AN ABSTRACT OF THE THESIS OF

ALBERT LOCK for the DOCTOR OF PHILOSOPHY
(Name of student) (Degree)
in Pharmacology (Toxicology) presented on 2/3/71
(Major) (Date)

Title: THE VAGAL INHIBITORY REFLEX IN THE RAT STOMACH

Redacted for privacy

Abstract approved:

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Electrical stimulation of the vagus nerves produces an excitatory contraction response in the isolated rat stomach. This contraction response was abolished by atropine to unmask the vagal inhibitory response (VIR) elicited during low frequency vagal stimulation. Bülbbring and Gershon (1967), after working on guinea-pigs and mice, hypothesized that both serotonin (5-HT) and acetylcholine (ACh) are neurotransmitters acting on the same ganglion cell in the vagal inhibitory pathway. This hypothesis for the role of 5-HT was tested in the rat after the VIR of the isolated stomach was characterized. The hypothesis was tested with 5-HT depletors, parachlorophenylalanine (PCPA) and reserpine, and a ganglionic blocking agent, hexamethonium (C6). If the hypothesized role for 5-HT is true, the depletion of 5-HT from stomach tissues should influence the VIR. Furthermore, if both 5-HT and ACh are neurotransmitters acting on the same ganglion cell in the vagal inhibitory pathway, the addition of C6
should only block the action of ACh but not the actions of 5-HT in the ganglia. Thus, vagal stimulation after the addition of C₆, in the presence of atropine, should still produce a VIR due to the action of 5-HT.

The VIR was present in all groups of rats tested and remained unchanged even though large amounts of 5-HT were depleted from the stomach tissues. The possibility still exists that the small amounts of 5-HT remaining after treatment with PCPA and reserpine could be sufficient to maintain the vagal inhibitory pathway. However, the VIR of the rat was completely abolished by C₆. These results indicate that serotonin is not involved either pre- or postganglionically in the vagal inhibitory pathway of the rat stomach.

Stimulation of the vagus nerves of the in situ rat stomach elicited a gastric relaxation response in contrast to the contraction response observed with the isolated stomach. This in situ relaxation response was elicited by stimulation of the intact vagi as well as by peripheral vagal stimulation. The in situ relaxation response was characterized and an effort was made to determine the relationship between the in situ relaxation response and the VIR. Some experimental conditions which may influence the in situ relaxation response were investigated. The influence of age and sex, lowered body temperature, starvation and partial evisceration by removal of the intestines, on the in situ relaxation response were examined. None of the above
conditions appeared to affect this response except the partial evisceration of the animal. The *in situ* relaxation response was abolished following the removal of the intestines and succeeding stimulation of the vagi elicited only gastric contractions. Subsequent treatment with guanethidine, reserpine and 6-hydroxydopamine (6-OHDA), all resulted in the reversal of the *in situ* relaxation response into a contraction response to vagal stimulation. Atropine abolished the contraction response to reveal the VIR. The evidence indicates that the *in situ* relaxation response is a composite response by three different gastric responses manifested in the following order of dominance: (1) an adrenergic inhibitory response, (2) a cholinergic excitatory response, (3) a non-adrenergic vagal inhibitory response.
The Vagal Inhibitory Reflex in
the Rat Stomach

by

Albert Lock

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1972
APPROVED:

Redacted for privacy

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Date thesis is presented

Typed by Opal Grossnicklaus for Albert Lock
ACKNOWLEDGEMENTS

Gratitude is extended to my family and friends, too numerous to mention individually, for their understanding, encouragement and moral support.

I would like to express special appreciation to my major professor and friend, Dr. Lavern J. Weber, for many hours of thought-provoking advice and enjoyable counsel throughout the course of this study.

Thanks and appreciation are extended to Drs. Robert E. Larson, Gregory B. Fink, Frederick L. Hisaw, Jr., Ronald H. Winters and Ian J. Tinsley for their helpful suggestions and efforts in planning my graduate program and preparation of this thesis.

Sincere thanks and appreciation are also extended to many of my fellow graduate students for their interesting and helpful discussions which have greatly influenced and stimulated my interest and thought.

Appreciation for invaluable technical assistance is extended to Miss Constance Lee Henderson.
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THE VAGAL INHIBITORY REFLEX OF THE RAT STOMACH

I. INTRODUCTION

This investigation was undertaken to study the vagal inhibitory reflex in the rat stomach. The possible role of serotonin as a neurotransmitter in the ganglia of the vagal inhibitory pathway was tested. Preliminary investigations indicated that the gastric response to in situ vagal stimulation was inhibitory while in vitro vagal stimulation was excitatory. An effort was made to determine the relationship of the relaxation response elicited by in situ stimulation of the vagi to the vagal inhibitory response (VIR).

A review of the literature on the vagal inhibitory reflex revealed that the mechanism and the physiological significance of this reflex is still unclear. Also, this phenomenon has not been as well characterized in the rat stomach as in other species.

The mammalian stomach is innervated by the parasympathetic and sympathetic divisions of the autonomic nervous system. The vagus nerves provide the parasympathetic supply to the stomach whereas the main sympathetic supply is provided by the splanchnic nerves.

According to the classical view, all preganglionic fibers in the ganglia are dependent upon acetylcholine (ACh) as the neurotransmitter as are the postganglionic parasympathetic nerve
terminals. The postganglionic sympathetic nerves are dependent upon norepinephrine (NE) as the neurotransmitter.

Acetylcholine (ACh) is present in the stomach wall and is released when the vagus nerves are stimulated to cause an excitatory contraction response. This cholinergic contraction response is abolished by atropine which is a competitive blocker of ACh (Dale and Feldberg, 1934).

Stimulation of postganglionic sympathetic fibers releases norepinephrine (NE) which causes an inhibition of gastric motility and a decrease in the tonus of gastric wall smooth muscles. The inhibitory response seen under appropriate conditions of vagal stimulation could be mediated by postganglionic sympathetic fibers that reach the vagus nerves from the sympathetic ganglia, or it could be a mechanism as yet not understood (Youmans, 1968).

The main sympathetic supply to the stomach descends from the hypothalamic region to the spinal segments T6 and T9 and passes through the ventral roots and white rami communicantes to the adjacent ganglia of the sympathetic trunk. Most of these fibers continue through the sympathetic trunk ganglia to synapse in the celiac, superior mesenteric and inferior mesenteric ganglia in the abdomen (Youmans, 1968). The postganglionic sympathetic fibers pass mainly from the celiac ganglia to the stomach along the left gastric artery (Lambert, 1965).
Although the vagus nerves contain mainly parasympathetic fibers to the stomach, some sympathetic and inhibitory fibers are also present (Texter et al., 1968). The vagus nerves synapse with neurons in the myenteric (Auerbach's) and submucosal (Meissner's) plexuses. The postganglionic fibers of these plexuses pass to other ganglion cells in the plexuses or to effector cells, i.e., secretory cells and smooth muscle fibers (Texter et al., 1968).

Many of the early studies on the effect of vagal stimulation on gastric motility were done during the latter part of the 19th century and the early decades of this century. After Bayliss and Starling (1899) suggested the classical hypothesis that gastric motility is regulated by a reciprocal action of the vagus and splanchnic nerves, Cannon and Lieb (1911) observed gastric relaxation after deglutition in dogs. They concluded that the relaxation response is a vagally controlled reflex since it was lost after bilateral vagotomy. They called this phenomenon "receptive relaxation." It was later suggested that the vagal inhibitory fibers may be part of a centrally induced reflex, called the vago-vagal inhibitory reflex by Harper et al. (1959), which is associated with Cannon and Lieb's "receptive relaxation" phenomenon (Martinson and Muren, 1963; Martinson, 1965; Jansson and Martinson, 1965).

While electrical stimulation of the vagus nerves generally produces muscular contractions, nerve stimulation may cause
muscle relaxation and inhibition of gastric movement (McSwinney, 1931). As early as 1889, Openchowski (as cited by McSwinney, 1931) working on rabbits, dogs and cats, suggested that the excitation of either an inhibitory or excitatory gastric response was dependent on the frequency and the strength of the electrical stimulation on the vagus nerves. Ten years later, Langley (1898) observed only gastric relaxations during electrical vagal stimulations after administration of atropine and curare to a rabbit in situ.

McSwinney and Wadge (1928) suggested that it was the initial level of smooth muscle tone which determines whether vagal stimulation will cause a gastric relaxation or a contraction response. Relaxation is manifested when the initial smooth muscle tone is high and contraction when the initial tone is low.

In 1936, Harrison and McSwinney, working on the cat and rabbit, proposed that the inhibitory effect on gastric motility during vagal stimulation may be due to adrenergic fibers located in the vagus nerves. However, the latent period of onset and the characteristics of the inhibitory response were unlike those of sympathetic relaxation responses. Thus, they expressed doubt as to whether the gastric relaxation obtained by vagal stimulation was partly or entirely mediated by adrenergic fibers. Nevertheless, the explanation that the vagal inhibitory response was due to adrenergic fibers in the vagus nerves was generally accepted and the vagal inhibitory
reflex received little attention for many years.

More recently, this hypothesis has been tested with more specific pharmacological agents in order to pharmacologically distinguish the two inhibitory systems. This has led to many inconsistent and variable results.

In 1956, Greeff and Holtz (cited by Martinson and Muren, 1963), using a guinea-pig stomach in vitro obtained gastric inhibition by vagal stimulation in the presence of atropine. They were able to abolish the inhibitory response with a sympatholytic drug, dihydroergotamine. They also abolished both the contraction and relaxation responses to vagal stimulation with the ganglionic blocking agent, hexamethonium.

However, using the cat stomach in situ, Martinson and Muren (1963) could not substantiate the effect of dihydroergotamine on the adrenergic inhibitory effect. They concluded that the adrenergic inhibitory effect of dihydroergotamine is doubtful in the cat in situ.

Then Greeff et al. (1962) working on the guinea pig stomach in vitro, reported a vagal inhibitory response after the addition of atropine which they called the "paradoxical vagus reversal effect." They reported that the inhibitory response was inhibited by a sympatholytic agent, Opilon (5-(2-Dimethylaminoethoxy)carvacol acetate) and an adrenergic neuronal blocking agent, bretylium. Three ganglionic blocking agents, hexamethonium (C6), azamethonium
and chlorisondamine, also inhibited the vagal inhibitory response. Furthermore reserpine and cocaine were also found to abolish the vagal inhibitory response. However, they reported that guanethidine, another adrenergic neuronal blocking agent, had no influence on the vagal reversal effect. They concluded from their data that the vagal inhibitory effect was due to an adrenergic response.

About the same time, Paton and Vane (1963), working with isolated stomachs of guinea-pigs, kittens, rats and mice also concluded that the vagal inhibitory fibers were adrenergic. They abolished the vagal inhibitory response with C6; reduced it with reserpine and TM 10 (choline 2,6-xylylether), a congener of bretylium, and enhanced the gastric relaxation with cocaine. They substantiated their conclusions by assaying stomach fluids before and after electrical excitation and found only acetylcholine (ACh) and "sympathin" neurotransmitter present.

However, the conclusion that the vagal inhibitory fibers are adrenergic in origin is incompatible with the interpretation by other investigators who could not substantiate the earlier studies and suggest that this reflex is non-adrenergic (Bennett et al., 1966a, 1966b; Burnstock et al., 1966; Martinson, 1965; Martinson and Muren, 1963). According to these investigators the relaxation responses elicited by non-adrenergic fibers can be distinguished from those of adrenergic fibers pharmacologically and also by their
time of onset and recovery periods. The typical sympathetically mediated inhibitory gastric response pattern can be elicited by a vagal stimulation of impulse frequencies of above 30 impulses per second, whereas the maximal non-adrenergic inhibitory response can be elicited at lower frequencies of approximately five impulses per second.

Burnstock et al. (1966) abolished the adrenergic inhibitory response with guanethidine and bretylium whereas the non-adrenergic inhibition obtained by low frequency vagal stimulation was not blocked by the above drugs. Martinson (1965) and Campbell (1966) reached the same conclusion after they found the vagal inhibitory fibers resistant to not only bretylium and guanethidine but also to alpha and beta adrenergic receptor blocking agents, phenoxybenzamine (POB) and nethalide respectively. This is substantiated by the conclusion that the vagal inhibitory fibers are non-adrenergic in origin.

Since the nature of the vagal inhibitory fibers is still controversial, the identity of the neurotransmitters mediating this reflex is likewise unclear. Bülbring and Gershon (1967) hypothesized that 5-hydroxytryptamine (5-HT, serotonin), along with acetylcholine (ACh), may be a neurotransmitter acting on the same ganglion cell in the vagal inhibitory pathway to the stomach. The following three observations suggested this hypothesis to them (Bülbring and Gershon, 1968). First, hexamethonium (C₆), a ganglionic blocking agent, only
partially blocks the relaxation response. This was interpreted to mean that another ganglionic transmitter, in addition to ACh, may be involved. Second, nerve terminals able to store 5-HT have been found in the myenteric plexus (Gershon and Ross, 1966). This location is suggestive of a neurotransmitter role for 5-HT in the myenteric plexus. Third, the effect of 5-HT on the in vitro guinea-pig stomach resembles the gastric response to vagal stimulation.

At the neuroeffector site, Burnstock et al. (1970) suggested that adenosine triphosphate (ATP) or a related nucleotide is the neurotransmitter substance released by non-adrenergic inhibitory fibers in the gastrointestinal tract. They used the same approach which Loewi (cited in Burnstock et al., 1970) applied to the cardiac vagus, i.e., stimulation of the nerve supply to a perfused organ and examination of the perfusate for substances which mimic the nerve action. They reported that ATP or some related nucleotide is the transmitter substance released during vagal stimulation of non-adrenergic inhibitory fibers. Su et al. (1971) confirmed the release of ATP upon activation of both non-adrenergic and adrenergic inhibitory nerves. They suggested that the features of ATP release, which may be unique to the non-adrenergic inhibitory neuroeffector system remains to be determined.

Baumgarten et al. (1970) identified three different types of neurons in the myenteric plexus: a cholinergic neuron, an adrenergic
type neuron and "p-type" neurons. The "p-type" fibers are named for their similarity to the polypeptide-storing neurosecretory nerves (Baumgarten et al., 1970). The nature of the substance stored in "p-type" fibers is still unknown. It is known that NE is bound to ATP in storage vesicles of sympathetic adrenergic nerves and adrenal medullary cells. If ATP is stored in "p-type" neurons, Baumgarten et al. (1970) suggests that they might be supposed to concentrate and store monamines.

The pharmacological agents used to distinguish the two inhibitory systems have variable effects on the ATP content of nerves associated with the inhibitory systems; this may be one of many factors contributing to the inconsistent conclusion of earlier investigators.

Many of the studies on the vagal inhibitory response reviewed thus far were done on in vitro stomach-vagus preparations or on in situ preparations where the vagus nerves were sectioned and stimulated peripherally. The vagus nerves also contain many afferent fibers which are concerned with the autonomic regulation of gastrointestinal function (Thomas and Baldwin, 1968). Some of the vagal efferent fibers may also be involved in the stomach's "receptive relaxation" phenomenon (Cannon and Lieb, 1911; Martinson and Muren, 1963; Martinson, 1965; Jansson and Martinson, 1965).

Gastric reflex relaxation in the cat, rabbit and dog can also be
produced by electrically stimulating a sectioned vagus nerve centrally while the opposite vagus nerve remained intact (Harper et al., 1959; Jansson, 1969; Ohga et al., 1969; 1970; Nakazato et al., 1970). As mentioned earlier, this reflex is called the vago-vagal reflex (Harper et al., 1959). Jansson (1969) suggests that this reflex originates, at least partially, in abdominal structures, with the efferent pathway consisting of inhibitory fibers. The inhibitory response to central vagal stimulation in the presence of C₆ was abolished while the response to peripheral vagal stimulation was reduced but never abolished (Ohga et al., 1969; 1970). They suggested that the afferent volley from the abdominal vagus nerves selectively induced reflex excitation of preganglionic elements in the efferent vagal inhibitory pathway. Thus, the inhibitory ganglion cells in this pathway may receive cholinergic preganglionic fibers and supply non-adrenergic postganglionic fibers to the stomach.
II. STATEMENT OF THE PROBLEM

This investigation was originally undertaken to test the hypothesis formulated by Bülbring and Gershon (1967) concerning the role of 5-HT in the vagal inhibitory pathway. Bülbring and Gershon (1967) confirmed the presence of non-adrenergic inhibitory fibers in the vagal inhibitory pathway to the stomach of guinea-pigs and mice. They also demonstrated that hexamethonium (C₆) decreased the magnitude of the vagal inhibitory response (VIR) in the guinea-pig but did not completely abolish it. They then proposed the hypothesis that both 5-HT and ACh, may be neurotransmitters acting on the same ganglion cells in the vagal inhibitory pathway to the stomach (Bülbring and Gershon, 1967; 1968). If this hypothesis is true, a depletion of 5-HT levels from stomach tissue should influence the vagal inhibitory response (VIR). Both parachlorophenylalanine (PCPA) and reserpine are known to decrease 5-HT levels from various tissues in the rat (Koe and Weissman, 1966; 1968; Erspamer, 1956). Therefore the role of 5-HT in the vagal inhibitory reflex of the rat stomach was investigated by using the 5-HT depletors, PCPA and reserpine, and a ganglionic blocking agent, C₆.

Recently, it was found possible to inhibit 5-HT synthesis
in vivo by the administration of PCPA which inhibits tryptophan hydroxylation by enzyme systems which convert tryptophan to 5-hydroxytryptophan (5-HTP). Since the 5-hydroxylation of tryptophan is the rate-limiting step in 5-HT formation (Udenfriend et al., 1957), prolonged blockade of the hydroxylating enzyme in vivo should cause depletion of tissue 5-HT (Koe and Weissman, 1966; 1968). Reserpine administered to animals causes a slow but progressive reduction of tissue 5-HT (Shore, Silver and Brodie, 1955; Pletscher, Shore and Brodie, 1955), as well as norepinephrine and other catecholamines (Carlsson, 1966). It is suggested that reserpine acts by inhibiting the transfer of the final products of 5-HT and catecholamine synthesis into the storage components, thus exposing these products to deamination by monoamine oxidases (Nickerson, 1970). However, reserpine is not a very effective depletor of amines in the stomach (Bennett, Bucknell and Dean, 1966; Erspamer, 1956). Since PCPA and reserpine deplete 5-HT by different mechanisms of action, they were administered together, as well as separately, in an attempt to further deplete 5-HT levels from rat stomach tissues.

Hexamethonium (C₆) is a ganglionic blocking agent against the actions of ACh liberated from presynaptic nerve endings within the autonomic ganglia (Volle and Koelle, 1970). Hexamethonium is a nicotinic blocking agent in the autonomic ganglia without producing
a concomitant change in the membrane potentials of ganglion cells (Volle and Koelle, 1970). If both 5-HT and ACh are neurotransmitters acting on the same ganglion cell of the vagal inhibitory pathway, the blockade of the ganglionic action of ACh by C₆ should not completely abolish the vagal inhibitory response since C₆ does not affect the action of 5-HT.

Little work has been done on the vagal inhibitory reflex of the rat. Paton and Vane (1963) reported an in vitro rat stomach vagal inhibitory response (VIR) in the presence of hyoscine and eserine. However, the gastric relaxation response showed a long recovery period of approximately 20 minutes which is not characteristic of the VIR described for other animals, but is more supportive of a sympathetically mediated gastric inhibitory response. Campbell (1966) reported that the VIR of the guinea-pig has a very brief delay of less than three seconds before recovery to the baseline which is abrupt in onset and develops rapidly.

In view of the controversy regarding the nervous mechanism responsible for the VIR, i.e., whether it is adrenergic or non-adrenergic, and also the discrepancy in the recovery characteristics of the VIR reported by Paton and Vane (1963) for the in vitro rat stomach, as opposed to those reported for guinea-pigs (Campbell, 1966), it was evident that the VIR must first be elucidated for that animal before the hypothesis proposed by Bülbring and Gershon (1967)
can be tested in the rat.

Species differences have been found regarding the occurrence and number of adrenergic nerves in tissues (Norberg, 1967) and this may account for the inconsistency between the VIR of the rat and guinea-pig.

In order to investigate experimental conditions which may cause the activation or alteration of the rat VIR, it was necessary to perform these experiments on the in situ rat stomach. In 1965, Holman and Hughes suggested that gastrointestinal smooth muscles of the rat, mouse, guinea-pig and rabbit are supplied with non-adrenergic inhibitory neurones. This in vivo inhibitory response remains to be clarified in the rat. Thus far, little or no work has been done on the VIR of the in situ rat stomach.

It was found that electrical vagal stimulation produced a gastric relaxation response in situ as opposed to only gastric contractions seen during vagal stimulation of the in vitro rat stomach. The question then arose as to whether the in situ rat gastric relaxation response was adrenergic or non-adrenergic or perhaps the result of a combination of opposing inhibitory and excitatory responses. An attempt was made to answer this question as well as trying to characterize the in vitro VIR and test the hypothesis proposed by Bülbring and Gershon (1967).
III. METHODS

Isolated Stomach - Vagus Nerve Preparation

The method for the in vitro stomach nerve preparation was similar to that described for guinea-pigs by Campbell (1966), with some modifications for the rat. Male Sprague-Dawley rats weighing between 200 and 300 grams were used. The animals were killed by crushing the spinal cord. They were then immediately exsanguinated. The stomach, with the first centimeter of the duodenum and the esophagus with the vagus nerves in the surrounding connective tissue were dissected out as a unit. The fat and pancreatic tissues were removed. A polyvinyl cannula (P. E. 240) was inserted through the end of the duodenum and fixed with a ligature tied around the pylorus. The end of the cannula extended well into the antrum. Then the stomach was washed out with warmed modified Krebs-Henseleit (Krebs) solution which was also used for the isolated organ bath. The Krebs solution consisted of sodium chloride (115.3 mM), potassium chloride (4.69 mM), calcium chloride dihydrate (1.36 mM), magnesium sulfate (1.16 mM), potassium dihydrogen phosphate (1.18 mM), sodium bicarbonate (22.14 mM) and glucose (7.88 mM). The salts and glucose were dissolved in doubly distilled water and stored at a temperature of about 4°C prior to use.
The vagi were carefully dissected from the esophagus and the stomach was then placed in a 100 ml Krebs bath, maintained at 37° C, through which oxygen was continuously bubbled. One ml of warmed Krebs solution was then introduced into the stomach via the pyloric cannula which was then connected to a Statham physiological low pressure transducer (model P23BB). A Gilson polygraph was used to record the changes in the intraluminal stomach pressure at a chart speed of 0.5 mm per second. All recordings were obtained using the same amplifier sensitivity set at 0.1 m. v. on the polygraph unless otherwise specified in the results. No attempt was made to quantify differences in magnitudes of gastric responses other than by gross observation.

The vagus nerves were electrically stimulated through a pair of silver electrodes. The vagi were stimulated with 10 second bursts of square wave pulses of 5 msec duration generated by a Grass stimulator (model S4K). The voltage and frequencies used were varied for each stomach until a maximal cholinergic response was obtained. Then atropine was added to block the classical cholinergic contractile response to low frequency vagal stimulation in order to unmask the vagal relaxation response. The final atropine concentration of the Krebs bath was $2 \times 10^{-7}$ g/ml.
In Situ Rat Stomach Preparation

Presurgical Procedures

Food was withheld from the rats employed in these experiments for 24 to 48 hours. The animals were housed in a suspended wire cage, thus, coprophagia did not complicate the status of the gastrointestinal tract. Water was available ad lib. At the time of the experiment, sodium pentobarbital (60 mg/kg/i.p.) was used for induction and maintenance of general anesthesia. The ventral areas of the neck, thorax and abdomen were shaved. The animal was then placed in dorsal recumbancy with its limbs extended on an operating board. The trachea was cannulated and the animal's respiration maintained by a small animal respirator (Phipps and Bird, Inc. no. 7088-600).

Abdominal Surgery

A midventral incision was made into the abdominal cavity extending from the xyphoid process to the lower abdominal region. The left lateral lobe of the liver was ligated and removed to facilitate stomach cannulation. A small transverse incision was made into the duodenum 1 cm caudal to the pyloric sphincter. A polyvinyl cannula (P.E. 240) was inserted through the transverse incision into the stomach and fastened in place between the incision and the pyloric
sphincter. The cannula was connected to a Statham physiological low pressure transducer (model P23BB) which was in turn attached to a Gilson polygraph. One ml of warmed tap water was introduced into the stomach via the cannula. A thin sheet of transparent plastic covering was placed over the abdominal cavity to minimize the loss of body heat and tissue dehydration.

Thoracic Surgery

The midventral incision was extended over the entire length of the sternum which was then cut longitudinally and separated by the use of a blepharostat retractor. Then the left and right phrenic nerves to the diaphragm were isolated and cut. The thoracic esophagus caudal to the lung was separated from the adjoining structures by blunt dissection. The two vagus nerve trunks were separated from the esophagus, using extreme caution to prevent excess trauma to them. A length of silk suture was tied loosely around the vagi in order to facilitate lifting of the nerves onto a pair of silver electrodes positioned above the esophagus and connected to a Grass stimulator (model S4K). The diaphragm was freed by cutting it along the lateral body walls. The blepharostat retractor was removed to minimize the thoracic opening size in order to prevent excess loss of moisture and body heat.
Serotonin Assay of Rat Stomach

5-HT Depletion Procedure

As discussed earlier, reserpine and parachlorophenylalanine (PCPA) both deplete 5-HT tissue levels but by different mechanisms of action. Thus the animals' 5-HT tissue levels were not only deplete by each drug separately, but some animals received both reserpine (Serpasil-Ciba) and PCPA (Aldrich Co.) concurrently intraperitoneally (i.p.) in an attempt to deplete the 5-HT levels even further. The PCPA was suspended in a 1% Tween 80 aqueous solution.

The rats were divided into four groups of 12 animals each for pretreatment. Group I, the control group, received only the 1% Tween 80. Group II received PCPA (200 mg/kg). Group III received reserpine (1 mg/kg). Group IV received both PCPA (200 mg/kg) and reserpine (1 mg/kg). The PCPA was administered once daily i.p. for five days. Reserpine was administered once daily, i.p., for five days; the final dose given four hours prior to sacrifice. Food was withheld from animals for 16 to 24 hours before sacrifice on day six but water was available ad lib throughout the pretreatment period. Animals were killed by crushing the spinal cord and immediately exsanguinated. Stomachs of six animals from each group were used to determine the quantity of tissue 5 HT. The stomachs from the
remaining six rats from each group were removed and used in testing for the presence of the vagal inhibitory reflex. The 5-HT content varies greatly within an individual stomach (Thompson and Campbell, 1967; Weber, 1970). Thus, the antrum and the corpus of the stomach were assayed fluorometrically along with the brain as a positive control (Udenfriend et al., 1958).

The 5-HT Extraction and Assay Procedure

In general, the following 5-HT extraction and assay procedure of Udenfriend et al. (1958) was used, including some modifications, as follows: one part brain tissue was homogenized in two parts 0.1N HCl while one part stomach tissue was used with 19 parts 0.1N HCl. A 2 ml aliquot of homogenate was transferred to a 50 ml centrifuge tube containing 2.5 ml borate buffer (pH 10), approximately 3 to 5 g NaCl and 15 ml n-butanol. The mixture was then shaken for 10 minutes and then centrifuged (International Equipment, Universal Model UV) for ten minutes at 1500 rpm. Then 10 mls of the n-butanol phase was transferred into another 50 ml centrifuge tube containing 10 ml heptane and 2.5 ml 0.1N HCl and shaken for ten minutes and then centrifuged at 1500 rpm for ten minutes. The heptane-butanol layer was aspirated and discarded. Two mls of the remaining acid layer was transferred to culture tube to which 0.6 ml of 12N HCl was then added. This mixture was transferred into a quartz cuvette for 5-HT analysis.
Serotonin analysis was conducted with a Fluoro-microphotometer (American Instrument Co.). The solution was activated using a narrow band filter of between 280 and 300 \text{nm} and the resultant fluorescence was measured at above 515 \text{nm}.

Reagent grade n-butanol (Baker Co.) and heptane (Baker Co.) were used in the extraction procedure without further treatment.

The borate buffer was prepared according to Udenfriend et al. (1958).
IV. RESULTS AND DISCUSSION

VIR in vitro

Guinea-pig Stomach

The evidence for the existence of an adrenergic and a non-adrenergic inhibitory innervation of the stomach is based largely on studies which used the isolated organs or tissues of the guinea-pig (Bülbring and Gershon, 1967; Campbell, 1966; Paton and Vane, 1963; Greeff et al., 1962). Therefore, for the purposes of these investigations, the vagal inhibitory response (VIR) was determined in the in vitro guinea-pig stomach preparation first for comparison with that of the rat stomach in vitro.

Figure 1 shows a guinea-pig stomach response to graded increments in impulse frequency per second during a vagal stimulation period of ten seconds. The impulse duration and voltages were kept constant at 5 msec and 6 v, respectively. Vagal stimulation elicited an abrupt contraction followed by a rapid recovery to the original baseline upon cessation of electrical stimulation. Frequently, the recovery of the contraction response occurred prior to the end of the ten-second stimulation period; followed by a slight rebound after-relaxation.

The nature of the gastric response to vagal stimulation depends,
in part, on the initial tone and distention of the stomach and also on
the frequency of vagal stimulation (Bülbring and Gershon, 1968). It
has been proposed that the vagus nerves contain both excitatory and
inhibitory fibers and with low stimulation frequencies, the excitatory
component is dominant and may mask any relaxation response
(Bülbring and Gershon, 1967; 1968). According to Bülbring and
Gershon (1968), the inhibitory component becomes more prominent
with increasing stimulation frequencies and the gastric response
becomes diphasic, consisting of a pronounced relaxation interrupted
by a rapid, short-lived contraction. However, this diphasic gastric
response was not observed by this investigator (Figure 1).

Figure 2 shows that the contraction response to vagal stimulation
was blocked by atropine to reveal an underlying, non-atropine sensi-
tive inhibitory relaxation response to a low frequency stimulation of
five impulses per second (5/s). This relaxation response is referred
to hereafter as the vagal inhibitory response (VIR).

The gastric relaxation response to dimethylphenylpiperazinium
iodide (DMPP, $5 \times 10^{-6}$ g/ml), and to 5-HT ($1 \times 10^{-7}$ g/ml), in the
presence of atropine, is shown in Figure 2. In fact, the 5-HT induced
relaxation response which was observed is one of the factors which
lead to the formation of the hypothesis by Bülbring and Gershon
(1967). However, as was subsequently observed, the addition of
5-HT to the rat stomach in vitro produced only contractions. Both
Figure 1. Guinea-pig stomach in vitro. Responses to vagal stimulations of graded increments in impulse frequency (no./s). Note the initial rapid contraction present in all responses and the concomitant absence of an initial relaxation response prior to contraction. Stimulation periods, 10 sec; pulse duration, 5 msec; voltage, 6 v.
Figure 2. Guinea-pig stomach in vitro. Responses to low frequency (5/s) vagal stimulation before and after atropine (2x10^{-7} g/ml), dimethylphenylpiperazinium iodide (DMPP, 5x10^{-6} g/ml) and 5-hydroxytryptamine (5-HT, 1x10^{-7} g/ml). Note the unmasking of the vagal inhibitory response after atropine and also the slow onset and recovery periods of the DMPP and 5-HT induced relaxations. (W = wash). Stimulation period, 10 sec; impulse duration, 5 msec; voltage, 6 v.
5-HT and DMPP, a ganglionic stimulant, produced gastric relaxation responses in the guinea-pig with slower onset and recovery times than in responses observed during low frequency vagal stimulations in the presence of atropine. The longer latency of onset and recovery periods are not consistent with the characteristics of the VIR observed during low frequency vagal stimulation.

Figure 3 shows the guinea-pig VIR before and after the addition of C6 and then atropine. Vagal stimulation using both low and high impulse frequencies, 5/s and 30/s respectively, produced contractions which were abolished by C6 (4x10^{-5} g/ml). Subsequent vagal stimulations elicited relaxation responses which were not abolished, even after doubling the concentration of the C6 (8x10^{-5} g/ml) as shown in Figure 3.

The gastric relaxation response to vagal stimulation in the presence of C6 does not substantiate reports by other workers (Paton and Vane, 1963; Greef and Holtz, 1956). Greef and Holtz (1956) showed that C6 reduced but did not completely abolish gastric contraction responses to vagal stimulation whereas Paton and Vane (1963) abolished the contraction response but did not produce a relaxation response in the guinea-pig. Atropine (2x10^{-7} g/ml) was then added to the bath solution and low frequency vagal stimulation (5/s) one minute later still produced a relaxation response. However, by eight minutes, this response was greatly diminished and it was
Figure 3. Guinea-pig stomach in vitro. Responses to low (5/s) and high (30/s) frequency vagal stimulations both before and after hexamethonium (C₆, 8x10⁻⁵ g/ml); plus responses 1, 8 and 15 minutes after further addition of atropine (2x10⁻⁷ g/ml). (W = wash). Note the relaxation responses after C₆ and their subsequent abolition within 15 minutes after further addition of atropine. Stimulation periods, 10 sec; impulse duration, 5 msec; voltage, 6v.
Atropine (1 min)
finally completely abolished by 15 minutes after the addition of atropine. The $C_6$ was then washed out (W) and vagal stimulation once again elicited a relaxation response (Figure 3). The irradication of the VIR by $C_6$, in the presence of atropine, suggests that ACh is the only ganglionic neurotransmitter in the vagal inhibitory reflex of the guinea-pig stomach. This observation with $C_6$, in the presence of atropine, does not substantiate reports by Paton and Vane (1963). They abolished the guinea-pig VIR with $C_6$ ($4 \times 10^{-5}$ g/ml) only and reported a persistent reduced relaxation response in the presence of both atropine ($2 \times 10^{-7}$ g/ml) and $C_6$ ($8 \times 10^{-7}$ g/ml). They attributed the persistent residual relaxation response to the excitation of post-ganglionic sympathetic fibers in the wall of the esophagus during vagal stimulation.

Since vagal stimulation, in the presence of $C_6$ alone, produced gastric relaxation responses (Figure 3) which were abolished after the addition of atropine. The possibility exists for a muscarinic inhibitory response mediated by ACh at the ganglia or at postganglionic muscarinic sites which is sensitive to atropine. Perhaps this possibility of a ganglionic muscarinic inhibitory pathway could be tested with a muscarinic ganglionic stimulant. $4$-(m-chlorophenylcarbamoyloxy)-2-butynyl-trimethylammonium chloride (McNeil-A-343), in the presence of $C_6$. 
Rat Stomach

**Determination of Values for Electrical Parameters**

Little work has been reported on the characteristics of the rat VIR. Thus, the values for the electrical parameters for vagal stimulation necessary to elicit the desired maximal excitatory and inhibitory responses were first determined.

The contraction response to vagal stimulation as a function of impulse frequency is shown in Figure 4. The maximal contraction response was attained at 9/s; further increases beyond 9/s resulted in a gradual decrease in contraction magnitude. However, the impulse frequency value for the maximal response among different preparations ranged between 7/s (Figure 6) to 15/s (Figure 5). Infrequently, contraction responses are followed by small after-relaxations with a long recovery period as illustrated in Figure 6.

Figure 7 shows that the maximal contraction was obtained with an impulse duration of 5 msec. The impulse frequency and voltage were held constant at 5/s and 8 v respectively. The vagus nerves were stimulated for ten seconds each time.

The gastric responses of the rat to increasing voltages of vagal stimulation are shown in Figure 8. The impulse frequency and duration were held constant at 5/s and 6 msec respectively. The maximal response was obtained by a 6 volt stimulation of the vagus nerves.
Figure 4. Rat stomach in vitro. Responses to vagal stimulations of graded increments in impulse frequency. Note the gradual decrease in contraction magnitudes beyond the maximal response, obtained at 9/s and also the absence of both initial relaxation responses and rebound after-relaxations. Stimulation periods, 10 sec; impulse duration, 5 msec; voltage, 6 v.
Figure 5. Rat stomach *in vitro*. Responses to vagal stimulations of graded increments in impulse frequency. Note the maximal contraction response, obtained at 15/s, and the absence of both initial relaxation responses and rebound after-relaxations. Stimulation periods, 10 sec; impulse duration, 5 msec; voltage 6 v.
Figure 6. Rat stomach *in vitro*. Responses to vagal stimulations of graded increments in impulse frequency. Note the maximal contraction response, obtained at 7/s, and the absence of an initial relaxation response. A slight rebound after-relaxation is present beyond stimulations of 3/s. Stimulation periods, 10 sec; impulse duration, 5 msec; voltage 6v.
Figure 7. Rat stomach in vitro. Responses to vagal stimulations of graded increments in impulse durations (msec). Note the maximal contraction obtained with impulse duration of 5 msec. Stimulation periods, 10 sec; impulse frequency, 5/s; voltage, 8v.
A. 5/s, 8v

Pulse duration (msec)
Figure 8. Rat stomach in vitro. Responses as a function of vagal stimulation voltage. Note maximal contraction obtained with 6v. Stimulation period, 10 sec; impulse frequency, 5/s; impulse duration, 6 msec.
B. 5/s, 6 msec

Voltage

2v  4v  6v  10v  20v  30v

10 sec
The maximal contraction responses are most often obtained with vagal stimulations using a 5 msec impulse duration and 6 volt intensity. The electrical parameters were adjusted to elicit a maximal response in each preparation.

Atropine abolished the rat gastric contraction response which revealed the VIR on vagal stimulation shown in Figure 9. Low frequency stimulation after atropine \((2 \times 10^{-7} \text{ g/ml})\) elicited a rapid gastric relaxation followed by a rapid recovery or cessation of vagal stimulation of the rat stomach. These VIR characteristics are similar to those reported for the guinea-pig (Campbell, 1966). In the presence of atropine, high frequency stimulation usually produced a profound relaxation followed by a prolonged recovery period. This response is considered a characteristic sympathetic inhibitory response which is obtained with high frequency vagal stimulation (Burnstock et al., 1966). This gastric relaxation response greatly resembles the response interpreted by Paton and Vane (1963) to be the VIR of the rat.

In this study, the maximal cholinergic contraction response and the VIR, seen after the addition of atropine, were elicited by using low frequency stimulations of less than 7/s. The sympathetic inhibitory responses were elicited using stimulation frequencies of 30/s or higher.

After the electrical parameter values for the maximal
Figure 9. Rat stomach in vitro. Responses to low (3/s) and high (30/s) impulse frequency stimulation both before and after the addition of atropine (2x10^{-7} g/ml). Note the abolition of the contraction response at 3/s after the addition of atropine which revealed the vagal inhibitory response and also the characteristic sympathetic inhibitory response obtained with high frequency (30/s) stimulation. Stimulation period, 10 sec; impulse duration, 5 msec; voltage, 6v.
contraction response were obtained with vagal stimulations of 5 msec duration and approximately 6 volts, the \textit{in vitro} rat stomach VIR was determined in the presence of atropine \((2\times10^{-7}\text{g/ml})\). Figure 10 shows the effect of various impulse frequencies on gastric relaxation. The maximal VIR frequency threshold is approximately analogous to those which elicited the maximal contraction response. However, Martinson and Muren (1963), working on the \textit{in situ} cat stomach, found that the maximal gastric contraction response had a shorter impulse duration threshold than required for the VIR. The maximal VIR was reached at approximately \(7/s\) to \(9/s\). The characteristic sympathetic inhibitory response was elicited with high frequency stimulation of \(30/s\) as shown in Figure 10. For this study, the relaxation responses elicited by impulse frequencies between \(7/s\) and \(30/s\) were considered responses elicited by a mixture of vagal inhibitory and sympathetic fibers. Thus, the impulse frequency used to elicit both the maximal contraction response and the VIR for this study was set at \(7/s\) and the maximal sympathetic relaxation response was elicited at an impulse frequency of at least \(30/s\).

\textbf{Experimental Factors Affecting the VIR}

After the electrical parameters necessary to characterize the gastric responses to vagal stimulation had been established in the rat, an effort was made to consider various experimental conditions
Figure 10. Rat stomach in vitro. Responses to vagal stimulations with variations in impulse frequency in the presence of atropine ($2 \times 10^{-7}$ g/ml). Note the maximal VIR at impulse frequencies of about 7/s to 9/s and the gradual transition to characteristically sympathetic inhibitory relaxation patterns of stimulations with higher impulse frequencies. Stimulation periods 10 sec; impulse duration, 6 msec; voltage, 6v.
which could affect the VIR of the *in vitro* rat stomach. The experimental conditions considered included the effects of acute and chronic atropine administration, prolonged starvation, 5-HT densensitization and pretreatment with 6-hydroxydopamine (6-OHDA).

Chronic Atropine Pretreatment

*Introduction.* Mozik *et al.* (1967) suggested a connection between the "pharmacological denervation" phenomenon and the development of tolerance to atropine in patients after prolonged atropine treatment. Chronic atropine treatment was found to decrease the level of ATP in the rat stomach wall (Mozik, Javor and Daroczy, 1970).

Since ATP has been suggested to be the neurotransmitter at the neuroeffector site of the vagal inhibitory pathway (Burnstock *et al.*, 1970), perhaps the development of tolerance to the effects of atropine is related to the decrease in ATP content in stomach walls caused by the chronic administration of atropine which may result in the reduction or abolition of the rat stomach VIR. Thus, atropine may have a dual role in the treatment of hypergastric motility, i.e., the blockade of cholinergic contractions which, in turn, leaves the action of the vagal inhibitory reflex unopposed resulting in a further decrease in gastric smooth muscle tonus.

*Experiment.* Three groups of six male rats of the same age were pretreated with atropine sulfate, 10 mg/kg, 1 mg/kg and saline
respectively. The doses were administered i.p. every 12 hours for 25 consecutive days.

**Results.** In general, vagal stimulation did not produce any significant differences in the relaxation responses among these three groups of rats. Furthermore, neither were these preparations significantly different in response from those stomachs obtained from rats which had been pretreated acutely with atropine.

**Prolonged Starvation**

**Introduction.** The effect of prolonged starvation on the VIR of the rat stomach was investigated. Different groups of rats were starved for periods ranging from one to 13 days while water was available ad lib.

**Results.** In general, prolonged starvation did not seem to have a significant influence on the VIR of the rat. During sustained electrical stimulation of the vagus nerves, gastric relaxation could be maintained for more than eight minutes in control animals without displaying fatigue in the relaxation response. Fatigue is the inability to maintain a relaxation response during sustained vagal stimulation. Fatigue was observed in some stomachs from rats which had been starved. Figure 11 shows a fatigue response occurring within a one minute period of sustained vagal stimulation. However, an attempt to consistently correlate the early onset of fatigue during sustained
Figure 11. Rat stomach *in vitro*. A relaxation response showing fatigue within a one minute sustained vagal stimulation period in a stomach from a rat previously starved for 5 days. Stimulation periods, 10 sec and 60 sec; impulse frequency, 5/s; impulse duration, 5 msec; voltage, 6v. Atropine (2x10^{-7} g/ml).
Atropine 5/s (60 sec)
Figure 12. Rat stomach in vitro. Responses to low frequency (5/s) stimulation after desensitization of stomach muscle to increments of 5-hydroxytryptamine (5-HT) concentrations, i.e., 10^{-8} g/ml, 10^{-7} g/ml, 10^{-6} g/ml and 10^{-5} g/ml) in the presence of atropine (2x10^{-7} g/ml). Note the lack of effect of desensitization on the vagal inhibitory response. Stimulation period, 10 sec; impulse duration, 5 msec; voltage, 6v.
vagal inhibitory pathway of the rat stomach.

"Chemical Sympathectomy" by 6-OHDA

**Introduction.** Recently, Votavova et al. (1971) reported a 91% decrease in norepinephrine (NE) content in the cat stomach after treatment with 6-hydroxydopamine (6-OHDA). Administration of 6-OHDA leads to a degeneration of adrenergic nerve endings and a consequent depletion of the amine stores (Thoenen and Tranzer, 1968). Furthermore, it has been found that 6-OHDA selectively destroys the adrenergic nerve terminals without affecting cholinergic nerve fibers, Schwann cells, or muscular cells (Tranzer and Thoenen, 1968). The adrenergic neurons present in the sympathetic ganglia are left intact (Laverty et al., 1965). Thus, 6-OHDA was used to test the possibility of the involvement of sympathetic fibers in the vagal inhibitory pathway.

**Experiment.** Six rats were pretreated with 6-OHDA with a dosage regimen of 2 x 34 mg/kg within a 24 hour period and seven days later with 2 x 68 mg/kg, i.v., as suggested by Thoenen and Tranzer (1968). A control group of six rats received only the vehicle, i.e., 0.001N HCl saturated with nitrogen, in equivalent volumes.

**Result and Discussion.** In four out of six rat stomachs tested, a VIR of reduced magnitude was observed during vagal stimulation.
Figure 13 shows one of the reduced VIRS seen after pretreatment with 6-OHDA, where vagal stimulation elicited the usual contraction responses which were abolished by atropine ($2\times10^{-7}$ g/ml).

The reduction in the magnitude of the VIR after the administration of 6-OHDA suggests sympathetic nerve involvement. However, little is known concerning synaptic relations in the ganglia of the myenteric and submucosal plexuses and adrenergic synapses found in the autonomic ganglia indicates that the autonomic system is anatomically more complex than previously thought (Norberg, 1967).

Baumgarten et al. (1970) reported that the "p-type" fibers are not affected by 6-OHDA. However, the possibility still exists that it may have an indirect effect somewhere within the complex myenteric plexus which can ultimately influence the VIR.

Watanabe (1971) studied adrenergic ultrastructure in the ganglia and found them to contain two main elements, one consists of a basket-like, simple nerve ending around some of the ganglion cell bodies, and the other consists of small polyhedral cells with short axonic processes. Then he suggested that they represent two different types of adrenergic mechanisms: (1) the true interneurons of the axon-collaterals of the postganglionic neurons which form adrenergic synapses with some of the postganglionic neurons and (2) the small, granule-containing cells releasing catecholamine in blood capillary. Malmfors and Sachs (1968) found that the degree of "chemical
Figure 13. Rat stomach in situ. Pretreated with 6-hydroxydopamine (6-OHDA); see text for dosage regimen. Responses to low (5/s and high (30/s) frequency stimulations both before and after the addition of atropine. Note the large reduction in the vagal inhibitory response. Stimulation periods, 10 sec; impulse duration, 5 msec; voltage, 6v.
sympathectomy" obtained with 6-OHDA is different in the vas deferens, which contain "short" adrenergic neurons (Falck, Owman and Sjöstrand, 1965), is different compared with that in the iris which is innervated with the ordinary type of "long" adrenergic neurons. Since little is known concerning the effect of 6-OHDA other than its action on adrenergic terminals, it is difficult to conclude that this is its exclusive effect in the animal.

**Test of Hypothesis for Role of 5-HT**

**Introduction**

Büllbring and Gershon (1967), after working on guinea-pigs and mice, hypothesized that 5-HT, along with ACh, may be a neurotransmitter acting on the same ganglion cell in the vagal inhibitory pathway. This hypothesis is illustrated in Figure 14 (top). The lower diagram in Figure 14 illustrates a classical parasympathetic cholinergic excitatory pathway. If their hypothesis is true, a depletion of stomach 5-HT levels should influence the VIR. Both reserpine and parachlorophenylalanine (PCPA) are known to decrease 5-HT levels from various tissues in the rat (Koe and Weissman, 1966; 1968; Erspamer, 1956). The role of 5-HT in the VIR of the rat stomach was investigated by using 5-HT depleters, PCPA and reserpine, and a ganglionic blocking agent, C₆.
Figure 14. Top diagram illustrates the role of 5-HT in the ganglion of the vagal inhibitory pathway as proposed by Bülbring and Gershon (1967). Bottom diagram illustrates the classical parasympathetic cholinergic pathway.
PCPA inhibits tryptophan hydroxylation by enzyme systems which convert tryptophan to 5-hydroxytryptophan (5-HTP). Since the 5-hydroxylation of tryptophan is the rate limiting step in 5-HT formation (Udenfriend et al., 1957), prolonged blockade of the hydroxylating enzyme in vivo should cause depletion of tissue 5-HT (Koe and Weissman, 1968). Reserpine also decreases tissue 5-HT level (Shore, Silver and Brodie, 1955), however, it is not a very effective depletor of 5-HT in the stomach (Bennett, Bucknell and Dean, 1966; Erspamer, 1956). It is suggested that reserpine acts by inhibiting the transfer of the final products of 5-HT into the storage components, thus exposing these products to deamination by monoamine oxidases (Nickerson, 1970). Since PCPA and reserpine deplete 5-HT by different mechanisms of action, they were administered individually, as well as concurrently, in an attempt to more completely deplete 5-HT from stomach tissues.

Hexamethonium (C₆) produces ganglionic blockade by occupying receptor sites on the ganglionic cells and stabilizing the postsynaptic membranes against the actions of ACh liberated from presynaptic nerve endings. There has been some disagreement concerning the extent to which the VIR is reduced by C₆. Greeff et al. (1962) and Paton and Vane (1963) reported that they abolished the VIR of the guinea-pig with C₆. However, Bülbring and Gershon (1967) found that C₆ greatly reduced but never abolished the VIR of the guinea-pig
stomach in vitro. If both 5-HT and ACh are neurotransmitters at the ganglia of the vagal inhibitory pathway, the addition of C₆ should only block the actions of ACh but not the actions of 5-HT in the ganglia (Volle and Koelle, 1970). Thus, electrical stimulation of the vagal inhibitory nerves after C₆, in the presence of atropine, should still produce a VIR due to the actions of 5-HT.

Procedure

Four groups of 12 rats were treated as follows: group I is the control group, group II received PCPA only, group III received reserpine only and group IV received both PCPA and reserpine. Group I, the control group, received only the vehicle, aqueous 1% Tween 80, in equivalent volumes. The respective drug dosage regimen has already been discussed in detail. Six rats from each group were used for the 5-HT assay and the remaining six from each group for the isolated stomach preparation.

Results

5-Hydroxytryptamine Assay

Table 1 contains the various concentrations of 5-HT tissue levels before and after treatment with PCPA and/or reserpine. The tissue concentrations of 5-HT are expressed as the amount
Table 1. The effect of parachlorophenylalanine (PCPA) and reserpine

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Treatment</th>
<th>Brain</th>
<th>Stomach</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tissue concentration</td>
<td>% depletion</td>
<td>Tissue concentration</td>
</tr>
<tr>
<td>I</td>
<td>6</td>
<td>Control</td>
<td>0.64 ± 0.02</td>
<td>0</td>
<td>10.7 ± 0.13</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>PCPA</td>
<td>0.08 ± 0.00</td>
<td>88</td>
<td>3.57 ± 0.22</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>Reserpine</td>
<td>0.08 ± 0.01</td>
<td>88</td>
<td>7.66 ± 0.55</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>PCPA + Reserpine</td>
<td>0.04 ± 0.00</td>
<td>94</td>
<td>1.8 ± 0.21</td>
</tr>
</tbody>
</table>

*a* tissue concentration of 5-HT, expressed as the amount of 5-HT in micrograms per gram of tissue ± the standard error.

*b* dosage, 200 mg/kg, i. p. × 5 days

*c* dosage, 1 mg/kg, i. p. × 5 days

*d* aqueous 1% tween 80 solution in equivalent volumes.

*n* is the number of animals in each group.
of 5-HT in micrograms (μg) per gram of tissue. Also the various tissues are expressed in relation to control tissue levels. The raw data is included in the appendix.

The results of the degree of 5-HT depletion were statistically analyzed with the least significant difference (LSD) test (P < .05). In the brain, the LSD test indicates the mean of group I is significantly different from the means of groups II, III, IV (P < .05). However, the means between groups II, III and IV are not significant (P < .05). Thus an additive effect by the combination of PCPA is questionable at a probability of less than 0.05. Therefore, in the rat brain, the effectiveness of 5-HT depletion by PCPA alone and reserpine alone is similar to the effectiveness of the two drugs together.

In the stomach, the LSD test indicates a significant difference between the control and the treated means in both the antrum and the corpus (P < .05). The LSD test indicates that the means between the PCPA and reserpine treated antrum and corpus tissues are significant (P < .05). The means between groups II and IV are significantly different (P < .05). The means between groups III and IV are also significantly different (P < .05). Therefore there is a significant additive effect of the PCPA and reserpine combination on the depletion of 5-HT levels from the stomach tissues.
The Effect of PCPA and Reserpine on the VIR

Half of the rats were used for the assay of 5-HT levels in various tissues. The remaining six rats from each group were used to test the VIR. It was found that the VIR of the stomachs from the treated groups remained unchanged from the VIR of the control group. This is illustrated in Figure 15. Thus, in spite of large amounts of 5-HT depletion from rat stomach tissues, especially after both PCPA and reserpine treatment, this had no apparent influence on the VIR of the rat stomach. This suggests that 5-HT is not involved in the VIR of the rat stomach.

The Effect of C₆ on the VIR

Hexamethonium (C₆) produces ganglionic blockade by occupying receptor sites in the ganglionic cells thus preventing the actions of ACh liberated from presynaptic nerve endings (Volle and Koelle, 1970). As illustrated in Figure 15, C₆ completely blocked the VIR of rat stomach in vitro in the control group as well as in the treated groups. The VIR response was recovered after the C₆ was removed by washing (W). This is contrary to the effect of C₆ in the guinea-pigs reported by Bülbring and Gershon (1967). The abolition of the VIR by C₆ strongly indicates that ACh is the only neurotransmitter at the ganglionic site.
Figure 15. Rat stomach in vitro. Responses to low frequency vagal stimulations in control, parachlorophenylalanine (PCPA) treated and PCPA-reserpine treated rat stomach before and after atropine (1x10^{-7} g/ml), followed by hexomethonium (C_6, 2x10^{-5} g/ml). Note the presence of the vagal inhibitory response (VIR) in all three preparations and also the complete abolition of the VIR after C_6. The VIR was recovered after the C_6 was washed away (W).
(a) Control

(b) Atropine PCPA Treated

Atropine
PCPA-Reserpine Treated

10 sec

s Atropine

C₆ s W s
High levels of 5-HT depletion from stomach tissues had no effect on the VIR and the addition of $C_6$ completely blocked the VIR. These two results combined to strongly suggest that ACh is the only ganglionic transmitter in the *in vitro* vagal inhibitory pathway of the rat stomach. Thus, the hypothesis proposed by Büllbring and Gershon (1967) for the role of 5-HT in the vagal inhibitory pathway could not be substantiated in the rat stomach.

The *In Situ* Relaxation Response

Introduction

When the vagus nerves in the rat were electrically stimulated *in situ* it was surprising that only gastric relaxation responses were elicited. This is illustrated in Figure 16. This gastric relaxation response of the rat is at variance to the expected classical cholinergic contractile responses to peripheral vagal stimulation recently reported for the *in situ* cat (Jansson, 1969; Martinson, 1964) and the *in situ* dog (Ohga *et al*.; 1969, 1970). However, the observation that electrical stimulation of the vagus nerves is capable of inhibiting gastric motility is not new and was reviewed as early as 1931 by McSwinney.
Figure 16. Rat stomach in situ. Responses to vagal stimulations of graded increments in impulse frequency. Note only relaxation responses to both low and high frequency stimulations. Stimulation period, 10 sec; pulse duration, 5 msec; voltage, 1.5 v.
The gastric relaxation response elicited by vagal stimulation in situ gave rise to the following questions. Why was the contraction response to vagal stimulation observed in vitro not elicited in situ? Since low frequency peripheral vagal stimulation in situ only elicited gastric relaxations in the rat instead of contractions as reported in other species in situ (Jansson, 1969; Ohga et al., 1969; 1970), how is this relaxation response related to the VIR? What are the factors which can influence this response and is this response a pathophysiological phenomenon? Which types of nerve fibers are involved in this relaxation response? An attempt was made to answer these and other questions concerning the in situ relaxation response. The gastric relaxation response to low frequency vagal stimulation of the rat in situ will hereafter be referred to as the in situ relaxation response.

Characterization of the In Situ Relaxation Response

Effect of Bilateral Adrenalectomy

As illustrated in Figure 16, the in situ relaxation response has a short latency of onset. Thus, the possibility that this response may be due to the indirect involvement by the release of catecholamines from the adrenal glands seemed unlikely (Martinson and Muren, 1963). The animal was, nevertheless, subsequently
bilaterally adrenalectomized to exclude the possibility of adrenal involvement. As illustrated in Figure 17, bilateral adrenalectomy had no apparent effect on the in situ relaxation response.

The short, rapid pulses recorded by the polygraph during the ten second periods of electrical stimulation were artifacts caused by striated muscle twitches from adjacent tissues. These artifact twitches are illustrated in Figures 16 and 17.

Central and Peripheral Nerve Components

The possibility also existed that the in situ relaxation response was due to the activation of a strong central inhibitory component via vagal afferent fibers which could override a contraction response observed in the in vitro rat stomach. This possibility was tested by first eliciting an in situ relaxation response by stimulating the intact vagi and then these nerves were sectioned and peripherally stimulated. If a central inhibitory mechanism was responsible for the in situ relaxation response, the peripheral stimulation of the vagi should eliminate this relaxation response.

Figure 18 illustrates gastric responses both to stimulation of the intact vagi and to peripheral stimulation of the sectioned vagi. The results show little difference in gastric responses to vagal stimulation under the two different conditions, thus eliminating the possibility that the in situ relaxation response was due exclusively
Figure 17. Rat stomach in situ. Responses to low frequency vagal stimulations before and after bilateral adrenalectomy. Note the lack of effect on responses after bilateral adrenalectomy. Stimulation period, 10 sec; pulse duration, 5 msec; voltage, 1.5 v.
A. *In Situ*

B. Adrenalectomized, *In Situ*
Figure 18. Rat stomach. The in situ and in vitro responses of the same stomach to both low (5/s) and high (30/s) frequency vagal stimulation. (a) in situ. Responses before and after central denervation. (b) in vitro. Responses before and after atropine (2x10^-7 g/ml). Stimulation periods, 10 sec; pulse duration, 5 msec; voltage, 1.5v (in situ) and 6v (in vitro).
(a) in situ

(b) in vitro

Cut Vagi

Atropine
to a central inhibitory mecahnism. The slightly stronger after-contraction to low frequency peripheral vagal stimulation, seen in Figure 18, could indicate the elimination of a weak central inhibitory component. Equally likely, however, is the possibility that it was caused by trauma on the nerves during sectioning which altered their sensitivity to subsequent electrical stimulations. Therefore, it is unlikely that a central inhibitory mechanism is responsible for the in situ relaxation response.

Comparison Between In Situ and In Vitro Responses

The stomach was removed from the rat and placed in an isolated organ bath after a control in situ relaxation response was elicited. This was done to substantiate the observations that vagal stimulation elicits a gastric relaxation response in the in situ rat in contrast to the in vitro rat stomach which responded with a contraction. Vagal stimulation of the same stomach in vitro now produced contraction responses. This is illustrated in Figure 18, where all the responses presented were derived from the same stomach. The addition of atropine (2x10^{-7} g/ml) abolished the in vitro contraction responses to reveal the underlying VIR (Figure 18). These results suggest the possibility that a strong, dominant in situ relaxation response was superimposed over an atropine-sensitive contraction response which, in turn, dominated a still weaker VIR. This may account for the
difference in the characteristics between the in situ relaxation response and the VIR. Apparently, components vital for the generation of the in situ relaxation response were lost after the stomach was removed from the rat and placed in an isolated organ bath. This left a strong contraction response remaining to manifest itself upon vagal stimulation over a weaker VIR. Thus, the in situ relaxation response is probably the resultant response of three different nerve components elicited in the following order of dominance: the in situ relaxation response, the contraction response and the VIR.

**Electrical Parameter Values for Vagal Stimulation**

Before the possible causes for the activation of the in situ relaxation response and the nerve mechanism responsible for this response can be further investigated, the values for the electrical parameters for vagal stimulation were first established. The values necessary to elicit the maximal in situ relaxation response were established to be an impulse frequency of from 3-7/s (depending on the individual preparation), with a 5 msec pulse duration at 1.5 v. The values for the sympathetic relaxation response, discussed earlier and illustrated in Figure 9, were established at an impulse frequency, with a 5 msec pulse duration at 1.5 v.
Figure 19 shows that a threshold impulse duration for the maximal in situ relaxation response was about 2 msec. After atropine (1 mg/kg) administration, the threshold impulse duration for the maximal response was about 25 msec, as shown in Figure 20. Since stimulation of longer impulse durations are needed to activate thinner nerve fibers than thicker ones, the ten-fold difference between impulse durations needed for the maximal relaxation responses before and after the administration of atropine may indicate that the in situ relaxation response is due to the activation of different nervous components of varying fiber sizes. An increase in the magnitude of the relaxation response after atropine was also noticed. This could be explained by the elimination of a contraction response by atropine, leaving two relaxation responses remaining unopposed which could produce a facilitated; thus larger, relaxation response on stimulation of the vagus nerves.

Possible Factors Which May Influence the In Situ Relaxation Response

The cause of reflex activation of the inhibitory neurons in the gastrointestinal tract in vivo has not been investigated (Holman and Hughes, 1965). The possibility exists that the in situ relaxation response may actually be a pathophysiological phenomenon. Therefore, some of the experimental conditions which may induce a
Figure 19. Rat stomach in situ. Responses to vagal stimulations with variations in impulse duration without atropine. Note the threshold obtained at 1 msec and maximal response at about 2 to 5 msec duration. Stimulation period, 10 sec; impulse frequency, 3/s and voltage, 1.5v.
Variations in impulse duration
Constant: 3/4, 1.5 v
Figure 20. Rat stomach in situ. Responses to low frequency (5/s) vagal stimulations with variations in impulse duration after the intrahepatic administration of atropine (1 mg/kg). Note the maximal relaxation response obtained at about 25 to 50 msec. Stimulation period, 10 sec and voltage, 1.5 v.
Constant: 5/s, 1.5v

Variations in impulse duration

Atropine

msec

0.05 0.5 5 10

25 50 75 150
pathophysiological in situ relaxation response in the rat were investigated. Some of the experimental factors examined were the effect of age and sex of the animals, the lowered body temperatures, the starvation times prior to experimentation, and the evisceration of the animals.

**Age and Sex**

The possible influence of age and the sex of adult rats on the in situ relaxation response was briefly investigated. There were no apparent differences observed between responses from young and old rats. Also, both male and female rats exhibit similar relaxation responses to vagal stimulation.

**Body Temperature**

The possible influence of lowered body temperature on the in situ relaxation response was examined. The rectal temperature of the rat during experimentation was measured and found to be about 4°C lower than the normal body temperature of 37°C. This was probably due to the loss of body heat from the general anesthesia and the surgically opened thoracic and abdominal cavities. Inhibition of gastric motility in rats after exposure to cold temperatures have been reported (Sudsaneh and Mayer, 1959; Mayer and Sudsaneh, 1959).

The influence of lowered body temperatures was investigated by
cervical transection of the spinal cord at the level of C7. Under this condition, the body temperature of the rat was lowered from $37^\circ C$ to $25^\circ C$ approximately 24 hours later. Figure 19 (top) shows that the lowered body temperature had no significant effect on the in situ relaxation response. Low frequency ($5/s$) vagal stimulation of the intact vagi, as well as after peripheral stimulation revealed only slight differences in the in situ relaxation response. This also eliminates an inhibitory component which could be activated centrally by the decrease in body temperature.

The effect of lowered temperature on the in vitro stomach was also examined. The above stomach was removed from the rat and placed in an isolated organ bath and maintained at $25^\circ C$. Low frequency ($5/s$) vagal stimulation elicited the expected in vitro gastric contraction response, followed immediately by a strong rebound contraction, shown in the bottom of Figure 21 (note the five-fold decrease in recording sensitivity used in order to include the after-contraction). High frequency ($30/s$) vagal stimulation produced a characteristic sympathetic relaxation response with the long latency of onset and recovery time. The addition of atropine ($2\times10^{-7} g/ml$) abolished the contraction response to reveal the VIR (Figure 21, bottom).

Therefore, a decrease in the temperature of both the in situ and in vitro stomach preparations did not greatly affect either the
Figure 21. Rat stomach. In situ and in vitro responses of the same stomach to vagal stimulations at 25°C. (a) in situ. Body temperature 25°C. Responses to stimulations before and after central denervation. (b) in vitro. Bath temperature, 25°C. Responses to stimulations before and after atropine (2x10^{-7} g/ml). Note the decrease in amplifier sensitivity 5 fold in order to include a large rebound contraction at 5/s. Stimulation period, 10 sec; pulse duration, 5 msec; voltage, 1.5v (in situ) and 6v (in vitro).
in situ relaxation response or the VIR in vitro. Thus, the possibility that the lowered body temperature during experimentation may be responsible for the activation of the in situ relaxation response appears unlikely. Figure 21 again illustrates the difference in gastric responses to vagal stimulations between the in situ and in vitro stomach preparations, in spite of the lowered temperatures in both cases.

**Starvation**

There is also the possibility that the in situ relaxation response was a pathophysiological phenomenon, activated by starvation of the rats prior to experimentation was investigated. A series of experiments were performed in an attempt to correlate the effect of various lengths of fasting periods to their influence on the in situ relaxation response. Some interesting observations were made, such as the elicitation of a continuous series of regular, repeating, cyclic responses, each resembling a VIR was obtained with a sustained, low frequency vagal stimulation in a starved rat. In general, the effort to correlate the influence of starvation periods to specific alterations in the in situ relaxation response was unsuccessful. Therefore more work is needed in this and other areas which may elucidate the activation of the in situ relaxation response.
Nerve Fiber Components Involved in the
In Situ Relaxation Response

In 1966, Jansson and Martinson reported, in cats, that gastric contractions elicited by activation of the vagal excitatory fibers were promptly and completely inhibited by intestinal distension. They suggest that this "intestino-gastric" inhibitory reflex operates by an adrenergic mechanism since it was blocked by guanethidine. Thus, the possibility exists that the in situ relaxation response may originate from nervous reflexes located in the gastrointestinal tract below the level of the stomach.

Partial Evisceration

The destruction of possible inhibitory fibers originating from below the stomach essential for the in situ relaxation response was investigated by partial evisceration of the animal.

The partial evisceration procedure was comprised of lifting out of the abdominal cavity the intestines and some structures associated with them, including the pancreas, from below the pylorus to the rectum. The extrusion of the intestines was ligated at the level of the abdominal opening and removed. The liver, spleen, stomach and kidneys were not directly affected by this procedure.

After partial evisceration of the rat, low frequency (5/s) vagal stimulation resulted in the reversal of the in situ relaxation
response into a contraction response, followed by a rebound contraction (Figure 22). High frequency (30/s) vagal stimulation elicited a small, transient contraction followed by a relaxation response (Figure 22).

As expected, atropine (1mg/kg) abolished the contraction responses to vagal stimulation in the partially-eviscerated animal, as shown in Figure 22 (middle), to reveal the persistent VIR. Peripheral stimulation did not produce significantly different responses than to stimulation of the intact vagi.

The absence of the in situ relaxation response after partial evisceration strongly suggests that this response is regulated by fibers below the level of the stomach. The presence of an inhibitory response seen after the administration of atropine also indicates that a set of inhibitory fibers is still intact in the vagus nerve trunks.

In view of the existence of a sympathetically controlled "intestino-gastric" inhibitory reflex (Jansson and Martinson, 1966) and the abolition of the in situ inhibitory response after partial evisceration of the animal, a strong possibility exists that the dominant relaxation response may be mediated by sympathetic fibers. This possibility was tested by employing a series of drugs which affect sympathetic nerve fibers and sympathetic responses.
Figure 22. Rat stomach **in situ**. Responses to low (5/s) and high (30/s) frequency vagal stimulation after evisceration, followed by administration (i.v.) of atropine (1 mg/kg) and peripheral stimulation after central denervation. Note the reversal of the **in situ** relaxation into a contraction response after evisceration. Stimulation period, 10 sec; pulse duration, 5 msec, voltage, 1.5v.
Alpha and Beta Adrenergic Blockers

The predominant effect of sympathomimetic agents on the gastrointestinal tract is inhibitory and mediated by both alpha and beta adrenergic receptors (Ahlquist and Levy, 1959). However, the nature and the extent of adrenergic control mediated by these receptors in vivo under various physiological conditions are poorly defined (Nickerson, 1970). Nevertheless, alpha and beta adrenergic receptor blocking agents were used to block the in situ relaxation response. This was an attempt to test whether or not the in situ relaxation response may be mediated by adrenergic fibers.

High doses of an alpha adrenergic blocking agent, phenoxybenzamine (POB, 10 mg/kg), and two beta blockers, propranolol (20 mg/kg) and dichloroisoproterenol (DCI, 2 mg/kg) were administered intraperitoneally (i.p.). The results of the three drugs administered separately to the same animal at least 20 minutes apart are shown in Figure 23. Both alpha and beta adrenergic blocking agents had little effect on the in situ responses to both low (5/s) and high (30/s) frequency vagal stimulations, as illustrated in Figure 23. The small influence by the above drugs on the in situ relaxation response suggests that this response is not mediated by adrenergic fibers. However, since these drugs also had little effect on the high frequency inhibitory response, which is supposedly
Figure 23. Rat stomach in situ. Responses to low (5/s) and high (30/s) frequency vagal stimulations before and after administration (i.p.) of phenoxybenzamine (POB, 10 mg/kg) followed by propanolol (20 mg/kg) and dichloroisoproterenol (DCI, 2 mg/kg). This illustrates the small effect of alpha and beta blocking agents on the in situ relaxation response. Stimulation period, 10 sec; impulse duration, 5 msec; voltage, 1.5 v.
caused by the activation of sympathetic fibers, it is more likely that these agents have a limited action on the adrenergic mechanism in the stomach under physiological conditions. Thus, other agents which affect adrenergic responses were administered in order to clarify the nervous mechanism primarily responsible for the in situ relaxation response.

Guanethidine

Guanethidine, an adrenergic neuronal blocking agent, was used to test for sympathetic nerve fiber involvement in the in situ relaxation response. The main effect of guanethidine is the inhibition of sympathetic nerve response; associated with a reduction in the release of norepinephrine (NE) from nerve terminals (Nickerson, 1970). The administration of guanethidine causes increases in gastrointestinal motility and this effect is commonly attributed to parasympathetic predominance after blockade of adrenergic fibers (Nickerson, 1970).

The effect of guanethidine on the in situ relaxation response was investigated. The animals were tested for the in situ relaxation response between two to six hours after administration of guanethidine (8 mg/kg, i.p.). This resulted in the replacement of the in situ relaxation response by a contraction response to low frequency (3/s) vagal stimulation, as shown in Figure 24, in half of the six rats.
Figure 24. Rat stomach *in situ*. Responses to both low (3/s) and high (30/s) frequency vagal stimulations after pretreatment with guanethidine (8 mg/kg, i.p.). Responses elicited before and after central denervation and the administration (i.p) of atropine (1 mg/kg). Note the reversal of the *in situ* relaxation to contraction response on low frequency stimulation. Stimulation periods, 10 sec; impulse duration, 5 msec; voltage, 1.5v.
tested. The remaining three rats retained their \textit{in situ} relaxation responses in spite of treatment with guanethidine, as shown in Figure 25.

An attempt was made to determine why guanethidine only affected half of the animals. The pretreatment time was correlated to the onset of the \textit{in situ} relaxation response reversal to a contraction response. It was found that the effect of guanethidine on the \textit{in situ} relaxation response was not manifested until about five hours after its administration. Figure 26 illustrates the gastric responses to vagal stimulation approximately 15 minutes and five hours after guanethidine administration. The \textit{in situ} response was present at least four hours after the administration of guanethidine but this relaxation response was reversed to a contraction response after five hours. Thus, the latent onset of action of guanethidine was probably responsible for the conflicting results seen in the earlier experiment with guanethidine. The sympathetic inhibitory response, elicited by high frequency (30/s) stimulation was weakened by guanethidine, but it was never completely abolished by it (see Figures 24, 25, 26).

The VIR was elicited after atropine (1 mg/kg) administration in spite of pretreatment with guanethidine. This supports the suggestion by earlier investigators that the VIR is not mediated by sympathetic fibers.
Figure 25. Rat stomach in situ. Pretreatment with guanethidine (8 mg/kg, i.p.). Responses to vagal stimulation before and after central denervation and atropine (1 mg/kg, i.p.) and curare (0.1 mg/kg, i.p.). Note the presence of the in situ relaxation response and its reversal to a contraction after central denervation. Stimulation period, 10 sec; impulse duration, 5 msec; voltage, 1.5 v.
Figure 26. Rat stomach in situ. Pretreatment with guanethidine (8 mg/kg, i.p.). Responses to both low (5/s) and high (30/s) frequency vagal stimulations 15 minutes and 5 hours after guanethidine administration followed by responses after atropine (1 mg/kg, i.p.). Note the reversal of the in situ relaxation response 5 hours after guanethidine administration to a contraction response to vagal stimulation. Stimulation period, 10 sec; impulse duration, 5 msec; voltage, 1.5v.
Guanethidine (15 min)

Atropine
Peripheral stimulation shortly after guanethidine elicited a contraction response as opposed to a relaxation response elicited prior to central vagal denervation (Figure 25). This may possibly be due to the loss of a central inhibitory component combined with a weakening peripheral sympathetic component in the stomach caused by the incomplete actions of guanethidine.

The possibility that some adrenergic fibers are selectively susceptible to the actions of guanethidine cannot be disregarded. Nevertheless, the reversal of the in situ relaxation response into a contraction response suggests that an adrenergic mechanism is involved.

**Reserpine**

Reserpine was used to further investigate the possibility that the in situ relaxation response is sympathetically mediated. This drug depletes stores of catecholamines and 5-HT from many organs, including the gastrointestinal tract. Tissue catecholamines are restored slowly, therefore repeated doses have a cumulative action (Nickerson, 1970).

Rats were pretreated with reserpine (5 mg/kg, i.p.) daily for five days. As shown in Figure 27, animals thus treated produced a greatly reduced in situ relaxation response to both low (5/s) and high (30/s) frequency vagal stimulation. Peripheral stimulation
Figure 27. Rat stomach in situ. Pretreatment with reserpine (5 mg/kg x 5 days, i.p.). Responses to low (5/s) and high (30/s) frequency vagal stimulation before and after central denervation. Note the greatly reduced in situ relaxation response after reserpine treatment. Stimulation period, 10 sec; impulse duration, 5 msec; voltage, 1.5v.
Cut Vagi
of the vagus nerves did not elicit a reversal of the reduced relaxation response into a contraction response, as seen after guanethidine in Figure 25. Reserpine had no effect on the VIR obtained after atropine administration (see Figure 15).

**6-Hydroxydopamine**

Since reserpine and guanethidine both reduced or abolished the relaxation response, this strongly suggests that the in situ relaxation is sympathetically mediated. This can be further tested by the use of 6-hydroxydopamine (6-OHDA) to "chemically sympathectomize" the rat. If the in situ relaxation is mediated by sympathetic fibers, the destruction of the nerve terminals of these fibers by 6-OHDA should abolish this response.

6-hydroxydopamine (250 mg/kg, i.v.) was administered to six rats about 36 hours prior to general anesthesia. It was found that low frequency (5/s) vagal stimulation of the control rats elicited in situ relaxation responses, whereas rats pretreated with 6-OHDA elicited contraction responses. The abolition of the in situ relaxation response by 6-OHDA is illustrated in Figure 28 (top). The sympathetic relaxation response elicited by high frequency (30/s) vagal stimulation was also greatly reduced after 6-OHDA. This suggests that the in situ relaxation response is mediated by sympathetic fibers.

Low frequency stimulation elicited a slightly smaller contraction
Figure 28. Rat stomach. Pretreatment with 6-hydroxydopamine (6-OHDA, 250 mg/kg, i.v.) 36 hours prior to experimentation. Responses to both low (5/s) and high (30/s) frequency vagal stimulation in situ and in vitro. (a) in situ. Responses before and after central denervation and bilateral adrenalectomy. Note the reversal of the in situ relaxation to a contraction response after 6-OHDA. (b) in vitro. Responses before and after atropine (2x10^-7 g/ml). Note the presence of the vagal inhibitory response (VIR) after atropine. Stimulation period, 10 sec; impulse duration, 5 msec; voltage, 1.5v.
with the nerves intact than after peripheral stimulation. High frequency (30/s) peripheral stimulation produced a contraction response followed by a latent prolonged rebound after-relaxation (Figure 28, top). Since the latency of onset of this relaxation response was of sufficient length to be caused by the indirect action of the adrenals, the animal was bilaterally adrenalectomized to test this possibility. The latent after-relaxation response was present after bilateral adrenalectomy of the animal, thus eliminating the possibility of an indirect hormonal influence by the adrenals to cause the latent after-relaxation in 6-OHDA treated animals. However, there is also the possibility of accessory chromaffin tissue involvement which cannot be denied. Thus, further work is needed to elucidate the mechanism for the after-relaxations following vagal stimulations.

The effect of 6-OHDA (250 mg/kg, i.v.) on the VIR was also examined by removing the stomach from the animal and placing it in an isolated organ bath. Figure 26 (bottom) illustrates a weak VIR in the presence of atropine (2x10^{-7} g/ml) after treatment with 6-OHDA. However, it is difficult to attribute the presence of the VIR exclusively to the lack of effect by 6-OHDA. It is possible that the single dose of 6-OHDA (250 mg/kg) (Furness et al., 1970) is not as effective as the multiple injections of 6-OHDA, i.v. (Thoenen and Tranzer, 1968) on the VIR. Further work is needed in defining the effective 6-OHDA dosage regimen for its possible effect on the VIR.
Discussion and Conclusion

Thus far, the in situ relaxation response was greatly reduced or abolished by guanethidine, reserpine and 6-OHDA. This strongly suggests that the in situ relaxation is mediated primarily by sympathetic nerve fibers. On the other hand, alpha and beta adrenergic blocking agents had no significant effect on either the low or high frequency relaxation response in the rat in situ. This was in spite of the administration of alpha and beta blocking agents in very high dosages. As mentioned earlier, the nature and the extent of their adrenergic control under physiological conditions are poorly defined (Nickerson, 1970). Furthermore, a very limited overall effect of alpha adrenergic blockade is indicated by the fact that phenoxybenzamine does not appreciably alter the rate of passage of barium sulfate along the human gastrointestinal tract (Nickerson, 1970). The limited action of alpha and beta blockers on the gastrointestinal tract could be due to the lack of availability of receptors sites to these agents. Thus, further work is needed to determine the overall effect of alpha and beta adrenergic blockers on the gastrointestinal tract in the in situ rat.

Gershon (1968) claims that the release of norepinephrine (NE) from terminals inside Auerbach's plexus affect smooth muscles only directly. Silva et al. (1971) showed that numerous adrenergic
terminals were present in the circular muscle layer in the myenteric plexus. However, Baumgarten et al. (1970) questions this conclusion because of the engulfment of axons by Schwann cell perikarya, the presence of a periganglionic connective tissue cell layer and by the action of degrading enzymes. Gershon's conclusion is also challenged by Paton and Vizi (1969) who found that NE acts directly on perikarya of intrinsic cholinergic neurons situated in the myenteric ganglia, thereby affecting the smooth muscle cells indirectly by reducing parasympathetic activity. Baumgarten et al. (1970) substantiated results by Paton and Vizi (1969) by electron microscopic detection of adrenergic varicosities close to the dendrites and stomata of the intrinsic neurons in the myenteric plexus. Thus, it is not surprising that the alpha and beta adrenergic blocking agents had no effect on the in situ relaxation response of the rat stomach since these agents are known classically only to block adrenergic receptors situated on the smooth muscle cells. The main effect of guanethidine, reserpine and 6-OHDA is on the preterminal sympathetic fibers. This supports the suggestion that NE acts on cholinergic neurons situated in the myenteric ganglia to reduce basal parasympathetic activity (Paton and Vizi, 1969; Norberg, 1967).

The results also show that the VIR was not affected by guanethidine or reserpine. This substantiates the conclusion by earlier investigators that the VIR is mediated by non-adrenergic fibers.
Since the effect of 6-OHDA is inconsistent, along with the possibilities that the dosage used may be inadequate or that this chemical may have other effects on nerve fibers not yet elucidated, the results of possible effects on VIR after 6-OHDA (250 mg/kg, i.v.) is questionable and should be interpreted with caution.
V. SUMMARY AND CONCLUSION

The vagal inhibitory response (VIR) of the isolated rat stomach was first characterized and the values for the electrical parameters for vagal stimulation were established. Then the hypothesis that both 5-HT and ACh are transmitters acting on the same ganglion cells in the vagal inhibitory pathway was tested in the rat stomach preparation. The role of serotonin in the VIR of the rat stomach was investigated by using the 5-HT depletors, PCPA and reserpine, and a ganglionic blocking agent, C₆.

There was no apparent influence on the VIR of the rat stomach in spite of substantial 5-HT depletion from the stomach tissues, especially after pretreatment with both PCPA and reserpine. The VIR was completely blocked after addition of C₆. The actions of 5-HT are not antagonized by either C₆ or atropine. If both 5-HT and ACh are neurotransmitters at the ganglia of the vagal inhibitory pathway, the addition of C₆ should only block the actions of ACh but not the actions of 5-HT. Thus, stimulation of the vagal inhibitory nerves after C₆ should still produce a vagal inhibitory response due to the actions of 5-HT.

The possibility still exists that the small amounts of 5-HT remaining after treatment with PCPA and reserpine could be sufficient to maintain the vagal inhibitory pathway. Nevertheless, the results
from 5-HT depletion by PCPA and reserpine and the abolition of the vagal inhibitory response after C₆ suggest that 5-HT in the rat is not involved either pre- or postangionically in the vagal inhibitory pathway. Thus, the hypothesis proposed by Bülbirng and Gershon (1967) for the role of 5-HT in the vagal inhibitory response could not be substantiated for the rat stomach.

When the vagus nerves were stimulated in situ, the rat stomach responded with an inhibitory relaxation response. In contrast, vagal stimulation elicited an excitatory contraction response after the stomach was removed and placed in an isolated organ bath. An effort was made to characterize the in situ relaxation response. The gastric inhibitory response to low frequency vagal stimulation in the rat in situ is referred to here as the in situ relaxation response, whereas the "non-adrenergic" inhibitory response, obtained after atropine administration, is called the vagal inhibitory response (VIR).

The apparent contradiction between the in situ relaxation response and the in vitro gastric contraction response to vagal stimulation once more emphasizes the need for caution in the interpretation of in vitro experimental results, especially where they are extrapolated to account for a physiological phenomenon.

An attempt was made to elucidate the basis for the discrepancy between the in situ relaxation response and the in vitro contraction response to vagal stimulation. Jansson and Martinson (1966) reported
the inhibition of excitatory responses of gastric smooth muscle by a reflex or direct activation of an adrenergic nerve fiber system. Since removal of the intestines may destroy reflex inhibitory fibers which could be responsible for the in situ relaxation response, the rat was partially eviscerated by removal of the intestines. Low frequency vagal stimulation after the partial evisceration elicited contraction responses which were abolished by atropine to reveal the underlying VIR.

The possibility that the in situ relaxation response is dominated by an adrenergic inhibitory reflex was tested with guanethidine, reserpine and 6-OHDA. All three of the above drugs reversed the relaxation responses into gastric contraction responses upon vagal stimulation. The above observations suggest that an adrenergic reflex is the dominant influence on the manifestation of the in situ relaxation response during vagal stimulation. Since the contraction response is abolished after atropine administration, it is probably cholinergic in origin. The underlying VIR was not affected by the partial evisceration, guanethidine or reserpine, thus its nervous origin is probably not under the direct influence of a classical sympathetic response. The effect of 6-OHDA on the VIR is inconclusive in the dose administered. Alpha and beta adrenergic receptor blocking agents had no effect on the in situ relaxation response. This is not surprising since the nature and the extent of adrenergic control
mediated by these receptors under various physiological conditions are poorly defined and the overall effect of adrenergic blocking agents in vivo are limited (Nickerson, 1970). The evidence suggest that the in situ relaxation response could be the result of three different gastric responses manifested in the following order of dominance: (1) an adrenergic inhibitory response, (2) a cholinergic excitatory response, (3) a non-adrenergic inhibitory response.

It is becoming evident that the autonomic system is anatomically and functionally more complex than indicated by the classical picture. According to Campbell (1970), there has been a tendency in the past to regard the rules as absolute and inflexible, resulting in generalizations that have attained an almost unquestionable classical status. Thus, the development of ideas about the autonomic nervous system have been impeded by an over-rigid adherence to theories formulated at the beginning of the century.

Furthermore, species differences have been found regarding the occurrence and number of adrenergic nerves in tissues (Norberg, 1967). Thus it is important to minimize the extrapolation of experimental results acquired from the guinea-pig, cat or other animal data to the rat. Although gastric response to vagal stimulation in the rat stomach resulted in similar patterns of response observed in the guinea-pig or cat stomach, one should not assume without reservation that they are of identical nervous origin. This is illustrated by the
in situ relaxation responses which contains not only two inhibitory relaxation responses of apparently different nerve origins, but furthermore, are also superimposed on an excitatory contraction response as well.

In conclusion, the evidence from this investigation indicates that ACh is the ganglionic neurotransmitter in the vagal inhibitory pathway of the rat stomach. The lack of effect on the VIR subsequent to 5-HT depletion from stomach tissues, and the abolition of the VIR by C₆ suggest that serotonin is not involved either pre- or post-ganglionically in the vagal inhibitory pathway of the rat. Perhaps this can be tested with the use of hemicholinium or botulinum toxin. Evidence also indicates that the in situ relaxation response in the rat is mediated predominantly by adrenergic nerve fibers. The neurotransmitter at the postganglionic inhibitory site is still controversial and remains to be elucidated.
BIBLIOGRAPHY


APPENDIX

SEROTONIN ASSAY DATA

Observations are expressed in terms of micrograms of serotonin per gram of tissue

<table>
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<tr>
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<th>Control</th>
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