feldman, Walsch et al, (1978) found that an intravenous infusion of exogenous G17, which produced a serum concentration of G17 similar to that following an intragastric infusion of peptones, resulted in a similar acid response in both cases. Thus it appeared that G17 in serum could account for all the gastrin-stimulated acid secretion. It is of interest that these workers reported that the mean rise in G17 after a meal was 13 pmol/l, remarkably similar to the mean rise in this study (15 pmol/l).

A further possible cause for the rapid fall in CASA levels may be the presence of enterogastrones entering the circulation and causing inhibition of net secretory potency.

In the hypergastrinaemic patients, as in the normal subjects, there was no correlation between the CASA levels and the immunoreactive gastrin concentrations. It was however notable that these patients' sera in general had markedly increased CASA levels, suggesting that gastrins were the major contributing factor to the CASA in these patients.

It is possible that the cytochemical bioassay may prove to be a useful additional test in the diagnosis of Zollinger-Ellison syndrome in certain "border-line" patients. An interesting example would be that of patient A.U. She had the Zollinger-Ellison syndrome by acid criteria but the serum gastrin was only slightly raised in the routine radioimmunoassay (G2). However her CASA was markedly raised, and the use of a second antiserum with a wider cross-reactivity showed grossly increased serum gastrin levels. This patient appeared to be secreting mainly sulphated gastrins which have only 7% cross-reaction with the G2 antiserum (Appendix 1) (Napier, 1978). However, before the cytochemical bioassay can be used as a diagnostic test, further work is required to establish the mechanism of acid hypersecretion in the face of normal serum gastrins. (Bugat, Walsch et al, 1976). The cytochemical bioassay offers interesting prospects for these studies and may provide a tool for a better understanding of the functioning of parietal cells.

CHAPTER 13

SUMMARY OF CONCLUSIONS

The aim of this study was to use the technique of quantitative cytochemistry to study the effects of acid secretagogues on carbonic anhydrase (CA) activity in the parietal cells of guinea-pig fundic mucosa.

A specific cytochemical stain for CA activity was employed and the density of the staining products was measured on a Vickers M85 scanning microdensitometer. The two major acid secretagogues, gastrin (2.3×10^{-15} to 2.5×10^{-12} mol/l), histamine (5.8×10^{-17} to 1.0×10^{-14} mol/l) and the analogue of acetylcholine, carbamylcholine, (1.2×10^{-12} to 1.0×10^{-9} mol/l) caused linear increases in CA activity. The peak activation of CA induced by all three agents occurred after 90 seconds treatment of the sections and then declined in all cases.

Gastrin had the greatest efficacy in that the maximal CA activity reached following stimulation was approximately 50% higher than with either histamine or carbamylcholine. However the most potent secretagogue by comparing the D_{50} of the dose response curves, was histamine, which was approximately 100 times more potent than gastrin and 40,000 times more potent than carbamylcholine. Gastrin stimulated CA activity was inhibited by acetazolamide (10^{-5}M), by boiling the tissue, and by anoxia.

The addition of subthreshold doses of gastrin (2.5×10^{-16}M) and histamine (1.0×10^{-17}M) to the dose response curves of histamine and gastrin respectively caused a significant left-shift of each of the curves.

Gastrin increased both the efficacy and potency of the histamine response whereas histamine increased the potency of the gastrin. This suggests that histamine and gastrin potentiate each other and is consistent with the work of Soll (1978a, b, 1980), who studied oxygen and (^{14}C)-aminopyrine uptake in isolated canine parietal cells.

The addition of a subthreshold dose of histamine to the carbamylcholine dose response curve also caused a shift to the left of the curve, which was not significant, whereas gastrin had no effect on the carbamylcholine response. Atropine (10^{-5}M) inhibited the effect of carbamylcholine but had no effect on histamine or gastrin-stimulated CA activity. The histamine H_2-receptor blocker cimetidine (10^{-5}M) caused marked inhibition of the histamine effect ($76 \pm 7.8\%$), and slight inhibition of the gastrin effect ($23 \pm 4\%$) but no inhibition of carbamylcholine-stimulated CA activity. This suggests that gastrin and histamine act via pathways independent of the cholinergic pathways. The small inhibition of the gastrin effect by cimetidine may be due to inhibition of the effect of endogenous tissue histamine, necessary for the full gastrin effect. The lack of potentiation between histamine and carbamylcholine in this system cannot be explained but there may be species variations.

Lanthanum chloride (10^{-6}M) which acts by blocking calcium channels in membrane, inhibited carbamylcholine-stimulated activity by $77 \pm 8\%$, gastrin-stimulated activity by $45 \pm 10\%$ and histamine by $2 \pm 9\%$. This differential effect suggests that the secretagogues act at individual sites of action, possibly by separate mechanisms.

However, the potentiation of the gastrin and histamine effects suggests that these receptor sites may be inter-dependent in that they require both sites to be filled to achieve maximal effects.

The technique was further used to compare the potency of the gastrins at a cellular level. It appeared that the carboxy-terminal decapeptide was necessary for full biological potency to be present. G34 had a similar potency to G17 but the slopes of the dose response curves were not similar, suggesting possible differences in modes of action. The amino-terminal fragment of G17 lacked all biological activity.

Lastly, the method was used as originally described for the cyto-chemical bioassay of gastrin (Loveridge, Bloom et al, 1974). The reservations that this assay is actually specific for gastrin have been stated. Nevertheless, removal of gastrin from serum by affinity chromatography and charcoal adsorption removed all CA-stimulating activity (CASA) and suggests that the serum gastrin is an important component of serum CASA.

CASA activity increased equivalent to 15 pmol/l gastrin after a meal, which is similar to the rise in G17 documented by Feldman, Walsch et al, (1978), whilst the peak CASA activity which occurred at 60 minutes after the meal coincided with the time at which it has been calculated that gastrin has its greatest effect on acid secretion (Richardson, Walsh et al, 1977). However there was no correlation between the CASA and immunoreactivity in the serum of normal subjects, either fasting or post-prandial nor in hypergastrinaemic patients, although in the latter the CASA was higher than in the normal subjects.

This suggests that the serum CASA does not totally reflect the biological activity of circulating gastrins, and this may be due to the presence of other acid secretagogues in the circulation. Alternatively, the biological activity of the various gastrins may not be accurately reflected by measurements of immunoreactivity. In view of the finding that the CASA was higher in the hypergastrinaemic patients, it should be measured in diseases of acid hypersecretion not due to raised gastrin secretion, where it may provide a useful diagnostic test.

This thesis raises many questions and the conclusions can thus be considered as preliminary. Some of these questions are discussed in the next chapter.

UNIVERSITY OF CAPE TOWN

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A. Extracts from the Regulations for the Ph.D. Degree:

(a) General:

The degree of Doctor of Philosophy is a research degree. Candidates must proceed through any one of the faculties of the University in acoordance with the regulations for the degree of Ph.D. and any relevant faculty by-laws with which candidates are expected to familiarise themselves.

(b) Form of Thesis:

Each copy shall be printed or typewritten or otherwise suitably reproduced and shall be bound.

(c) Literary Presentation:

The literary presentation of the contents must be of a satisfactory standard as must also their statistical presentation if they include statistical argument or material.

(d) Content:

The thesis may embody only the original work of the candidate, with such acknowledged extracts from the work of others as may be pertinent. In presenting a thesis a candidate shall also submit a declaration by himself regarding the extent of which the thesis represents his own work, both in concept and execution. In those faculties where the by-laws so permit, and if the supervisor so approves but not otherwise, a candidate may publish work done by him under supervision as a candidate for the degree. Such published work may be included in or comprise the thesis in those cases where the thesis so presented contains a complete and connected account of all significant stages of the work of the candidate under supervision for the degree.

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In addition to his thesis a candidate may submit as collateral testimony any work published by him, on the understanding that such collateral testimony shall be considered by the examiners as elucidating or supporting the thesis but not as forming part of his work for the degree. In no case, however, may a candidate be given credit for any work that has been accepted for a degree at any university.

(f) Text of Thesis:

The text of the thesis must be prefaced by a brief summary of its contents indicating in what way it constitutes a contribution to knowledge.

B. The/ .....

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Examiners are requested to be guided by the provisions below and by the requirement that the thesis must make a distinct contribution to the knowledge of the subject and afford evidence of independent critical power in the handling and interpretation of material either known or newly discovered.

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The supervisor's report may, at the discretion of the relevant Committee of Assessors, as appointed by the Ph.D. Board, be sent to the examiners for their information along with the thesis. The report will be a general report on the candidates work, not concerned with assessing the quality of the work, but providing background information about the circumstances under which the thesis has been written.

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Examiners are requested to submit (i) a short concise report on the thesis (long reports are not required) and in addition (ii) a standard report on the attached form reflecting their final verdict.

It is desirable that the examiners should add to their recommendation a brief summary of the considerations that have led to it in terms intended for the guidance of members of the Committee of Assessors and Ph.D. Board who are not necessarily specialists in the subject of the thesis. Provision for such a summary is made on the reverse of the attached standard report form. (This summary should not be confused with the concise report requested under (i) above.)

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The examiners' reports on a candidate are submitted to the relevant Committee of Assessors for consideration and recommendation to the Ph.D. Board. If there is lack of consensus amongst the examiners, the Committee of Assessors may consult with the examiners (usually three in number), the supervisor and the head of the department or request the candidate to submit to an oral or other examination as considered necessary before reporting to the Ph.D. Board.

F. Report to Candidates and Confidentiality of Examiners' Reports:

It is the University's practice, in normal circumstances, to make the contents of the external examiner's report available in full to the candidate when the examination has been completed and the Ph.D. Board has decided whether or not to award the degree. However, discretion to vary this practice rests with the Board.

If the decision of the Ph.D. Board is to award the degree, it is also the University's normal practice to disclose the names of the examiners to the candidate when the result of the examination is known.

LKD/jm
17th July, 1980

CHAPTER 14

CRITIQUE AND FURTHER QUESTIONS

This thesis describes preliminary experiments which have explored the effects of the acid secretagogues on gastric parietal cell carbonic anhydrase (CA) activity. Dose-dependent increases of CA activity in response to gastrin, carbamylcholine and histamine have been demonstrated. The independent sites of action of the secretagogues are suggested by the specific inhibition of the carbamylcholine effect by the anticholinergic agent atropine and of the histamine effect by the histamine H_2-receptor blocker. The effect of gastrin, in contrast, is only slightly inhibited by cimetidine which may be due to inhibition of the effect of endogenous tissue histamine, as gastrin and histamine have been shown to potentiate each other. Carbamylcholine however, appears to act via a relatively independent cholinergic mechanism. Lastly, gastrin and carbamylcholine but not histamine activation of CA appears to depend upon calcium flux, again suggesting separate sites of action.

Thus this work in some respects confirms Soll's hypothesis (1978a, b, 1980), namely, that these agents have separate but interdependent receptors on the parietal cells. However this work differs from the findings of Soll in several respects. Firstly the gastrin effect at low concentrations did not depend upon the presence of a phosphodiesterase inhibitor or histamine. Secondly, Soll showed potentiation between histamine and carbamylcholine and although the data in this work tend to support this, the differences were not statistically significant.

Whilst these differences may relate to species differences, they open up further questions along which lines this work should proceed. The most important of these is to determine the physiological relevance of CA activation in these studies and whether it truly relates to physiological acid secretion. There is great difficulty in explaining the effects of these secretagogues at concentrations lower than that which normally elicits acid secretion in vivo. Thus the rise in gastrin levels after a meal causing submaximal acid secretion is in the range of 10 x 10^{-12}M. This concentration however, causes maximal CA activation in vitro. Furthermore, histamine requires even higher concentrations to cause an effect in vivo but in vitro, activates CA at lower concentrations than gastrin.

In addition, endogenous histamine occurs in gastric mucosa in microgram/gram wet wt. quantities and although the histamine concentration in the extracellular space is not known, it has been estimated that it may be present in the nanomolar range. Thus the dose effect noted here at lower concentrations of histamine, is difficult to explain. It is possible that the above estimate of histamine levels is not correct because of the rapid tissue degradation of this substance, whilst the reason for the effect being seen at low concentrations in vitro may be that this preparation provides direct access to the parietal cells, which may reduce degradation. Furthermore the concentrations of gastrin at tissue level are not known and these may be below the circulating blood levels. Alternatively, perhaps in this in vitro preparation there is an absence of inhibitory substances which may tend to desensitise the effects in vivo.

However, information on these subjects is scant, and further work is required. A suggested explanation for the in vitro effects at low concentrations of secretagogues is that water may be absorbed into cells as a result of loss of cell membrane integrity, leaving the actual concentrations much higher than this level. Whilst this is unlikely, monitoring the concentrations of an extra-cellular marker such as inulin or mannitol may offer further insight into this problem.

The transient nature of the secretagogue effects also needs to be explained. An auto-feedback mechanism has been postulated but this is speculative. The answers to both these two major questions may relate to the preparation per se. The question whether this preparation is viable, whether the cells are intact, and how long the tissue may be kept while remaining apparently functional, remain unanswered. It is even possible that the transience of the response represents a "last gasp" effect of dying tissue. Alternatively intracellular negative feedback mechanisms may be operative and further work should be done to explore the nature of both the "on" and "off" mechanisms of CA activation.

One might argue that parietal cells which are snap-frozen and then sectioned, are disrupted and not truly viable. Some evidence that this is not the case has been presented, notably the lack of a response during anoxia, and the effects of lanthanum on the different acid secretagogue actions. Furthermore, the work of Chayen and colleagues (Chayen, Bitensky and Butcher, 1973), lends support to the contention that this particular method of freezing maintains relative cellular integrity.

One must nevertheless ask the question whether these cells can be regarded as functionally viable or whether an isolated cellular event is being monitored in non-intact cells? Related to this argument is the fact that adenylyl-cyclase may stimulate cyclic-AMP production in non-intact cells. Thus an altered specificity may occur in broken cell preparations and may not have predictive value regarding the effects seen in intact cells. Thus until one is able to be sure that the cells are intact, one cannot make the assumption that this reflects stimulation of parietal cell acid secretory mechanisms.

Possible methods of answering these problems include the measurement of intracellular electrolyte concentrations and both transepithelial and intracellular electrical potentials, as an index of membrane integrity and thus the viability of the cells. However the best manner of showing the inter-relationship between physiological acid secretion and CA activation in this system may be to devise a parallel system where other indices of acid secretion such as oxygen uptake could be simultaneously monitored.

Thus this work is still inconclusive and opens up many questions in which direction further research should proceed. These include:

1) A clear demonstration of the viability or non-viability of these sections. If the latter is found, then an explanation as to the clearly specific acid secretagogue responses is required. This will have great relevance in the understanding of receptor interactions and mechanism of action of these agents.

2) Further experiments to determine the relationship between CA activation in this system and acid secretion by the parietal cell.

3) Further work to explain the transience of the secretagogue effect. If this is indeed an auto-feedback effect it is clearly of great physiological interest relating to the function and control of CA activity in parietal cells.

4) Further experiments to determine in full the nature of the CASA of serum. Although the experiments described here tend to suggest that gastrin is the predominant factor contributing towards CASA, measurements of histamine and acetylcholine in serum are required to ensure that these factors had not undergone degradation during the collection of the samples and subsequent tests.

5) Lastly, if the above questions are answered, there are several directions in which one could expand this study. The effects of acid inhibitors, including the prostaglandins and gut peptides on the parietal cells, and in addition the effects of calcium, c-AMP and other intracellular messengers may be studied.

Furthermore, the use of this technique, as in this thesis, to study secretagogue potencies and the CASA of serum should be further expanded, with special reference to diseases of gastric acid secretion, such as duodenal ulceration, especially the group of acid hyper-secretors with normal serum gastrin concentrations. This technique may thus help identify the presence of acid secretagogues other than those enumerated here and so thus lead to a greater understanding of the pathophysiology of these diseases, enabling us to rationalize our approach to prevention and treatment of this world-wide problem.

APPENDIX I

A: RADIOIMMUNOASSAY OF GASTRIN

Characteristics of Standards, Radiolabel and Antiserum

Immunoreactive gastrin was measured by modification of the method described by Yalow and Berson (1970). The standard used was synthetic human nonsulphated heptadecapeptide gastrin (SHG-17-I) which was freshly prepared for each assay and dissolved in 0.02M sodium barbital buffer pH 8.4, in concentrations ranging from 3-400 pmol/l. Radio-labelled (^{125}I) -G-17-I was purchased from CEA Sorin, France, and had a specific activity of 1000-1300 µCi/µg as determined by comparison of displacement of antibody-bound label by serial dilutions of the radiolabelled and cold gastrin.

Two separate antisera were used:

(a) Antiserum 2604/7 was a gift from Dr. J. Rehfeld of the Institute of Medical Biochemistry, Aarhus. It was raised by the immunization of a rabbit with human gastrin-I (2-17) conjugated to bovine serum albumin (Rehfeld, Stadil and Rubin, 1972) and was used in the assay in a final dilution of 1:100,000.

(b) Antiserum G2 was similarly raised in our laboratory (Napier, 1978), and used in the assay in a final dilution of 1:500,000. The characteristics of the two antisera are compared in the Table A. Bombesin was received by courtesy of Dr. Erspamer, Farmitalia, Milan, and VIP by courtesy of Dr. Said, Dallas, Texas.

The standard curves using antisera G2 and 2604/7 are shown in Fig.A The data was fitted to a logit/log plot of $B/_{Bo}$ versus hormone concentration and concentrations of gastrin obtained on a Hewlett Packard computer by linear regression analysis and substitution in the equation for a straight line.

Assay Procedure

The assay procedure was similar for both antisera. To each tube was added 700µl sodium barbital buffer pH 8.4, 100µl of sample or standard, 100 µl antiserum and 100µl of the radio-labelled hormone (approximately 5000 c.p.m.). The tubes were allowed to incubate for 24 hours at 4°C and free antigen was separated from antibody-bound antigen by precipitation with 500µl of 4% charcoal treated with dextran (0.4%) and centrifugation. The tubes were counted for 10 minutes or 10 000 counts. The non-specific binding of label to charcoal was subtracted from each value. The initial binding, the inter-assay coefficient of variation and the recovery of known amounts of NHG-I added to serum is shown in Table A.

B. PRODUCTION OF GASTRIN-FREE SERUM

(a) Charcoal-treatment of Serum

Activated charcoal added to serum adsorbs peptides and small molecules. In a 2% suspension the charcoal will strip the majority of gastrin-17 and 34 from serum but not Big-big gastrin (BBG) (Yalow and Wu, 1973). 20mg. of activated charcoal (Norit A) was added to 1 ml. of normal fasting serum. This was stirred for 1 hour at room temperature and left overnight to stand at 4°C. The suspension was then filtered and centrifuged. Gastrin immuno-reactivity measured by the G2 antiserum was reduced from 17.5 pmol/l to 12.5 pmol/l after treatment.

(b) Affinity Cromatography

Affinity chromatography is a type of adsorption chromatography in which the bed material has affinity for the substance to be isolated. The adsorptive properties of the bed material are obtained by covalently coupling an appropriate ligand binding substance to an insoluble matrix. The coupled ligand is able to bind the required substance from the samples which can then be eluted by altering the eluting fluid after the non-bound substances have passed through the column. Alternatively, if sufficient passes of the sample are made through the column one may obtain a sample free of the bound substance.

In order to remove gastrin-like immunoreactivity from serum, G9 antiserum, which was prepared by inoculation of a rabbit with SHG-17-I coupled to whelk haemacyanin (Napier, 1978), was coupled to CN-Br activated Sepharose 48 (Pharmacia Fine Chemicals, Uppsala, Sweden), using carbadimide as the coupling agent by the method of Cuatrecasas (1970). This antiserum recognized both synthetic and natural human gastrins G17 and G34.

A column of antibody-sepharose complex was poured into a 2ml plastic syringe with a glass wool base. One ml of fasting serum diluted 1:8 in 0.02M barbital buffer pH 8.4 was passed through the column in a continuous circulation over a period of 8 hours at 22°C by peristatic pump (8 passes). The diluted serum was then assayed by radio-immunoassay using the G2 antiserum and was found to be free of gastrin-like immunoreactivity.

TABLE A

COMPARISON OF THE RADIOIMMUNOASSAYS AND ANTISERUM CHARACTERISATION

	Antiserum 2604/7	Antiserum G2
Final Titre	1: 100 000	1:500 00
Standard	SHG-17-I	SHG-17-I
ID_{50} (pmol/litre)	46.7±1.9	50.2 ± 1.5
Detection limit (pmol/litre)	4.0	8.0
Initial binding (%)	38.3 ± 2.2	50.1 ± 0.73
Nonspecific binding (%)	1.8	1.8
Intraassay coefficient of variation (%)	8.3	6.8
Interassay coefficient of variation (%)	13.8	17.2
Recovery of 40pmol/l SHG-17-I added to serum	55%	85%
ID_{50} as % of standard		
NHG-17-I	61.9	100
NHG-17-II	46.4	7%
NHG-34-I	31.3	10%
CCK Octapeptide	1	10
Synthetic gastrin 8 - 17	37	100
Synthetic gastrin 1 - 13	0.001	0.02
Caerulein	0.7	5
Bombesin	0	0
Insulin	0	0
Glucagon	0	0
V.I.P.	0	0

The ID_{50} and initial binding is given as the mean ± SEM of 6 consecutive assays

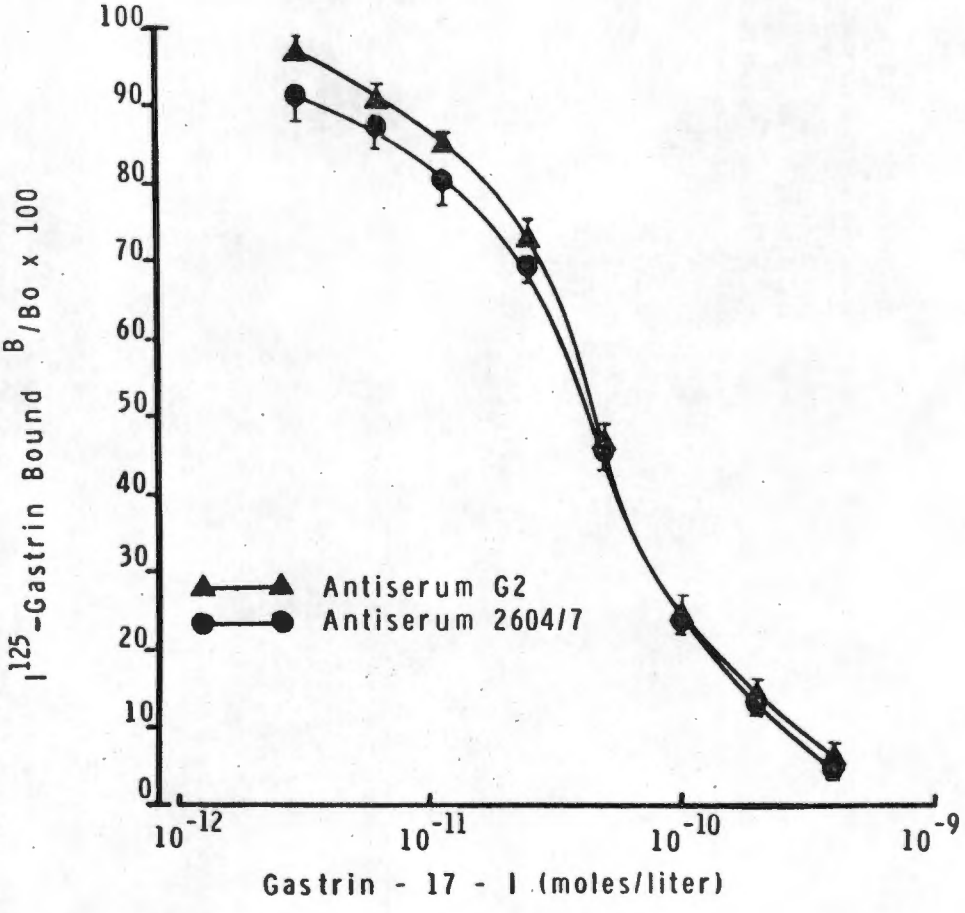

FIG. A Radioimmunoassay standard curves of gastrin using antiserum 2604/7 and G2

APPENDIX 2

STATISTICAL METHODS : (Snedecor and Cochran, 1967)

A MEAN

$$\bar{X} = \Sigma \frac{X}{n}$$

Where X = the observations
= = the sum of
n = the number of observations

B STANDARD DEVIATION (SD)

$$SD = \sqrt{\frac{\Sigma(X-\bar{X})^2}{n}}$$

C STANDARD ERROR OF THE MEAN

$$SEM = \sqrt{\frac{SD}{n}}$$

D COEFFICIENT OF VARIATION (CV)

$$= \frac{SD}{\bar{X}} \times 100$$

E EQUATION OF A STRAIGHT LINE

$$\hat{y} = \hat{a} + \hat{b}\,x$$

or

$$\hat{y} = \hat{c} + \hat{m}\,x$$

F CORRELATION COEFFICIENT

$$r = \sqrt{\frac{\Sigma(Y^2 - \bar{Y})^2}{\Sigma(Y - \bar{Y})^2}}$$

G SLOPE

$$\hat{b} \text{ (or } m) = \frac{n\Sigma XY - \Sigma X \Sigma Y}{n\Sigma X^2 - (\Sigma X)^2}$$

H INTERCEPT

$$\hat{a} \text{ (or } c) = \frac{\Sigma Y - \hat{b}\,\Sigma X}{n}$$

I STD DEVIATION SLOPE

$$S_b = \sqrt{\frac{\Sigma(Y - \hat{Y})^2}{n-2}} \qquad \sqrt{\frac{1}{(X - \bar{X})^2}}$$

J STD ERROR OF ESTIMATE

$$Sxy = \sqrt{\frac{\Sigma(Y - \hat{Y})^2}{n-2}}$$

K INDEX OF PRECISION

$$= Sxy/_b$$

L CONFIDENCE LIMITS

The confidence intervals about a calibration line represent parabolas above and below the lines with the uncertainties being smallest near the mid-point of the data used to establish the line and expanding near the extremes.

$$Y = \hat{a} + \hat{b}\,x \pm Sxy + \sqrt{\frac{1}{n} + \frac{(X - \bar{X})^2}{X^2 - n\,\bar{X}^2)}}$$

M STUDENT'S t-TEST

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(SD_1)^2}{n_1} + \frac{(SD_2)^2}{n_2}}}$$

N A - I & M were calculated on the Hewlett Packard statistics pac (9162 - 0050 Digital Cassette) on a Desktop Calculator Model 9830A.

J, K and L were calculated by a programme designed specifically for this thesis (see programme 1, page 204).

Comparison of regression lines for variance and parallelism was done by analysis of covariance (Snedecor and Cochran, 1967). (see programme 2, page 205).

In the determination of p , significance was accepted at the 5% level ($p \leq 0.05$).

(1). PROGRAMME FOR INDEX OF PRECISION AND 95% CONFIDENCE LIMITS

```
10 REM INDEX OF PRECISION
20 FIXED 3
30 DISP "HOW MANY SAMPLES";
40 INPUT L
50 A=0
60 FOR I=1 TO L
70 DISP "ENTER EXPECTED"I;
80 INPUT H
90 DISP "ENTER OBSERVED"I;
100 INPUT O
110 B=(O-H)↑2
120 A=A+B
130 NEXT I
140 E=SQR(A/(L-2))
150 PRINT "SXY="E
160 DISP "ENTER SLOPE";
170 INPUT M
180 PRINT "INDEX OF PRECISION="E/M
190 PRINT
200 DISP "CONFIDENCE LIMITS? Y=1;N=0";
210 INPUT C
220 IF C>0 THEN 240
230 GOTO 10
240 DISP "ENTER CALCULATED 0.005PG/ML";
250 INPUT F
260 PRINT "CONFIDENCE LIMITS"
270 GOTO 670
280 IF L=12 THEN 490
290 IF L=11 THEN 510
300 IF L=10 THEN 530
310 IF L=9 THEN 550
320 IF L=8 THEN 570
330 IF L=7 THEN 590
340 IF L=6 THEN 610
350 IF L=5 THEN 630
360 IF L=4 THEN 650
370 IF L>12 THEN 780
380 PRINT
390 PRINT "MOLES/LITRE","                MEAN","CONFIDENCE LIMITS"
400 FOR I=1 TO 4
410 X[I]=I-1
420 Y[I]=F+(M*X[I])
430 S[I]=E*T*(SQR((1/L)+((X[I]-B1)↑2)/(A9-(L*B1↑2))))
440 FIXED 1
450 PRINT "2.5*10E"I-16,Y[I];Y[I]-S[I]"TO"Y[I]+S[I]
460 NEXT I
470 PRINT
480 GOTO 10
490 T=2.228
500 GOTO 380
510 T=2.262
520 GOTO 380
530 T=2.306
540 GOTO 380
550 T=2.365
560 GOTO 380
570 T=2.447
580 GOTO 380
590 T=2.571
600 GOTO 380
610 T=2.776
620 GOTO 380
630 T=3.182
640 GOTO 380
650 T=4.303
660 GOTO 380
670 DISP "HOW MANY POINTS AT 5PG?";
680 INPUT A1
690 A6=A1*9
700 DISP "AT 0.5PG?";
710 INPUT A2
720 A7=4*A2
730 DISP "AT 0.05PG?";
740 INPUT A3
750 A9=A6+A7+A3
760 B1=((A1*3)+(A2*2)+A3)/L
770 GOTO 280
780 DISP "ENTER T"L-2"DEGREES FREEDOM";
790 INPUT T
800 GOTO 380
810 END
```

(2). PROGRAMME TO DETERMINE PARALLELISM BY ANALYSIS OF COVARIANCE

```
10 REM SIGNIFICANCE OF PARALLELISM
20 DISP "ENTER EQUATION 1(SLOPE,INTERCEPT)";
30 INPUT S1,E1
40 DISP "HOW MANY POINTS IN 1?";
50 INPUT N1
60 A=D=X=Y=H=0
70 PRINT "REGRESSION LINE 1----------"N1"POINTS"
80 PRINT "Y="S1"X+"E1
90 PRINT "OUTER","INNER"," X","OBSERVED Y","EXPECTED Y"
100 DISP "HOW MANY OUTER LOOPS?";
110 INPUT P1
120 FOR I=1 TO P1
130 PRINT
140 DISP "HOW MANY INNER LOOPS?";
150 INPUT P2
160 FOR L=1 TO P2
170 DISP "ENTER X,Y ("L")";
180 INPUT X[I,L],Y[I,L]
190 D[I,L]=(X[I,L])↑2
200 H[I,L]=(X[I,L])*(Y[I,L])
210 D=D[I,L]+D
220 X=X[I,L]+X
230 Y=Y[I,L]+Y
240 H=H[I,L]+H
250 A[I,L]=(S1*X[I,L])+E1
260 A=A+((A[I,L]-Y[I,L])↑2)
270 PRINT (I-1),L,X[I,L],Y[I,L],A[I,L]
280 NEXT L
290 NEXT I
300 PRINT
310 PRINT
320 DISP "ENTER EQUATION 2(SLOPE,INTERCEPT)";
330 INPUT S2,E2
340 DISP "HOW MANY POINTS IN 2?";
350 INPUT N2
360 PRINT
370 B=C=Q=R=Z=0
380 PRINT
390 PRINT "REGRESSION LINE 2----------"N2"POINTS"
400 PRINT "Y="S2"X+"E2
410 PRINT "OUTER","INNER","X","OBSERVED Y","EXPECTED Y"
420 DISP "HOW MANY OUTER LOOPS?";
430 INPUT N5
440 FOR J=1 TO N5
450 PRINT
460 DISP "HOW MANY INNER LOOPS?";
470 INPUT N6
480 FOR G=1 TO N6
490 DISP "ENTER X,Y("G")";
500 INPUT Q[J,G],R[J,G]
510 B[J,G]=(S2*Q[J,G])+E2
520 PRINT J-1,G,Q[J,G],R[J,G],B[J,G]
530 B=B+((B[J,G]-R[J,G])↑2)
540 C[J,G]=(Q[J,G])↑2
550 Z[J,G]=(Q[J,G])*(R[J,G])
560 C=C+C[J,G]
570 Q=Q+Q[J,G]
580 R=R+R[J,G]
590 Z=Z+Z[J,G]
600 NEXT G
610 NEXT J
620 PRINT
630 S3=((H-(X*Y/N1))+(Z-(R*Q/N2)))/((D-(X*X/N1))+(C-(Q*Q/N2)))
640 N3=N1+N2
650 E3=((Y+R)/N3)-(S3*((X+Q)/N3))
660 PRINT "POOLED REGRESSION LINE----------"N2+N1"POINTS"
670 PRINT "Y="S3"X+"E3
680 PRINT "DEVIATION SUM OF SQUARES EQUATION 1=A="A
690 PRINT "DEVIATION SUM OF SQUARES EQUATION 2=B="B
700 A5=A/(N1-2)
710 B5=B/(N2-2)
720 IF A5>B5 THEN 820
730 PRINT "VARIANCE F="B5/A5
740 PRINT "LOOK UP F FOR ("N2-2";"N1-2") DEGREES OF FREEDOM"
750 F5=((D-(X*X/N1))*(C-(Q*Q/N2)))*((S1-S2)↑2))/((D-(X*X/N1))+(C-(Q*Q/N2)))
760 PRINT "BETWEEN SLOPES SUM OF SQUARES=C="F5
770 F6=F5*((N3-4)/(A+B))
780 PRINT
790 PRINT "PARALLELISM F="F6
800 PRINT "LOOK UP F FOR (1,"(N3-4")")DEGREES OF FREEDOM"
810 END
820 PRINT "VARIANCE F="A5/B5
830 PRINT "LOOK UP F FOR ("N1-2","N2-2")DEGREES OF FREEDOM"
840 GOTO 750
```

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