72-3411

MAO, Chi-chiang, 1942-

IS CYCLIC AMP THE INTRACELLULAR MEDIATOR OF GASTRIC ACID SECRETION IN THE MAMMALIAN STOMACH?

The University of Oklahoma, Ph.D., 1971
Physiology

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

IS CYCLIC AMP THE INTRACELLULAR MEDIATOR OF GASTRIC ACID SECRETION IN THE MAMMALIAN STOMACH?

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

CHI-CHIANG MAO

Oklahoma City, Oklahoma

1971

IS CYCLIC AMP THE INTRACELLULAR MEDIATOR OF GASTRIC ACID SECRETION IN THE MAMMALIAN STOMACH?

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TO MY WIFE AND MY ENTIRE FAMILY

ACKNOWLEDGMENTS

The author wishes to take this opportunity to express sincere appreciation to Dr. Eugene D. Jacobson for contributing to the author's professional growth during the past four years. Dr. Jacobson's guidance, stimulation and financial support greatly aided the completion of this study. His patience and help in preparing this dissertation are gratefully acknowledged.

Special gratitude is due to Dr. Linda L. Shanbour for her concern, encouragement and technical instructions in both animal surgery and biochemical assays upon which the entire study relied.

The author is also greatly indebted to Dr. Daniel S. Hodgins for his expert advice on biochemical aspects of the study and for generous use of his equipment.

Appreciation is also due to Mr. Alvin C. K. Chang, Mr. Lenard Lichtenberger, Dr. Louis Bussjaeger and Mr. Bernhard Dittmann for their invaluable assistance in surgical preparation during the course of the study.

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IS CYCLIC AMP THE INTRACELLULAR MEDIATOR OF GASTRIC ACID SECRETION IN THE MAMMALIAN STOMACH?

CHAPTER I

INTRODUCTION

Secretion of hydrochloric acid by the stomach was first reported by William Pront in 1824 and by Beaumont in the next decade (1833). Since then, the problems of formation and secretion of gastric acid have been subjected to a great deal of study with many hypotheses having been proposed. At present there exists no satisfactory picture of the metabolic mechanism of acid secretion by the stomach.

Recently, Harris and his coworkers (1969) proposed an exciting hypothesis which describes the initial biochemical reactions involved in the secretory response of the frog stomach to secretagogues. This postulate is that gastric acid secretion is mediated by adenosine-3',5'-monophosphate, or cyclic AMP, which has been recognized as a unique intracellular mediator of many hormones. This research was undertaken for two reasons: 1) the hypothesis has the potential of providing an important breakthrough in our comprehension of the subcellular events involved in gastric acid secretion; and 2) the hypothesis has not been validated in the mammalian stomach.

Mechanisms of Acid Secretion by the Stomach

The gastric mucosa transfers hydrogen ions from the plasma at a concentration of 10^{-5} mN to gastric juice at a concentration of 160 mN. Chloride ion is transported from the plasma at a concentration of 110 mN into gastric juice at concentration of 170 mN. There is a potential difference (PD) across the gastric mucosa, which varies among species, but the secretory side is always negative with respect to the serosal side. Therefore, it is obvious that both H^+ and Cl^- ions are moved by uphill transport, the former against a vast concentration gradient, the latter against both concentration and electrical gradients.

Mammalian gastric mucosa is composed of several major secretory cell types: mucus producing cells, chief cells and parietal cells. The gastric juice normally collected is a mixture of the outputs from these different cell types. The cell of origin of each major component of gastric juice has been well-established. Acid is secreted by parietal cells (Bradford and Davies, 1950); pepsinogen, which later is activated to pepsin, is derived from chief cells (Bowie and Vineberg, 1953); and mucus containing various glycoproteins is the product of mucus secreting cells. Besides these major components, electrolytes probably have their origins in several types of cells. Apart from this, there is little information as to which cells secrete the various ions. The major electrolytes found in normal gastric juice include these ions: chloride (Cl^-), hydrogen (H^+), sodium (Na^+), potassium (K^+) and traces of calcium, phosphate, bicarbonate, etc. Because the gastric juice has several sources, its composition is variable. However its approximate isosmolality with respect to the plasma is steadily maintained. During the course of se-

cretion the concentrations of electrolytes in the juice have been found to be a function of the secretory rate of the stomach. At a low rate of secretion, gastric juice resembles an isotonic saline solution with a low concentration of K^+. At a high rate of secretion, Na^+ is gradually replaced by H^+ as the major cation, while K^+ concentration is still maintained relatively constant.

Gastrophysiologists have long been intrigued by the variations in the ionic composition of gastric secretion. Hollander, influenced by earlier ideas of Pavlov, suggested a two-component hypothesis (Hollander, 1932; 1952). The essential element of his thesis was that variation in gastric acid concentrations depends on changes in the proportion of two components, a pure acid portion from the parietal cells, and a nonacid or alkaline portion probably originating in the surface epithelial cells, the mucous cells of the neck, and/or the chief cells. Sodium has its sole source from alkaline secretions, whereas chloride exists in both acid and alkaline portions, but at higher concentration in the latter. When the acid secretory rate increases, the proportion of alkali decreases and so does the pH of the mixture. Although the existence of nonparietal secretions has been supported both by direct (Pevsner and Grossman, 1955; Hollander, 1963; Webster *et al*., 1958; James and Pickering, 1949; Ihre, 1938) and indirect (Fisher and Hunt, 1950; Lifson *et al*., 1943) evidence, the poor rectilinear relations between concentrations of secreted H^+, Cl^- and Na^+ have been criticized. Several investigators demonstrated a curvilinear relationship in the region of high acid concentrations (Linde and Öbrink, 1950; Gudiksen, 1950; Cooke and Grossman, 1965).

Rosemann in 1907 suggested that the gastric glands extract a solution from plasma containing sodium chloride of constant concentration and convert a varying proportion of this solution to hydrochloric acid. The degree of conversion depends upon the intensity of the secretory stimulation. Rosemann's major point was that chloride was secreted at a constant rate independent of the increase of H^+ secretion. This observation was confirmed by Cooke and Grossman (1965) at the low rates of acid secretion. However, when acid concentration was greater than 130 mN, the concentrations of acid and chloride varied together. This observation might be explained by the effect of dehydration, as Rosemann suggested (1907), or by an osmotic effect on the secretion, as pointed out by Durbin and Moody (1965). It should be noted that the key step in Rosemann's theory, namely conversion of NaCl to HCl was never elucidated.

Teorell proposed a "Diffusion Theory" in 1933 (Heinz and Öbrink, 1954). In this theory, the variation in acidity of the gastric juice depends upon a diffusion process. The primary secretion in gastric tubules, isosmotic with plasma, consists of constant concentrations of HCl and KCl. At low rates of secretion the back-diffusion of H^+ from lumen to plasma exceed that of Cl^- owing to the differences in their respective mobilities and the permeability of the mucosa to these two ions. In order to maintain electrical neutrality, Na^+ ions move into the lumen from plasma. The net result is ion exchange between H^+ and Na^+. The diffusion rate of H^+ will depend on the mucosal surface area, concentration and volume of gastric secretion and the degree of mixing. The curvilinear relation between the concentrations of Cl^- and H^+ predicted by this hypothesis was well supported (Linde and Öbrink, 1950; Gudiksen,

1950; Cooke and Grossman, 1965). It has been shown (Davenport et al., 1964; Davenport, 1967), however, that H^+ in the gastric lumen does not penetrate the normal mucosa to any great extent in exchange for Na^+.

Based upon the anatomical arrangement of the gastric glands in the mammalian stomach, Hirschowitz proposed a 4th explanation for the variations of ionic composition in gastric juice (Hirschowitz, 1960; Hirschowitz, 1961). He split the gastric secretory process into two mechanisms: primary and secondary. The function of the former is to secrete a solution containing Na^+, K^+ and Cl^-. The secondary mechanism is the exchange reaction of H^+ for Na^+. In this respect, Hirschowitz's view resembles the one suggested by Rosemann (1907). Hirschowitz further considered that the gastric tubule comprises the basic unit of the mucosa, the gastron, which accomplishes both secretory mechanisms. He suggested that chief cells at the base of the gastric tubules are the site of origin of primary secretion. When this solution flows through the middle region of the tubule where the parietal cells are located, a varying proportion of Na^+ is exchanged for H^+. This hypothesis has not yet been tested. Furthermore, only the mammalian gastric glands have both parietal and chief cells. In the acid secreting stomach of other vertebrates, such as birds, reptiles, amphibians and fish, a single cell type, usually designated the "oxyntic" or "oxynticopeptic" cell functions as both the parietal and chief cell of mammals (Ito, 1967).

In view of the preceding discussion, it is apparent that none of the hypotheses mentioned is able to explain fully the observed ionic variations of gastric juice in man and in animals. At present, it seems justifiable to say that after a pure acid juice is secreted from the

parietal cells, it will be diluted or neutralized by some nonacid secretions from another cell type. Some H^+ may diffuse across the mucosa in exchange for Na^+. In some cases, contamination from saliva, duodenal content and food during the collection of gastric secretion also accounts for the variation of electrolyte content. In the future, a quantitative combination of two or more hypotheses might prove to be more useful in understanding this problem.

In 1834, Donne first recorded a potential difference across the wall of the stomach. Success in developing two tissue preparations, namely the isolated frog mucosa by Delrue in 1930 and the chambered segment of canine stomach by Rehm in 1944, facilitated further research on the electrophysiology of the stomach.

Following the discovery of the gastric potential, there was little agreement on its origin until 1951. Hogben (1951) utilized the voltage clamp technique of Ussing and Zerahn (1951) in the isolated frog mucosa. In this procedure, the natural potential difference across the mucosa was brought to zero by passing an external current through the membrane. In the absence of a concentration gradient, the intensity of the external current (or "short circuit current") needed for maintaining a zero potential reflected the net active transports of ions across the membrane. Combining these results with those obtained from isotopic flux studies (Hogben, 1955), Hogben was able to demonstrate conclusively that net transport of Cl^- in excess of secreted H^+ was responsible for the short-circuit current. In other words, the equivalents of net Cl^- transport were the sum of short-circuit current and secreted H^+ ions. This conclusion was confirmed later by Forte _et al_. (1963) when they replaced

all the Cl^- in the salt solution bathing the isolated mucosa with another anion, isothionate, which is not readily secreted by the mucosa, the polarity of gastric potential reversed from the normal. This showed that the chloride pump is electrogenic and is necessary for the naturally occurring potential difference across the mucosa. An important observation in the isotopic study made by Hogben (1955) was that the leakage of Cl^- back from the secretory side was more than twice that necessary to account for the total membrane conductance. Apparently, part of the Cl^- moving from the secretory to nutrient solution was nonconducting, probably in the undissociated state. This prompted the author to suggest his "carrier" hypothesis for chloride transport in the gastric mucosa (Hogben, 1955).

The secreting mucosa of the dog maintains a fairly constant spontaneous potential and a mucosal resistance which are relatively independent of the rate of H^+ secretion (Rehm and Hokin, 1948). The short-circuit current also does not depend on secretion. Similar observations have been reported for the isolated frog mucosa (Crane _et al_., 1946). As the mucosal short-circuit current is constant and independent of the rate of H^+ secretion, variations in H^+ secretion demand corresponding variation in the rate of Cl^- secretion. Thus the active transport of chloride is a part of the integral system of acid secretion.

The nature of the mechanism for Cl^- transport is completely unknown. Nevertheless, its wide range of specificity has been well documented by several research groups. Külz first showed that the stomach secreted other halogens when they were introduced into the animal by intravenous injection (Külz, 1886). Lipschitz (1931) found a higher accu-

mulation ratio of gastric concentration and plasma concentration for iodide (I^-) than for Cl^- and bromide (Br^-). Davenport's group (Davenport and Fisher, 1940; Davenport, 1943) showed that the concentrations of I^- in the gastric juice were not dependent on the rate of secretion. Heinz et al. (1954) further studied the problem with cats by replacing plasma Cl^- with a wide range of Br^- concentrations. The accumulation ratio of Cl^- between concentrations in gastric juice and plasma increased with increasing Cl^- concentrations in plasma, while the ratio of Br^- decreased with a gradual increase of plasma concentrations. This different behavior of Br^- and Cl^- in gastric secretion was attributed to their different affinities for a carrier material which was probably responsible for active transport of anions in gastric cells. With a pure kinetic approach, Durbin studied the interaction between Cl^- and other anions such as Br^-, I^- and thiocyanate in gastric acid secretion from isolated frog stomach (Durbin, 1964). He found that the relation between acid secretory rates and anion concentrations in nutrient solutions closely resembled Michaelis-Menten Kinetics. Lineweaver-Burk plots denoted a linear relationship between acid secretion and permeable anion concentrations in bathing solutions.

This finding greatly strengthened the hypothesis that there is a membrane carrier for anion transport in gastric mucosa. The affinity of Br^- for the postulated carrier appeared strongest among the three halogens tested, with Cl^- showing next high affinity (Durbin, 1964). This means that the gastric mucosa transports Br^- more readily than Cl^-.

Thiocyanate is known as an inhibitor of gastric acid secretion. In the Durbin study (1964) thiocyanate acted as a competitive inhibitor

of Cl^- transport. It is likely that thiocyanate ions compete with Cl^- for the transport carrier. In the presence of high concentrations of thiocyanate, Cl^- transport from nutrient to secretory side was greatly reduced, and H^+ transport was hindered owing to a lack of electrical balance.

Based upon the preceding discussion, it appears that Cl^- is actively extruded from gastric cells into the lumen of the stomach. The continuous source of Cl^- is the circulating plasma which is hardly a limiting factor under physiological conditions. When Cl^- is secreted into the lumen, the plasma Cl^- will tend to diffuse down the concentration gradient into the gastric cells (Forte _et al_., 1963) and assure a constant supply of Cl^- for transport.

In the case of H^+ secretion in the stomach, the situation is obviously different. During the actively secreting state, H^+ is secreted into the stomach lumen with acid at a concentration of approximately 160 mEq/l. Because a neutral pH must always be maintained in plasma and cells, the perpetually perplexing question for the gastrophysiologist has been how the gastric cells generate H^+ and actively transport H^+ against a million-fold chemical gradient. Numerous hypotheses have been proposed to meet the quandry in the past 150 years, but none satisfactorily explained the problem.

In 1948, Conway and Brady (1948) first introduced a new concept, the "redox theory," for H^+ generation in the gastric cells. Similar proposals by several other investigators followed almost immediately (Crane and Davies, 1948; 1948a; Davies and Ogston, 1950). Despite divergent views on the details, they concurred that H^+ production by the stomach

involves an oxidation-reduction reaction at the surface of the parietal canalicular border.

According to this theory, atomic hydrogen in an energy-producing substrate is oxidized to produce H^+ by an electron acceptor. The H^+ is extruded out of the cell membrane bordering the gastric cavity. Meanwhile, the electron acceptor turns to the inner face of a membrane and delivers its carried electron to molecular oxygen. The oxygen is subsequently reduced to hydroxyl ion (OH^-). The hypothetical electron acceptor (or "metal catalyst" after Conway) could very well be a cytochrome-like compound. The electron transferring sequence has not yet been proven. The electrons might be split off from the hydrogen atom and be accepted by some organic coenzymes such as DPN (diphosphopyridine nucleotide) or FAD (flavin adenine dinucleotide) prior to the involvement of cytochrome materials (Conway, 1953).

The cellular respiratory process responsible for the liberation of H^+ in acid secreting cells is probably not different from the conventional cellular respiratory system except for the separation of two products of the process, H^+ and OH^- ions, instead of their combination to form water. This separation of positive and negative charges represents the crucial point of the redox theory (Robertson, 1960).

How electrons move in the cytoplasm resulting in eventual acceptance by molecular oxygen remains controversial (Lundegårdh, 1940; Crane and Davies, 1948; Geissman, 1949; Chance and Williams, 1956; Chance, 1959). It seems reasonable to speculate that the separation of H^+ and electrons occurs in a particular lipoid membrane in which the electron carriers might be arranged into close proximity with one another.

Because of the low permeability of the membrane for H^+, H^+ once formed will be released into one side of the membrane while the electron passes through the array of carriers and finally combines with oxygen on the other side of the membrane to form OH^- in the presence of water (Robertson, 1960).

The mitochondrial membrane is likely to be the best locus for these reactions, since the electron transport system and the accompanying oxidative phosphorylation are intimately associated with mitochondria. Strong suggestive evidence also comes from ultramicroscopic studies. First, the parietal cells are particularly rich in mitochondria. Other indications are that the mitochondrial membrane is not always separated from other cellular membranes. Geren and Schmitt (1954) showed that the mitochondria in a lobster nerve could connect with the axon surface of the Schwann cell. If this should also be the case in the parietal cell, the produced H^+ in the mitochondria may be transported through the connection to the openings of the secretory surface in the canaliculi (Robertson, 1960). This possibility is attractive and should be subjected to further investigation.

It seems clear now that a Cl^- pump is responsible for the PD across the gastric mucosa and constantly transports Cl^- from serosal to secretory side. A question thus arises: Is the favorable electric potential set up by the Cl^- pump the driving force for H^+ secretion? The answer was given in the work of Heinz and Durbin (1959). They replaced all Cl^- in the nutrient solution bathing an isolated frog mucosa with sulfate ion which is not actively transported by the tissue. They found that H^+ secretion was maintained at about two-thirds of the normal rate.

Furthermore, the PD was reversed in sign and the short-circuit current displayed characteristics of an active H^+ pump. Therefore, H^+ can be actively transported by gastric mucosa independent of Cl^- secretion. This conclusion was supported by Shanbour and Rehm (1971).

In 1859, Brücke assumed that if an acid was liberated in the gastric tubules, an alkaline fluid of corresponding strength must be elaborated simultaneously in the opposite direction into the blood (Davies, 1948). This assumption has subsequently been proved correct by many investigators (Bulger _et al._, 1928; Hanke _et al._, 1931; Gray _et al._, 1940; Teorell, 1951). Furthermore, it was noted that carbon dioxide (CO_2) is essential for normal acid secretion because of its ability to form a buffer system in the parietal cells and to neutralize the OH^- left behind during acid secretion. It has been repeatedly shown with isolated frog gastric mucosa that, in the absence of external supplies of CO_2, acid is secreted at greatly reduced rates (Delrue, 1933; Gray and Adkison, 1941; Davies and Longmuir, 1948; Weaver, 1951; Hogben, 1953). Similar results have been demonstrated in intact animals (Apperly and Crabtree, 1931; Browne, 1932; Fürst, 1950). _In vitro_, ulceration and perforation were observed in the mucosal tissue if the rate of acid secretion was high (Davies and Longmuir, 1948). These observations provided evidence that CO_2 produced by gastric metabolism during acid secretion is stoichiometrically insufficient for neutralization of alkali within the secretory cells.

It has been known for years that the uptake and release of CO_2 in blood cells are catalyzed by an enzyme, carbonic anhydrase. This enzyme is capable of catalyzing the reaction, $CO_2 + H_2O \rightleftarrows H_2CO_3$.

Davenport and his coworkers showed that carbonic anhydrase exists in very high concentrations in the parietal cells (Davenport, 1939; 1940). This observation implies, but does not guarantee, that this enzyme does actively function in acid secreting cells. It is now believed that carbonic anhydrase in the parietal cells catalyzes the formation of carbonic acid which dissociates into H^+ and bicarbonate (HCO_3^-). H^+ derived from this reaction is used for neutralizing the OH^- produced in the acid secretory process, while HCO_3^- diffuses across the serosal membrane of the parietal cells into the blood and causes the "alkaline tide" (Hanke *et al.*, 1931). Because carbonic anhydrase exists at such a high level in the parietal cells, a very high degree of inhibition of the enzyme is necessary to hinder acid secretion (Davies and Roughton, 1948). This inhibition has been shown both *in vitro* (Davenport and Jensen, 1948; Davies *et al.*, 1951) and *in vivo* (Janowitz, 1952) with various carbonic anhydrase inhibitors. Today, the carbonic anhydrase system in the parietal cells has been recognized as a protective acid-base balance measure against damage from the alkaline by-product of acid secretion.

Cellular respiration is critical for acid secretion. *In vitro*, the lack of an oxygen supply or use of specific inhibitors of respiration cause immediate cessation of acid secretion (Crane *et al.*, 1946; Davenport, 1947). A linear relationship between acid secretion and O_2 consumption by gastric tissue has been well-established. The efficiency of acid secretion in terms of the molar ratio of H^+/O_2 has received much recent attention. This ratio has particularly interested the gastrophysiologists because it is directly related to the basic assumption of the redox hypothesis of acid formation. As discussed previously, with

every H^+ generated from atomic hydrogen of a substrate precursor, one electron is concomitantly released into the electron carriers and eventually transferred to O_2. One molecular O_2 can theoretically take up 4 electrons and be reduced to 4 OH^- in the presence of water. In other words, there will be 1 molecule of O_2 consumed for every 4 free H^+. However, so far, a wide range of values has been found by different investigators (Crane and Davies, 1948; Davies and Ogston, 1950; Davies and Roughton, 1948; Teorell, 1949; Davenport and Chavre, 1952; Davenport, 1952; Davenport and Chavre, 1953). This great discrepancy may be attributed to the difficulties in measuring O_2 consumption (Davies, 1957), differences in experimental conditions (Forte and Davies, 1963) and probably the heterogeneity in the cellular composition of mucosal tissue. This controversy is largely unsettled. Nevertheless, the experimental evidence seems generally to arrive at a ratio of about 2, although a ratio higher than 4 would not necessarily exclude a redox pump (Hogben, 1960).

Since O_2 supply is absolutely required for sustained acid secretion in _in vitro_ preparations, the acid secretory process appears to depend upon aerobic metabolism of the parietal cells. Davenport and Chavre (1952) reported that at low partial pressures of O_2, the average ratio of H^+ secreted to O_2 consumed increased. They concluded that anerobic glycolysis can also contribute energy to a certain extent for acid secretion under such a circumstance. Many substances are capable of stimulating acid secretion _in vitro_, such as glucose, acetoacetate (Davenport and Chavre, 1951), lactate and pyruvate (Davenport and Chavre, 1950), and fatty acids (Alonso _et al_., 1967). But none of these materials have been

shown to be essential for acid secretion. The hypothesis that the acid secretory process depends on the mediation of energy-rich phosphates was supported by evidence that azide and 2,4-dinitrophenol, which were known to interrupt the coupling of oxidative phosphorylation, inhibit acid secretion without affecting the O_2 uptake (Davenport and Chavre, 1953; Davies, 1951). Forte _et al_. (1965) studied the ATP content in bullfrog gastric mucosa. They found a substantial correlation between the rates of acid secretion and the concentrations of ATP in the mucosa. Thiocyanate inhibited acid secretion but had no effect on ATP content of mucosa (Forte _et al_., 1965). It is interesting to compare this latter finding with the experiments done by Kasbekar and Durbin on gastric adenosine triphosphatase (ATPase). Kasbekar and Durbin reported (1965) a membrane-bound ATPase from bullfrog gastric mucosa. This enzyme has distinct characteristics from other ATPases related primarily to Na^+ and K^+ transport. Gastric ATPase was stimulated by HCO_3^- and halide ions but reversibly inhibited by thiocyanate ions. Therefore the results supported the view of Forte _et al_. (1965) that thiocyanate inhibits acid secretion by preventing the utilization of ATP by the secretory mechanism. Based upon the experimental observation, Durbin and Kasbekar (1965) put forward an ATPase hypothesis which includes ATPase as an integral part of the HCl secretory mechanism. Like other hypotheses, it is supported but unproven.

In summary, the mechanism of acid secretion in stomach is unclear. What we can be certain of is that the formation of hydrochloric acid is the result of the active transport of two ions, H^+ and Cl^-. The transport processes of H^+ and Cl^- can be independent of one another, but

during acid secretion they normally operate in parallel. Water seems to be pulled into the lumen by a passive force, an osmotic gradient established by active transport of H^+ and Cl^-. It is not known how the two ionic transports interrelate during acid secretion. The nature of the transport mechanisms for two ions are likewise obscure. Rehm et al. argued that H^+ and Cl^- may be secreted from different cellular origins, probably surface epithelial cells for H^+ and parietal cells for Cl^- (Rehm et al., 1953). This hypothesis is unlikely (Davies, 1957) and there is strong evidence suggesting that ionic transport responsible both for electrical and acid secretory phenomena originates in the parietal cells (Davenport and Allen, 1959; Durbin and Kasbekar, 1965). Active transport processes require energy. For acid secretion, high energy phosphate bonds may play a central role. However how this high energy producing system is geared into the ionic transport mechanism remains an interesting subject for future investigation.

The Biochemistry of Adenosine 3',5'-Monophosphate (Cyclic AMP)

While investigating the effect of catecholamines and glucagon on the activation of liver phosphorylase, Rall, Sutherland, and Berthet (1957) discovered a heat-stable factor capable of stimulating the conversion of inactive phosphorylase to its active form. This factor was detected in the presence of either epinephrine or glucagon in the "boiled extract" of an incubation mixture which included the low-speed particulate fraction of liver homogenate, adenosine triphosphate (ATP) and magnesium ions. In the absence of epinephrine and glucagon, the "boiled extract" had little effect on phosphorylase activation. The response to

the hormones in liver homogenates could be separated into two phases: (1) the formation of an active factor in particulate fractions in the presence of epinephrine or glucagon, and (2) the stimulation of homogenates by liver phosphorylase in the supernatant fractions, in which the hormones themselves had no effect. At the same time, Cook *et al*. (1957) independently reported finding a new product during hydrolysis of ATP in the presence of barium hydroxide. Subsequent studies showed the new substances reported by both groups of investigators to be identical, (Sutherland and Rall, 1957; 1958) with a structure of a mononucleotide of adenylate with a phosphate group esterified at carbons 3' and 5' of the ribose moiety. Therefore, it was given the name of adenosine 3',5'-monophosphate, or cyclic AMP. The structure was later confirmed by the use of a variety of enzymatic and chemical degradations, as well as direct determination of molecular weight with ultracentrifugation (Lipkin *et al*., 1959). Cyclic AMP proved not only to be stable during 30 minutes of boiling, but to be dialyzable and resistant to acid and alkali (Sutherland and Rall, 1958). Not long afterward, two enzymes were discovered which directly influenced the level of cyclic AMP in tissues: adenyl cyclase and cyclic nucleotide phosphodiesterase.

Adenyl Cyclase

Adenyl cyclase is widely distributed in nature, having been found in all animal tissues studied to date and in a variety of unicellular organisms--the possible exception being canine red blood cells (Klainer *et al*., 1962). In all cases, the enzymic activity seems to be restricted to low-speed particulate fractions. In addition, most of the activity has been associated with the plasma membrane (Davoren and

Sutherland, 1963; Øye and Sutherland, 1966; Pohl *et al*., 1969; Birnbaumer and Rodbell, 1969). A soluble adenyl cyclase has been identified in *Brevibacterium liquefaciens* (Hirata and Hayaishi, 1967), although its exact location within the intact bacterium remains uncertain.

Adenyl cyclase catalyzes the formation of cyclic AMP from ATP. The cofactor known to be required for this reaction is a divalent cation. Magnesium may be the natural cofactor, but manganese is also effective (Sutherland *et al*., 1962). Pyrophosphate is formed stoichiometrically with cyclic AMP in cell-free systems.

The chemical structure of adenyl cyclase is poorly understood. Sutherland and his coworkers (1962) suggested that it might be a lipoprotein, based on their finding that the enzyme could be solubilized from the particulate fractions of mammalian cerebral cortex, heart, skeletal muscle, and liver with a detergent, Triton. Generally, adenyl cyclase activity has been found to be highly labile, especially in highly concentrated salt solutions (e.g., KCl) or in sucrose solution. However, when the particulate extract is prepared in hypotonic solution, considerable activity is retained for long periods after freezing and storage at -20°C or -70°C. The particulate preparation has also been lyophilized to dry powder form and stored *in vacuo* in the cold with full retention of activity (Sutherland *et al*., 1962). Nevertheless, due to its particulate nature and lability, adenyl cyclase has proven extremely difficult to purify and characterize extensively.

The adenyl cyclase systems in some tissues exhibit specific and sensitive responses to a variety of hormones. There are two alternative explanations for this: either (1) the different hormones stimulate a

single enzyme via different receptors, or (2) a separate adenyl cyclase exists for each of the hormones. Birnbaumer et al. (1970) studied the hormonal specificity in fat-cell ghosts with the four most active lipolytic hormones: corticotrophin (ACTH), epinephrine, secretin, and glucagon. They reported that propanolol, a beta-adrenergic blocking agent, inhibited the stimulatory activity of epinephrine but failed to affect that of ACTH or glucagon. DCB [1-(2,4-dichlorophenyl)-1-hydroxy-2(t-butylamino)ethane], a beta-adrenergic agent, stimulated adenyl cyclase, but its effect was not additive with the effect of epinephrine. An ACTH analogue inhibited only the stimulatory action of ACTH. Pretreatment of the adipose tissue with trypsin completely deprived glucagon of its stimulating effect, caused partial loss of ACTH and secretin activity, and failed to affect epinephrine and fluoride activity. Judging from these observations, the authors concluded that a single adenyl cyclase system exists in adipose tissue, with distinctive and specific receptors or binding sites for different hormones. A single adenyl cyclase system with different receptors has also been postulated for many other tissues, such as cat liver (Makman and Sutherland, 1964), rat heart (Murad and Vaughan, 1969), and cat heart (Levey and Epstein, 1969).

In all adult mammalian tissues studied thus far, fluoride has been reported to stimulate adenyl cyclase very effectively in broken-cell preparations. Unlike hormones, fluoride apparently lacks selectivity in enhancing adenyl cyclase activity in different tissues. In 1958, Rall and Sutherland attributed the fluoride effect on cyclic AMP formation in dog liver homogenate to the maintenance of substrate concentration (ATP) by inhibition of ATPase (Rall and Sutherland, 1958). Later, they sug-

gested that fluoride also stimulates adenyl cyclase activity (Sutherland *et al.*, 1962). Although the stimulatory effect of fluoride on adenyl cyclase in broken-cell preparations may sometimes be striking, it has not been demonstrated in any intact tissues. The nature of fluoride stimulation in broken-cell preparations, as well as the failure of fluoride to stimulate adenyl cyclase in intact cells, are unexplained at present. The two events appear related, so that an answer to one question might help clarify the other.

In some tissues, combinations of maximally stimulating concentrations of hormones and fluoride do not produce additive effects, indicating that only one adenyl cyclase system is affected. For example, in fat-cell ghosts, fluoride does not potentiate the maximal stimulating effect of lipolytic hormones on cellular cyclic AMP formation (Birnbaumer and Rodbell, 1969). When the incubation conditions were varied, Birnbaumer and Rodbell were able to show several differences between the effects produced by fluoride and by the homrones upon the enzyme. Their results suggested that hormones and fluoride activate a single enzyme through different processes. On the other hand, both fluoride and norepinephrine activate adenyl cyclase in pineal homogenates, with many shared characteristics apparent. Nevertheless, norepinephrine plus amounts of fluoride that are in themselves maximally effective have been shown to further increase the rate of cyclic AMP formation (Weiss, 1969). This indicates that either (1) fluoride and norepinephrine activate separate catalytic sites of a single enzyme, or (2) two stimulants increase the activity of two enzymes in the pineal gland.

Cyclic Nucleotide Phosphodiesterase

Like adenyl cyclase, phosphodiesterase is widely distributed in nature. This enzyme is known to hydrolyze cyclic AMP at the 3' position, thereby producing 5'-AMP. Divalent metal ions are also essential for the activity of the enzyme. The conversion of cyclic AMP to 5'-AMP by phosphodiesterase is the only well-known physiological mechanism for terminating the action of the cyclic nucleotide. The existence of this enzyme was first demonstrated by Sutherland and Rall (1958) in the beef heart. In addition to being identified in mammalian tissues, the enzyme has been detected in many unicellular organisms, such as yeast (Cheung, 1966), a bacterium (Brana and Chytil, 1966), and slime mold (Chang, 1968), as well as in several marine organisms (Yamamoto and Massey, 1969). The only cells to date which have been shown to lack the enzyme are avian erythrocytes (Drummond and Perrott-Yee, 1961). All mammalian tissues examined have shown cyclic nucleotide phosphodiesterase activity. The highest activity has been measured in brain tissue (Drummond and Perrott-Yee, 1961; Butcher and Sutherland, 1962).

The subcellular distribution of phosphodiesterase activity in mammalian tissues has been demonstrated in both soluble and particulate fractions (2500 x g). The particulate fractions of homogenates of the various canine tissues vary in activity, ranging from 10% to 90% of the total activity initially present in crude homogenates (Butcher and Sutherland, 1962). The characteristics of the particulate activity in all cases were almost identical with those of the soluble form. Butcher and Sutherland (1962) carried out the fractionations of phosphodiesterase activity in beef heart with ammonium sulfate and DEAE (Diethylaminoethyl)

cellulose. They were able to purify the soluble enzyme 153-fold. Most of the work on the properties of phosphodiesterase has been done with the soluble enzyme.

In contrast to adenyl cyclase, phosphodiesterase is a stable enzyme. Storage at -20°C for a month usually causes no appreciable changes in activity. Divalent cations are absolute requirements for full enzymic activity in all tissues studied. Mg^{++} and Mn^{++} are most effective; other divalent ions, such as Co^{++}, Ni^{++}, and Ba^{++}, meet the requirement much less effectively. Some divalent ions, like Ca^{++}, Cu^{++}, Hg^{++}, and Zn^{++}, may actually be inhibitory (Cheung, 1967; 1970). EDTA completely abolishes the enzymic activity, although activity can be fully restored by the addition of Mg^{++} or Mn^{++}. Based upon these findings, Cheung suggested that the active form of phosphodiesterase might be a metal-enzyme complex (Cheung, 1967). Phosphodiesterase from most tissues has a pH optimum between 7.5 and 8.0. In exceptional cases, such as the enzymes from dog heart (Nari, 1966) and fish brain (Yamamoto and Massey, 1969), the pH optimum ranges between 8.5 and 9.0.

The molecular structure of phosphodiesterase is unknown. The only identifying clue comes from experiments using p-hydroxymercuribenzoate and beta-mercaptoethanol. The former inhibits enzymic activity whereas the latter reverses the inhibition, seeming to indicate that phosphodiesterase possesses sulfhydryl groups essential for enzymic activity. The molecular weight of phosphodiesterase has not been firmly established, although a value of approximately 300,000 is suggested by the data of several investigators studying different tissues (Cheung, 1970; Chang, 1968; Menahan _et al._, 1969).

Cheung (1969) was able to resolve the enzymic activity into two active interconvertible peaks on a Sepharose 4B column. The first peak, of seemingly higher molecular weight, apparently consisted of the molecules of phosphodiesterase aggregated by intermolecular disulfide linkages. The second peak, with a smaller molecular weight, probably contained the reduced enzyme with the sulfhydryl groups. On the other hand, trypsin was found to activate purified phosphodiesterase and to reduce the molecular weight from the value determined before treatment with trypsin. Since molecular size could be changed under different conditions, Cheung (1970) proposed that phosphodiesterase may be composed of subunits giving rise to different subunit structures and thus different molecular weights under varying conditions.

The Michaelis-Menten constant (K_m) of phosphodiesterase has varied in the different tissues studied. At least in the brain tissues of some animals, two different K_m values have been reported (Brooker et al., 1968; Cheung, 1970). Whether the two K_m values represent two species of the enzyme or both K_m values apply to a single species is unknown.

Many substances are known to inhibit phosphodiesterase activity. Butcher and Sutherland (1962) first demonstrated the inhibitory effects of the methylxanthines, such as theophylline, caffeine, and theobromine. In addition, papaverine inhibits phosphodiesterase activity (Kukovetz et al., 1969; O'Dea et al., 1970; Triner et al., 1970). Since methylxanthines are not readily metabolized in tissues, they have been used extensively in endocrine studies in which cyclic AMP is believed to play a role. However, phosphodiesterase from a slime mold (Chang, 1968) and

Escherichia coli (Brana and Cytil, 1966) was not inhibited by caffeine and only slightly inhibited by theophylline.

A rat brain phosphodiesterase was also inhibited by pyrophosphate, several nucleotide triphosphates (including ATP), and some intermediate organic acids in the citric acid cycle (Cheung and Salganicoff, 1966; Cheung, 1967). The strong inhibition by ATP and pyrophosphate at approximately physiological concentrations prompted the speculation that phosphodiesterase might exist *in vivo* in a greatly inhibited state (Cheung, 1966). In addition, the activity of the enzyme was largely depressed upon purification. Based upon his pioneering work, Cheung suggested that the loss of activity was due to removal of a protein cofactor from the enzyme during the purification process (Cheung, 1970). A parallel distribution of phosphodiesterase and its protein cofactor was indicated by subcellular fractionation of bovine brain cortex, cerebellum, hypothalamus, and other tissues. Phosphodiesterase cofactor has also been isolated from rat, porcine, and human brain and bovine heart. Cross-activation studies have shown that the activators (or cofactors) lack tissue specificity. On the basis of *in vitro* studies, apparently the activity of phosphodiesterase can be regulated by three distinct factors: triphosphate nucleotides, divalent metal ions, and the protein activator of the enzyme. The importance of these individual factors in the control of phosphodiesterase activity and their interaction in *in vivo* systems remains to be elucidated.

Biological Importance of Cyclic AMP

Since the discovery of cyclic AMP in 1957, many investigators have demonstrated it to be an extremely versatile agent. Not only has

this simple nucleotide been implicated in numerous cellular functions, it has been established as a unique intracellular mediator of a wide variety of hormones. A few hormonal actions mediated by cyclic AMP have been successfully studied at the level of enzymic activities--a well known example being phosphorylase activation by glucagon and epinephrine in the liver and by epinephrine in muscle. These hormones appear to stimulate adenyl cyclase in liver and muscle to increase cellular cyclic AMP formation. This increased cyclic AMP level initiates a "cascade" effect on activation of an enzyme system. In the liver, cyclic AMP is known to convert an inactive enzyme, phosphorylase-b-kinase, to its active form. This active kinase catalyzes subsequent activation of phosphorylase-b to phosphorylase-a (Sutherland and Robison, 1966). Krebs et al. (1966) presented evidence that increased cyclic AMP formation in skeletal muscle by epinephrine and glucagon stimulated a particular receptor earlier than phosphorylase-b-kinase. That receptor may be another kinase which is responsible for phosphorylation of phosphorylase-b-kinase.

Glycogen synthetase and phosphofructokinase are two additional enzymes whose relationship to cyclic AMP has received detailed attention. Glycogen synthetase exists in two forms: one is dependent upon glucose-6-phosphate (the D form), and the other is independent of glucose-6-phosphate (the I form). The inactivation of glycogen synthetase from the I to D form is catalyzed by a kinase, which is under the control of cyclic AMP (Huijing and Larner, 1966; Dewuff and Hers, 1968; Bishop and Larner, 1969). Phosphofructokinase is a key enzyme in the glycolytic process. In some species, at least, it may exist in both the active and inactive forms. The activation of this enzyme absolutely requires the presence

of cyclic AMP. A kinase probably also participates in the activation of phosphofructokinase, with cyclic AMP stimulating the process through regulation of the kinase activity (Mansour, 1966; Stone and Mansour, 1967). Furthermore, cyclic AMP antagonizes the inhibitory effect of ATP on the activated phosphofructokinase. This effect of cyclic AMP may also contribute to the activation of phosphofructokinase (Stone and Mansour, 1967).

The precise mechanisms whereby cyclic AMP influences many hormonal functions are unknown. Ignorance of discrete biochemical reactions extends even to those biological processes which have been shown to be mediated by cyclic AMP, such as lipolysis in adipose tissue in response to various hormones (Vaughan and Steinberg, 1965), steroidogenesis in adrenal gland and corpus luteum in response to ACTH and the gonadotrophins (Hilf, 1965; Marsh et al., 1966), thyroid hormone production stimulated by TSH (Gilman and Rall, 1966; Ensor and Munro, 1967), and positive inotropic response of the heart to catecholamines (Sutherland et al., 1968).

In order to identify cyclic AMP as an intracellular mediator of any given hormone, Sutherland and his collaborators have proposed four essential criteria, which can be established by four types of experiments. Specifically, (1) adenyl cyclase in broken-cell preparation should respond to the same hormone which is effective in the intact tissue; (2) the level of cyclic AMP in intact tissue should change appropriately in response to hormonal stimulation; (3) the hormone which stimulates adenyl cyclase should be potentiated by drugs which inhibit phosphodiesterase activity, such as theophylline and caffeine; and (4) the hormonal effect may be mimicked by exogenous cyclic AMP (Sutherland et al., 1968).

Many hormones appear to be part of a two-messenger system Sutherland *et al.*, 1968). According to this concept, the hormones acting as the first messengers travel from their cells of origin to the target tissues where they stimulate membrane-bound adenyl cyclase to promote the formation of intracellular cyclic AMP. The increased concentration of cyclic AMP then activates special cellular mechanisms responsible for particular physiological responses, according to the characteristics of the cells involved. Cyclic AMP receives the biological signals coming from the hormones (the first messengers) and transmits them to the cellular machinery to produce hormonal effects in the cells. This two-messenger concept assumes cyclic AMP to be the second messenger.

Despite a poor understanding of the mechanisms of action of cyclic AMP in most hormonal functions, the discovery of the participation of cyclic AMP in hormonal processes does seem to lead us one step closer to understanding how hormones influence their target tissues.

Effect of Cyclic AMP on Gastric Mucosa

Although cyclic AMP has been intensively studied in a number of mammalian tissues, especially in muscle, liver and brain, little is known about the role of the nucleotide in gastric secretory processes.

Orloff and Handler (1962) observed that cyclic AMP and theophylline mimicked the hormonal effect of vasopressin on the toad bladder *in vitro*. Each agent caused an increase in the permeability of toad bladder to water and in the rate of active transport of sodium (expressed in a change of the short circuit current).

In cooperation with Butcher and Sutherland, they further demonstrated that incubation with either arginine-vasopressin or theophylline

resulted in a significant increase in the concentration of cyclic AMP in the toad bladder and that when the two agents were employed together, a synergistic effect on the increase in cyclic AMP level was apparent (Handler et al., 1965). The results of these experiments supported their original hypothesis that cyclic AMP is the intracellular mediator of the effects of vasopressin. The effect of theophylline on ion transport in toad bladder was interpreted as inhibition of phosphodiesterase, thereby increasing cyclic AMP levels.

The discovery that cyclic AMP participates in producing the effect of vasopressin on sodium and water transport in toad bladder was the first evidence that the cyclic nucleotide plays a part in ion transport across cell membranes. The pioneering work of Orloff and Handler essentially initiated the subsequent studies of cyclic AMP effects on acid secretion in the stomach.

Harris and Silen (1964) reported that imidazole largely inhibited spontaneous acid secretion from isolated frog gastric mucosa and depressed acid secretion by more than half during stimulation with histamine in the same preparation. Intravenous infusion of imidazole in a dog with a Heidenhain pouch also significantly decreased acid secretion during constant histamine stimulation (Harris and Silen, 1964). Subsequently, imidazole was shown not only to inhibit histamine-stimulated acid secretion from isolated frog mucosa but also to reduce active sodium transport by toad urinary bladder (Alonso et al., 1965). The inhibitory effect of the imidazole on acid secretion could not be ascribed to its buffering capacity or to competitive inhibition of histamine (Harris and Silen, 1964; Alonso et al., 1965). Since imidazole was found

in vitro to stimulate the phosphodiesterase that inactivates cyclic AMP (Sutherland and Rall, 1960), the effect of imidazole on ion transport may be related to declining concentrations of intracellular cyclic AMP.

In another investigation, Alonso and Harris drew attention to the effects of methylxanthines on isolated frog gastric mucosa (Alonso and Harris, 1965). Their results indicated that all three methylxanthines studied (theophylline, theobromine and caffeine) were capable of increasing acid secretion from the intact isolated mucosa and of increasing oxygen consumption in the minced mucosal tissue, provided that the bathing solutions contained chloride ions. Theophylline was the most potent of these three compounds in stimulating acid secretion and oxygen consumption. Both theophylline and histamine produced a large increase in mucosal short circuit current which preceded the rise in H^+ transport and declined as the H^+ flow began to increase. On a molar basis, the mucosa was less sensitive to theophylline than to histamine. However, at both maximally effective concentrations, theophylline yielded a significantly greater response than histamine (Alonso and Harris, 1965). The significance of this finding is unclear.

Because the methylxanthines appeared to affect both acid secretion and metabolic rate concomitantly, a causal relation between these two responses was suspected. In an attempt to dissociate secretory and metabolic responses to supposed increases in cyclic AMP, Alonso and Harris substituted an impermeant ion, glucuronate, in the solution bathing the *in vitro* frog submucosal surface. They found that oxygen consumption of the tissue remained unchanged even with histamine or caffeine which stimulated oxygen consumption when chloride was present (Alonso

and Harris, 1965). Durbin (1954) previously showed that substitution of glucuronate for chloride in the bathing solution essentially eliminated both H^+ flow and short circuit current. Therefore, it seems that histamine and the methylxanthines act primarily on the ion transport process, and the increase in oxygen consumption appears secondary to ion secretion.

Since both methylxanthines and imidazole directly influence cellular concentrations of cyclic AMP, the results of the preceding experiments suggested a role for cyclic AMP in acid secretory function. In order to obtain more evidence for this hypothesis, Harris and Alonso examined the effect of exogenous cyclic AMP on isolated frog mucosa bathed with solutions containing either chloride or glucuronate (Harris and Alonso, 1965). With chloride in the bathing solution, cyclic AMP increased acid secretion in the mucosa from one species of frogs, but failed to evoke secretion in the mucosa of another species. In the second species, however, cyclic AMP impeded the natural decline in secretion that occurred in untreated mucosa during incubation. The replacement of chloride solution on the submucosal side with glucuronate solutions resulted in either complete cessation or great reduction of H^+ flow, even with cyclic AMP in the bathing solutions. Beside the secretory effect, cyclic AMP exerted a profound influence on the tissue metabolic rate. With both glucuronate and chloride solutions, cyclic AMP significantly increased oxygen consumption of the mucosal tissue. The latter finding suggests that cyclic AMP affects cellular metabolism independent of H^+ and Cl^- transport. In other words, it may be possible that cyclic AMP primarily affects metabolic activity in the mucosa and increases secre-

tion only secondarily or that cyclic AMP could cause separate effects on metabolism and membrane transport of hydrochloric acid (Harris and Alonso, 1965). It is not surprising that cyclic AMP should be related to energy metabolism in the secretory stomach, since the nucleotide plays a documented role in carbohydrate and lipid catabolism in many tissue (Robison et al., 1968).

In frog gastric mucosa, Nigon and Harris (1968) found that exogenous cyclic AMP did not stimulate but only inhibited the decline in phosphorylase activity observed during the incubation. However, in another investigation (Alonso et al., 1968), cyclic AMP significantly increased glycogenolysis in mucosa incubated in chloride solutions and to a lesser degree in glucuronate solutions. Therefore, the authors concluded that the small changes in phosphorylase activity shown previously were physiologically important.

The difference of magnitude in glycogenolysis in two solutions was similar to the difference in oxygen consumption of mucosal tissues incubated with chloride versus glucuronate during addition of cyclic AMP. From these results Alonso et al. (1968) suggested that cyclic AMP may have a direct effect on glycogenolysis that is not tightly coupled to the effect on acid secretion.

According to their calculation, Alonso et al. (1968) reported that only half of the measured oxygen consumption could account for glycogen utilization either in the mucosa exposed to cyclic AMP or the untreated mucosa. This implies that carbohydrate is not the only energy source for gastric mucosa. Their hypothesis is compatible with previous observations that fatty acids or triglycerides also provide energy for

acid secretion (Alonso et al., 1967). Thus, it is likely that cyclic AMP may accelerate both lipid and carbohydrate metabolism in gastric mucosa. A part of the energy increment would be devoted to ion transport processes. Most recently Harris' group reported a relationship between cyclic AMP and gastric acid secretion which was noted when theophylline was incubated with frog mucosal tissues; tissue cyclic AMP concentration measured by a method based on phosphorylase activation was significantly increased. There were two strong arguments supporting the hypothesis: first, the concentration of methylxanthines that affect acid secretion correlated remarkably with the concentrations used to increase the mucosal cyclic AMP level; second, the increase in tissue cyclic AMP in response to theophylline paralleled the rise in acid secretion as far as the time course was concerned (Harris et al., 1969).

The results with isolated frog gastric mucosa may be summarized as follows: There is strong evidence that cyclic AMP is involved in gastric acid secretion in frog stomach. The mechanism by which cyclic AMP regulates gastric acid secretion and the relation of enhanced metabolic activity to secretory processes is uncertain. Furthermore, a question raised by Harris' experiments remains unanswered. If the secretory effect of histamine and theophylline was due to their ability to raise intracellular cyclic AMP concentrations, why wasn't the metabolism increased independently of secretion? This independence of the metabolic response was observed when exogenous cyclic AMP stimulated secretion.

As early as 1944, Roth and Ivy (1944) demonstrated that caffeine was a potent stimulant of gastric secretion in the cat and in man. Caffeine also potentiated histamine-stimulated acid secretion (Roth and Ivy,

1944a). Years later it was demonstrated that caffeine and theophylline failed to stimulate gastric acid secretion in dogs, although these agents potentiated the effects of histamine (Robertson and Ivy, 1949). These findings were also reported by other investigators (Roth and Ivy, 1944; Merendino *et al*., 1945; Robertson *et al*., 1950). However, theophylline was found to initiate secretion of acid gastric juice in man either by oral or intravenous administration (Krasnow and Grossman, 1949).

These *in vivo* results were obtained before cyclic AMP was discovered. With our present knowledge about cyclic AMP, the *in vivo* effects of these methylxanthines on acid secretion seemed to fit nicely with the cyclic AMP hypothesis of gastric acid secretion, excepting only the action of these methylxanthines on gastric acid secretion in the dog.

Another approach to the problem was employed by Levine and his collaborators (1967). They studied the effects of acute and continuous intravenous infusion of cyclic AMP on histamine stimulated gastric acid secretion in conscious dogs with gastric pouches and in man. Unexpectedly in both species, the volume responses of secretion to histamine were depressed by cyclic AMP to one-third of the original values. Gastric mucosal blood flow was concomitantly decreased as measured by aminopyrine clearance in the dog. Two alternative mechanisms may account for the action of cyclic AMP on acid secretion in conscious dogs and man. Since gastric mucosal blood flow was decreased along with the decline in secretion, cyclic AMP may constrict blood vessels primarily, thereby depriving the gastric mucosa of its energy supply and secondarily reducing energy-dependent transport of ions. This possibility was supported by the fact that acid secretion and blood flow were not specifically affected

by cyclic AMP, i.e., other adenine nucleotides (including 2',3'-AMP and 5'-AMP), in comparable doses to cyclic AMP, were found to produce similar effects (Levine et al., 1967). The other mechanism may be direct inhibition by cyclic AMP on the parietal cell secretory machinery. Since mucosal blood flow has been shown to correspond directionally to changes in acid secretion (Jacobson et al., 1966; Jacobson et al., 1967), the change in mucosal blood flow observed with infusion of cyclic AMP may be secondary to the decrease in acid secretion. Based upon the information from Levine et al's study, it is not possible to determine which explanation is correct.

Cyclic AMP has been found to be poorly transportable through the plasma membranes of many tissues. It is not certain that the infused cyclic AMP in Levine et al's experiment could pass through the parietal cell membrane and produce an inhibitory effect in the cell. Furthermore, cyclic AMP is a nonspecific initiator of many physiological processes in an in vivo system (Levine and Vogel, 1966; Levine et al., 1968). It would also be possible that the infused cyclic AMP triggered the release of some other humoral factors which were responsible for the inhibition of acid secretion.

Recently, Perrier and Laster (1970) measured mucosal adenyl cyclase activities. They found that in vitro histamine at 10^{-4} M consistently stimulated gastric mucosal adenyl cyclase of guinea pig stomach, whereas choline esters and gastrin had no effect. These findings do not support the cyclic AMP hypothesis for gastric acid secretion, since gastrin and choline esters, as well as histamine, are potent secretagogues. If cyclic AMP is a final common mediator for acid secretion,

adenyl cyclase in parietal cells ought to respond to all secretory stimulants.

Studies of the relationship between prostaglandins and gastric acid secretion have yielded evidence both in favor of and against the role of cyclic AMP in secretory function of the stomach. Prostaglandins are a group of highly active, lipid soluble unsaturated hydroxy acids present in most if not all mammalian tissues. They have been found to have a wide spectrum of biological actions. The physiological roles of prostaglandins remain to be established.

It has been shown that prostaglandins inhibit several hormonal effects such as the effect of vasopressin on the toad bladder (Orloff et al., 1965) and rabbit kidney tubules (Orloff and Grantham, 1967), and the effect of epinephrine on adipose tissue (Butcher and Baird, 1968). These antagonistic actions were associated with a decrease in the intracellular accumulation of cyclic AMP. The effect of prostaglandins on gastric secretion has been studied in the amphibian (Way and Durbin, 1969), the rat (Shaw and Ranwell, 1968; Main, 1969), the dog (Robert et al., 1968, Jacobson, 1970) and in man (Horton et al., 1968; Wilson et al., in press). In all cases except one in man (Horton et al., 1968), prostaglandins produced profound inhibition of gastric acid secretion stimulated by various means. The mechanism by which prostaglandins inhibit acid secretion is largely unknown. A direct inhibitory effect on the acid secretory cell has been suggested (Way and Durbin, 1969; Jacobson, 1970). More specifically, adenyl cyclase has been considered as a possible cellular site for the action of prostaglandins. In other words, prostaglandins may inhibit the secretory process by reducing

cyclic AMP formation because the secretory effect of exogenous cyclic AMP on isolated frog mucosa was not reduced by the prostaglandins (Way and Durbin, 1969). However, in rat stomach prostaglandin E_1 did inhibit acid secretion in response to added cyclic AMP (Ramwell and Shaw, 1968). Thus, prostaglandins may also interfere with the cyclic AMP action in the cell.

Surprisingly, Perrier and Laster reported (1970) that prostaglandins activated adenyl cyclase in guinea pig gastric mucosa. This seems to argue against the contention that acid inhibition by prostaglandins involved cyclic AMP. It should be noted that this report by Perrier and Laster was in preliminary form. Furthermore, since gastric mucosa is nonhomogeneous, the stimulatory effect of the prostaglandins might be occurring in a cell type other than the parietal cells, a situation resembling that in adipose tissue (Butcher and Baird, 1968). A test of this possibility would not be possible until an isolated parietal cell preparation could be developed.

Aims of the Investigation

A logical division of molecular events in gastric secretion involves three sequential steps which may not be independent of one another: (1) activation of the acid producing system, i.e., the effects of secretagogues on the parietal cells; (2) generation of hydrogen ions and chloride ions for formation of acid; and (3) active transport of the ions across the cellular membrane.

It appears reasonable to divide the gastric secretion process into the three steps cited above, although the initial activating step is hardly understood.

There has been practically no effort directed toward bridging the gap between extracellular controls of parietal cell function and the intracellular secretory mechanism.

Recently, the historical discovery of cyclic AMP has turned a new page in the development of endocrinology. A number of hormones have been shown to stimulate the functions of their target tissues indirectly via activation of adenyl cyclase to produce cyclic AMP. The increase in intracellular cyclic AMP in turn promotes the expected cellular response. Thus cyclic AMP functions as a transmitter of the hormonal message from the outside to the inside of the cells.

Naturally occurring neurohumoral substances which provoke gastric secretion, such as acetylcholine, gastrin and histamine are either charged compounds or polypeptide with a sizable molecular weight. They may not be able to penetrate the plasma membrane of the parietal cells and stimulate secretion of HCl within the cell. An alternative mechanism not requiring penetration of chemical stimuli through the parietal cell membrane would be their triggering a chain reaction which started on the extracellular side of the membrane; the best candidate for initial membrane receptor for secretagogues would be the adenyl cyclase system. Harris and his collaborators were the first group of investigators to test this hypothesis. Their findings have evoked great interest, because of the potential value of their hypothesis in explaining the metabolism of acid secretion by the stomach. Our comprehension of gastric acid secretion would be greatly advanced were cyclic AMP established as the final common mediator of gastric acid secretion, and adenyl cyclase shown to be the cell membrane receptor which transduces the message from

extracellular secretagogues.

The conclusions of Harris and his collaborators (Harris _et_ _al_., 1969) depend almost entirely upon their experiments, which focused upon the cyclic AMP degrading system, namely examining the effects of phosphodiesterase inhibitors on acid secretion and cyclic AMP concentration in the frog gastric mucosa. They did not study actual enzymatic levels responsible for tissue cyclic AMP content with acid secretagogues or phosphodiesterase inhibitors. However, the effect of secretagogues on adenyl cyclase and phosphodiesterase must be regarded as one of the crucial points in testing the present hypothesis.

Recently, Perrier and Laster (1970) reported that histamine, a secretory stimulant, and prostaglandin E_1, a secretory inhibitor, both stimulated gastric mucosal adenyl cyclase activity of guinea pig with similar potencies, whereas choline esters and gastrin (secretory stimulants) were without effect on the enzyme. Unfortunately, their abstract presented no data. At present, therefore, before the hypothesis of Harris and his coworkers can be accepted, a systematic study of the hypothesis is apparently needed.

The research of this dissertation was designed primarily to focus on measurements of acid secretory rates and the activities of adenyl cyclase and phosphodiesterase in canine gastric mucosa. In addition, the effect of exogenous cyclic AMP on gastric acid secretion was determined, employing intra-arterial infusions of dibutyryl cyclic AMP to maximize penetration of the nucleotide into the parietal cells (Posternak _et_ _al_., 1962).

By using above approaches the present investigation was ad-

dressed to answering five questions: (1) Can substances which inhibit gastric phosphodiesterase activity *in vitro* stimulate acid secretion when injected into intact animals intravenously or intra-arterially? (2) Are there parallel changes in phosphodiesterase activity and acid secretory rate during the administration of phosphodiesterase inhibitor? (3) Does the classical acid secretagogue, histamine, affect gastric mucosal adenyl cyclase activity *in vitro*? (4) Does intravenous infusion of histamine yield directional changes in gastric adenyl cyclase activities and acid secretory rates? and (5) Can dibutyryl cyclic AMP initiate acid secretion in intact animals when given by intraarterial infusion?

CHAPTER II

METHODS AND MATERIALS

In order to meet the objectives set for this investigation, three major experimental approaches were designed: (1) the effect of theophylline and papaverine on gastric phosphodiesterase activity *in vitro* and *in vivo*, as well as the acid secretory response of the stomach to these drugs; (2) the effect of histamine on adenyl cyclase activity *in vitro* and the relationship between acid secretion and adenyl cyclase activity during the *in vivo* administration of the secretagogue; and (3) the acid secretory response to increased concentrations of cyclic AMP in gastric cells induced by intraarterial infusion of dibutyryl cyclic AMP.

Animal Preparation

Mongrel dogs of either sex weighing from 15-20 kg were used. Food was withdrawn for 24 hours before use. Each dog was anesthetized with an intravenous injection of chloralose (55 mg/kg dissolved in a solution containing 0.5 gm of ethyl carbamate per ml of saline). The dog was maintained in a supine position under anesthesia. A femoral artery and vein were cannulated, the former connected with a Sanborn pressure transducer for monitoring the blood pressure during the experiment and the latter for drug infusion and supplemental anesthestic.

A midline abdominal incision was made with an electrical cautery

device. A wedge of stomach wall was excised from the greater curvature in the region of the gastroepiploic vessels, but its blood supply was maintained intact (Fig. 1). The wedge was brought out of the peritoneal cavity and secured between a Lucite ring and a partitioned chamber. The mucosal surface of the stomach flap was enclosed and divided by the chamber into two equal halves, each with its own blood supply. On the top of the chamber, there was one large window for each half of the stomach flap, designed to allow the obtaining of biopsies of mucosa. One outlet from each half of the chamber permitted collection of gastric juice (Fig. 1). The chamber was fixed in a position slightly above the anterior peritoneal wall for the duration of each experiment.

For experiments with intraarterial infusion, a branch of the splenic artery leading to the spleen was cannulated so that the infusate could be administered in the direction of the stomach tissue. The experiments were begun about 30 min after finishing all surgical procedures.

Chemical Assays

Assay of Cyclic Nucleotide Phosphodiesterase Activity

The assay was performed in three separated steps: enzymatic reactions, inorganic phosphate determination and protein determination.

Principles of the assay.

Enzymatic reactions. The reaction system was modified from the method of Butcher and Sutherland (1962), which included two enzymic reactions taking place in a single incubation mixture. The mixture was composed of cyclic AMP, an excess of snake venom containing a potent 5'-nucleotidase and Tris buffer with Mg^{++} ions. When the tissue preparation

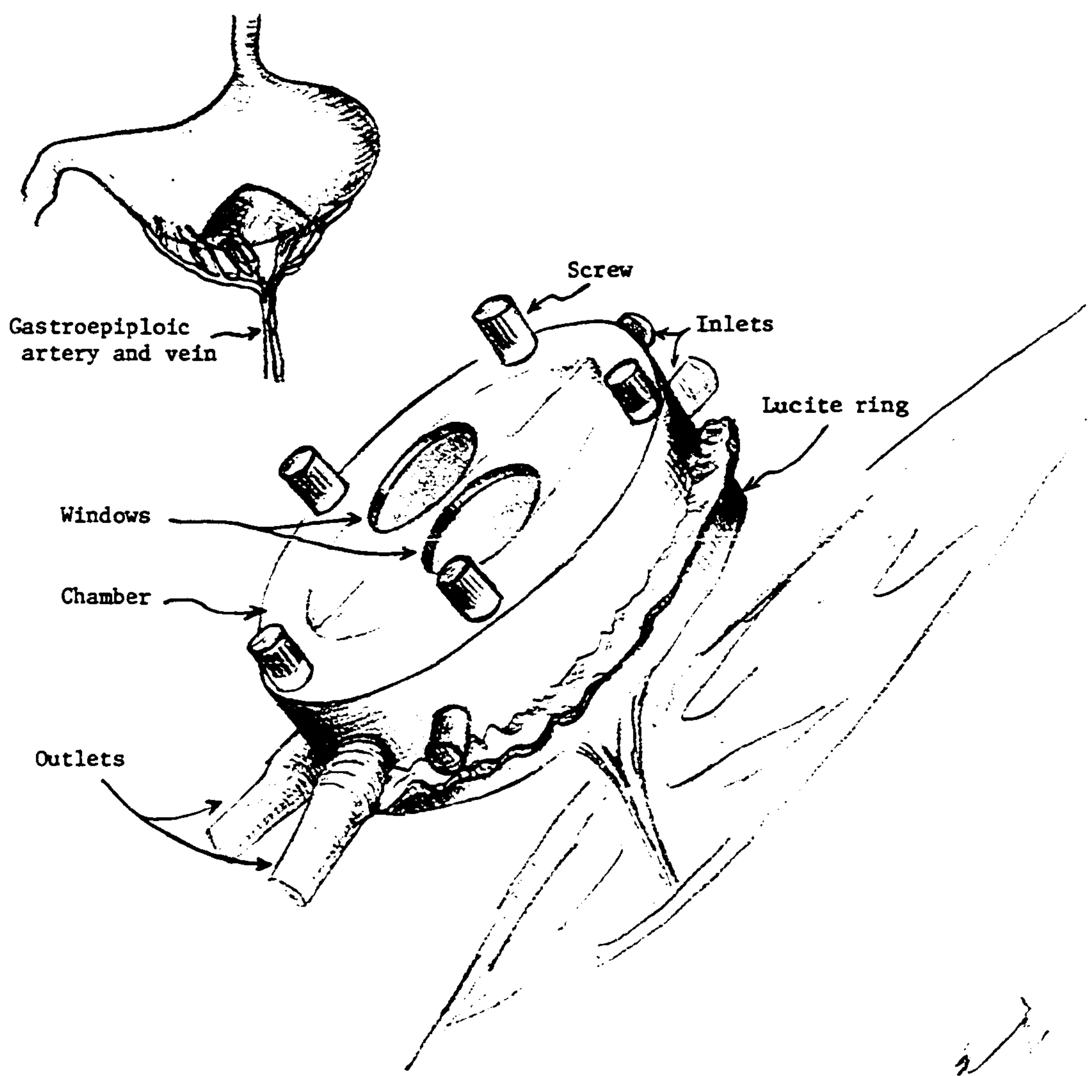

Figure 1. Chambered segment preparation of the dog stomach.

containing the phosphodiesterase activity to be assayed was added into the reaction mixture, it cleaved the 3'-ester bond of cyclic AMP to produce 5'-AMP. In the presence of 5'-nucleotidase, the 5'-AMP formed in the phosphodiesterase reaction was immediately converted to adenosine and inorganic phosphate. The cyclic AMP concentration in the reaction mixture was chosen to reveal the full activity of phosphodiesterase. The quantity of snake venom used assured a complete conversion of formed 5'-AMP to adenosine and phosphate. Thus the increase in phosphate concentration in the mixture in the presence of a given amount of tissue reflected the enzymic activity of phosphodiesterase.

Inorganic phosphate determination. The determination was done according to a method described by Buell et al. (1958). The principle involved was based upon the fact that phosphomolybdic acid is easily reduced to yield a highly colored compound which can be measured colorimetrically. A phosphate reagent containing ammonium molybdate and ascorbic acid in sulfuric acid was used. In the solution, molybdic acid was formed through the reaction of ammonium molybdate and sulfuric acid. When standard KH_2PO_4 or samples containing an unknown quantity of inorganic phosphate was added to the reagent, the inorganic phosphate reacted with molybdic acid to form phosphomolybdic acid. This complex acid was readily reduced by the reducing agent, ascorbic acid. The intensity of the blue color displayed by reduced complex acid was linearly proportional to a wide range of the concentrations of inorganic phosphate existing in the mixture.

Protein determination. The principle was similar to the one for the inorganic phosphate determination described previously. The

solution, Folin's phenol reagent, contained mainly phosphomolybdic and phosphotungstic acids. Both complex acids could be reduced in alkaline condition by phenol or oxybenzol compounds to form color substances, which resemble the corresponding unreduced compounds in all respects, except that of color. Taking advantage of this fact, Wu (1922) first proposed to determine protein concentration by using the Folin phenol reagent, for proteins generally contain aromatic amino acids such as tyrosine and tryptophan, which can reduce phosphomolybdictungstic acids to form color products. The blue color obtained is not stable in strong alkaline condition. It was found that the best alkali for the purpose was sodium carbonate (Folin and Denis, 1912). Lowry _et_ _al_. (1951) subsequently modified Wu's method by pretreating the proteins with alkaline copper solution prior to the reaction with phenol reagent. They found that copper treatment of protein was advantageous not only because in the presence of copper alkaline treatment resulted in a 3- to 15-fold increase in color and acceleration of color development at room temperature, but also because copper minimizes the differences in extinction coefficients originated in the reactions of different proteins with the Folin reagent. The origins of the copper effects are unknown.

Working solutions and reagents.

1) Tris-Mg^{++} buffer, pH 8.3: The buffer was made by placing 6.055 gm of Tris (Tris[hydroxymethyl]aminomethane) and 739.5 mg of $MgSO_4 \cdot 7\ H_2O$ in a 1-liter graduated beaker and filling with approximately 900 ml of deionized water. A magnetic stirrer was used to accelerate the dissolution and mixing. The pH of the solution was measured with a glass electrode on a Sargent-Welch pH meter. After the solids were completely

dissolved, the pH was titrated to 8.3 with 10.2 N HCl. The solution was then poured into a 1-liter graduated cylinder and was filled to exactly 1 liter with water. The final solution contained 50 mM Tris and 3 mM $MgSO_4 \cdot 7\ H_2O$. The pH of the solution was once again checked when the magnetic stirrer was in use. The solution was stored in the refrigerator.

2) Cyclic AMP solution, 2.5 mM: 32.92 mg cyclic AMP disodium salt were weighed out on a Mettler semi-micro balance and dissolved in 40 ml of Tris-Mg^{++} buffer. The solution was stored in a freezer. New solution was made for every month.

3) Snake venom, 1 mg/ml: The solution was made fresh each time it was used, by dissolving a sufficient amount of snake venom powder in Tris-Mg^{++} buffer to make a concentration of 1 mg/ml.

4) Theophylline solutions: 0.1753 gm of aminophylline was dissolved in 20 ml of Tris-Mg^{++} buffer to form a theophylline stock solution with a concentration of 41.7 mM. A series of five 20-ml test tubes, each with 5 ml Tris-Mg^{++} buffer were prepared, 5 ml of the theophylline stock solution were transferred to the first of the serial tubes. After the solution in the first tube was well mixed, 5 ml of it was pipetted into the second tube. The same procedure was repeated until serial concentrations were made, each concentration being 50% diluted as compared to the previous one. The concentrations of these 6 solutions were: 41.7, 20.8, 10.4, 5.2, 2.6 and 1.3 mM, respectively. They were stored in a freezer until they were used.

5) Papaverine HCl solutions: 0.1879 gm of papaverine HCl was dissolved in 20 ml of warm deionized water to form a papaverine HCl solution of 25 mM. The rest of the procedure was the same as that used in

preparing theophylline solutions, except that warm deionized water always replaced Tris-Mg^{++} buffer for papaverine, because papaverine HCl is readily precipitated in an alkaline pH. The concentrations of the papaverine solution were 25, 12.5, 6.25, 3.13, 1.56 and 0.78 mM, respectively. They were stored in the freezer.

6) Standard phosphate solutions: A graded series of concentrations of the standards were made by dissolving 65.3 mg of KH_2PO_4 in 200 ml of Tris-Mg^{++} buffer and diluting to the appropriate concentrations with the buffer in the manner described above. These standard solutions were kept in a refrigerator for long time periods without an appreciable change developing in the standard phosphate curve.

7) Trichloroacetic acid (TCA), 2.5% (w/w): 2.5 gm TCA was dissolved in 97.5 ml deionized water.

8) Trichloroacetic acid (TCA), 55% (w/w): 110 gm TCA was added to 90 ml deionized water. The solution was stored in a glass bottle.

9) Sulfuric acid, 0.2 N: 10 ml of 36 N H_2SO_4 was diluted to 1800 ml by adding acid into deionized water.

10) Phosphate reagent: The reagent was prepared freshly for each experiment by dissolving a sufficient amount of ammonium molybdate and ascorbic acid in 0.2 N H_2SO_4 to achieve a concentration of 0.1% for the former and 3% for the latter. The glassware used for this reagent was washed thoroughly with acid before use. The color of the reagent was a transparent light yellow.

11) Cupric EDTA reagent: 250 mg of EDTA ([Ethylenedinitrilo] tetraacetic acid), disodium cupric salt was dissolved in 200 ml of deionized water. Simultaneously, 500 ml of deionized water was added to

another beaker with 20 gm of sodium carbonate powder and 4 gm of sodium hydroxide pellets. After the constituents of the two solutions were completely dissolved, the two solutions were mixed in a 1-liter volumetric flask and filled to the mark with additional water. The reagent was stored at room temperature.

12) Phenol reagent, 1 N: Commercial 2 N solution was diluted one-fold with deionized water and stored at room temperature.

13) Bovine serum albumin, 1 mg/ml: 10 mg of bovine serum albumin powder were dissolved in 10 ml of Tris-Mg^{++} buffer by gently rotating the bottle. The solution was stored frozen.

Working procedures.

A. Treatment of tissue samples. For *in vitro* study, a sufficient amount of canine mucosa was pealed off from the fundic area of the dog stomach. The mucosal tissue was immediately immersed into a beaker of ice cold distilled water. This procedure not only provided quick cooling of the tissue, but also washed off the residual blood which adhered to the tissue. The washed tissue was subsequently blotted with a piece of gauze sponge. The residual submucosal tissue was carefully trimmed away with a pair of fine scissors. The mucosal tissue was then frozen in liquid nitrogen. After completely freezing, the tissue was transferred and stored in the freezer until it was used.

In *in vivo* experiments, biopsy samples of mucosa were taken from one half of the chambered segment with a biopsy instrument at definite time intervals. The rest of the treatment was the same as for the tissue in the *in vitro* study.

Enzymatic preparation of phosphodiesterase was made by grinding

the mucosal tissue in Tris-Mg^{++} buffer with a polytron for 1 min. In order to have a c^mplete broken cell preparation, the tissue solution was further homogenized in a Kontes Duall glass homogenizer for about 1 min. The final concentration of the preparation was approximately 80 to 100 mg wet weight of the tissue per ml.

B. Enzymatic reactions. Enzymatic incubation took place in small culture tube sized 10x75 mm. For each experiment, a batch of culture tubes were prepared and divided into two groups: a) Sample group: In this group, each tube contained 100 microliter (μl) of snake venom, 200 μl of enzyme preparation and 300 μl of Tris-Mg^{++} buffer or the same volume of the various concentrations of testing substances. Every two tubes had exactly the same content. b) Standard phosphate group: 400 μl of each standard KH_2PO_4 solution was pipetted into a tube containing 100 μl of Tris-Mg^{++} buffer and 100 μl of snake venom. No tissue was added.

All tubes were then warmed up in a water bath at 37°C for 5 min. For starting the reaction, in the sample group, 400 μl of cyclic AMP solution were pipetted into one of every two tubes with the same content. The other tube received 400 μl of Tris-Mg^{++} buffer to serve as the internal control. All tubes in the standard group recieved 400 μl of Tris-Mg^{++} buffer. After adding the final constituent, the content of the tubes was mixed by a vortex shaker. The total incubation volume in each tube was 1 ml. All tubes were allowed to incubate at 37°C for 30 min before the reactions were arrested by adding 200 μl of 55% TCA.

For assaying the phosphodiesterase activity from biopsy samples, exactly the same procedures were carried out, but no exogenous testing

substances was used.

C. Inorganic phosphate determination. The denatured proteins in the reaction mixture were precipitated by centrifuging at 3,000 x g in an International centrifuge (Boston, Mass.) for 15 min. 0.5 ml of supernatant from each tube was transferred to another set of culture tubes. 0.5 ml of 2.5% TCA served as a blank. The color reaction was initiated by adding 1.5 ml of phosphate reagent, mixing and incubating for 20 min at 37°C. Optical densities (OD) were measured at 700 mμ in a spectrophotometer (Gilford Co., Oberlin, Ohio) against the TCA blank. The OD readings from 6 graded phosphate standards formed a calibration curve when plotted against standard phosphate concentrations on a linear scale.

D. Protein determination. The chemical procedure for protein determination was undertaken according to a modified method originally reported by Lowry *et al*. (1951). Both albumin standards and the samples were diluted to 1 ml in a serial 10-ml test tubes. 5 ml of cupric EDTA reagent were added to each tube. The contents of the tubes were mixed and incubated for approximately 30 min at room temperature. 0.5 ml of 1 N phenol reagent was subsequently pipetted into each tube with immediate mixing. All tubes were allowed to stand at room temperature for exactly 30 min. Their OD's were then read at 700 mμ in the spectrophotometer against a water blank. A calibration curve was constructed by plotting the OD readings from 6 graded protein standards on a linear scale against the concentrations of the standards.

E. Methods of calculations. In the phosphate determination, the OD differences between the samples and their own internal controls

represented the true OD derived from that produced by inorganic phosphate in the enzymatic reactions. Based upon the phosphate standard curve, the concentration of enzymatically produced phosphate was known. This calculated phosphate concentration was multiplied by the total volume of 1.2 ml (1 ml of reaction mixture plus 0.2 ml of 55% TCA) to obtain the total inorganic phosphate produced by the action of phosphodiesterase. The protein concentrations of the samples were calculated based upon the values taken from the protein standard curve. Therefore, the amount of protein used in each sample was known. The total inorganic phosphate produced was divided by the amount of tissue protein used in the reaction and by the incubation tissue (30 min) to obtain the specific activities of mucosal phosphodiesterase in the samples. All calculations were done with a desk computer.

Assay of Adenyl Cyclase Activity

Principle of the assay.

Enzymatic reaction. The principle was based on the fact that exogenous ATP can be converted to cyclic AMP by tissue adenyl cyclase. By using radioactive C^{14}-ATP as the substrate, a minute quantity of cyclic AMP which has been converted from ATP, can be detected by highly sensitive radioactive scintillation counting.

Purification of labeled cyclic AMP. In order to separate radioactive cyclic AMP produced in the adenyl cyclase reaction from excess ATP and possible contamination from 5'-AMP, two steps of purification were carried out: column chromatography and Ba^{++}-Zn^{++} precipitation. Both steps took advantage of the characteristics of the cyclic structure, namely the single phosphate group in a cyclic AMP molecule which is

esterized to the ribose moiety with two ester bonds, thereby leaving one ionized group on the phosphate. In contrast, both ATP and 5'-AMP have more ionizable groups in their molecules. When the supernatant of the enzymatic reaction mixture was placed on the top of a cation exchange column (AG-50,X8) and eluted with deionized water, ATP readily came out of the column in the first 2 ml fractions. The majority of cyclic AMP was recovered in the following 2 ml fractions; 5'-AMP (if any) began to show up in the 5th ml fraction.

Ba^{++}-Zn^{++} precipitation was first employed by Krishna to purify the cyclic AMP fraction. He found that barium hydroxide ($Ba[OH]_2$) and zinc sulfate ($ZnSO_4$), when combined in use, were able to precipitate quantitatively a variety of nucleotides except cyclic compounds (Krishna et al., 1968). Consequently, the combination of column chromatography and Ba^{++}-Zn^{++} precipitation has proved to be a powerful method to purify cyclic AMP from ATP and other products formed during the incubation with crude adenyl cyclase preparation.

Working solutions and reagents.

1) Tris-Mg^{++} buffer: The buffer was made by the procedure described previously for the assay of phosphodiesterase, except that the pH was adjusted to 7.4.

2) Cyclic AMP solution, 0.6×10^{-2} M: 0.0395 gm of cyclic AMP disodium salt were dissolved in 20 ml Tris-Mg^{++} buffer.

3) AG50-X8 resin suspension, 50% (v/v): The commercial resin (200-400 mesh) was washed in 0.2 N HCl. Deionized water subsequently replaced the acid and the washing procedure was repeated until the pH of the suspension became identical to that of deionized water. 50% (v/v)

suspension was finally made.

4) C^{14}-ATP solutions: C^{14}-ATP powder obtained from New England Nuclear Co. 250 μci with specific activity of 29.2 mci/mmole were packed in a small sealed bottle. Before C^{14}-ATP was used, it was subjected to chromatographic purification. 0.5 ml of Tris-Mg^{++} buffer was used to dissolve the ATP powder in the bottle. The solution was placed on the AG50-X8 column (0.4 x 3 cm) prepared by pipetting 2 ml of resin suspension into a glass column of 22 cm in length, including a 35 ml reservoir. Another 0.5 ml of buffer which was used to wash the radioactive bottle was also pipetted into the column. The total 1 ml elution was collected. The column was further eluted with an additional 1 ml of buffer. This second fraction was collected and mixed with the first ml elution to form the purified C^{14}-ATP stock solution. 5 μl of it in 10 ml of aquasol (scintillation fluid) were counted in a liquid scintillation counter (Nuclear-Chicago Co.). By knowing the total counts of the stock solution and its specific activity (same as original commercial product), the concentration of the stock solution could be calculated.

Two C^{14}-ATP working solutions: (a) C^{14}-ATP solution #1 (0.5 μci/0.1 μmole, 20 μci/ml) and (b) C^{14}-ATP solution #2 (1 μci/0.1 μmole, 20 μci/ml) were prepared by diluting the stock solution with sufficient nonradioactive ATP and Tris-Mg^{++} buffer. All C^{14}-ATP solutions were stored in a deep freeze.

5) Zinc sulfate ($ZnSO_4$), 8.0% (w/v): 16 gm of zinc sulfate powder were dissolved in deionized water to a final volume of 200 ml.

6) Barium hydroxide ($Ba[OH]_2$), 7.2% w/v): deionized water was added to a 200-ml volumetric flask containing 14.4 gm of barium hydroxide

crystals. The solution was vigorously stirred by a magnetic stirrer until all crystals dissolved. Because barium hydroxide easily absorbs carbon dioxide to form an insoluble precipitate, the flask was tightly stoppered when not in use.

7) Histamine2HCl, 4×10^{-2} M: 73.6 mg of histamine2HCl powder was dissolved in 10 ml Tris-Mg^{++} buffer. Various concentrations of histamine solutions were made by diluting this stock solution with the addition of Tris-Mg^{++} buffer.

8) Sodium fluoride (NaF), 4×10^{-2} M: 33.6 mg of sodium fluoride powder were added to 20 ml of Tris-Mg^{++} buffer.

9) Aquasol (scintillation fluid).

Working procedures.

A. Treatment of the tissue. The procedures carried out were similar to those for the phosphodiesterase assay, except that the concentration of tissue in the homogenate was approximately 150 mg of wet weight of tissue per ml of Tris-Mg^{++} buffer for canine enzymic preparation.

When guinea pig gastric adenyl cyclase was assayed, the mucosal surface of the stomach was washed sith ice cold water and blotted with filter paper. The mucosal tissue was scraped from outer muscularis mucosa and immediately homogenized in cold Tris-Mg^{++} buffer of pH 7.4 with a Kontes Duall glass homogenizer. The final concentration of the guinea pig enzymic preparation was about 300 mg of wet weight of tissue per ml of buffer.

B. Enzymatic reaction. The reaction took place in a small culture tube (10 x 75 mm). 100 μl of incubation medium of Tris-Mg^{++} buffer

(Tris, 50 x 10^{-3} M and $MgSO_4$, 3 x 10^{-3} M) contained: cyclic AMP (1x5 x 10^{-3} M); C^{14}-ATP (either 1 mM or 0.5 mM); various concentrations of testing substances; and tissue (0.7 - 0.8 mg of dry weight). All samples were duplicated for each experiment. The incubation was allowed to continue for various amounts of time at 37°C before the reaction was terminated by immersing the tubes in a boiling water bath for 5 min. All tubes were chilled in ice water and then centrifuged for 15 min at 3,000 x g.

C. Purification of produced radioactive cyclic AMP. The supernatant of the reaction mixture was chromatographed on AG50-X8 column prepared as previously described. The column was eluted with deionized water. The first 2 ml of elution contained almost 100% of C^{14}-ATP put on the column and were collected for determination of ATP recovery. The following 2 ml of eluate contained 75 to 80% of the total amount of cyclic AMP in the reaction mixture. With the cyclic AMP fraction, a volume of 3 ml was reached by adding 1 ml of deionized water. 250 µl each of $ZnSO_4$ and $Ba(OH)_2$ solution were added and thoroughly mixed. Without intervening centrifugation, the addition of $ZnSO_4$ and $Ba(OH)_2$ solution was repeated. The mixture was then centrifuged at 3,000 x g for 25 min.

D. Scintillation counting, optical density measurement and tissue measurement. 2 ml of the supernatant of the precipitated solution were added to 15 ml of aquasol in a scintillation counting vial. The radioactivity was measured in the liquid scintillation counter. 0.5 ml of supernatant were used for checking the optical density (OD) in the spectrophotometer against a water blank at 260 mµ. The recovery of cyclic AMP was computed by treating the OD of a standard as the 100% re-

covery. The OD standard contained 4 ml of deionized water and the same amount of cyclic AMP as was used for the sample. For quantitative analysis, all adenyl cyclase activities were expressed in terms of the amount of labelled cyclic AMP produced per mg of dry weight of the tissue used in enzymatic reaction. The dry weight of the tissue was obtained by pipetting 25 µl (the same volume as used for enzymic reaction) of tissue homogenate into a small culture tube whose weight had been recorded and by drying the tissue in a vacuum dessicator for two days. The weight of the combination (dry tissue plus tube) was subtracted by the weight of the empty tube to obtain the weight of dry tissue.

E. Method of calculations. The calculations of the data were performed using the desk computer which had been programmed with the following formula:

$$A.C.A. = \frac{T_v}{\overline{V}_c} (cpm\text{-}BC) \cdot \frac{1}{R_c} \cdot \frac{1}{E_f} \cdot \frac{1}{F_{dpm}} \cdot \frac{1}{SA} \cdot 10^6 \cdot \frac{1}{E_{nz}}$$

A.C.A.: Adenyl cyclase activity in terms of pico moles of cyclic AMP produced per mg of dry weight of tissue.

T_v: Total volume (ml) of supernatant after Ba^{++}-Zn^{++} precipitation.

$\overline{V}_c$: Volume for scintillation counting, i.e., 2 ml.

cpm: Counts per minute of 2 ml of supernatant.

BC: Background counts (cpm) of the cyclic AMP fraction from a sample without addition of tissue.

R_c: Recovery of cyclic AMP calculated from optical density.

E_f: Efficiency of the scintillation counter.

F_{dpm}: A conversion factor of 2.22×10^6 which converts dpm (disintegration per min) to µci (microcuri).

SA: Specific activity of C^{14}-ATP used in terms of μci per μmole.

E_{nz}: Dry weight (mg) of the tissue used in the reaction.

Experimental Designs

In Vitro Study of Phosphodiesterase Inhibitors

For each experiment, 3 groups of the culture tubes were prepared as follows: a) Theophylline group. In this group, each tube contained 300 μl of a theophylline solution, 100 μl of snake venom and 200 μl of enzymic preparations; b) Papaverine group. Each tube contained 50 μl of a papaverine solution and 250 μl of Tris-Mg^{++} buffer. The other constituents were the same as in the theophylline group; c) Standard phosphate group. The standards were set up as described previously.

The rest of the chemical procedures for all 3 groups were carried out according to the method stated in the section of *Chemical Assay*.

In Vivo Study of Phosphodiesterase Inhibition and Acid Secretion

Intravenous administration of phosphodiesterase inhibitors. The solutions of theophylline and papaverine HCl were prepared freshly for each experiment. Theophylline solution was made by dissolving a sufficient amount of aminophylline powder in 10 ml of saline to provide a dose of 0.016 gm of theophylline per kg of body weight. Papaverine solution was prepared similarly to provide a dose of 6 mg per kg.

The experiments were begun with a one-hour control period, without using any treatments on the dog other than monitoring the blood pressure. During the experiment, at the end of every 30 min, one biopsy was

taken and secretion (if any) was drained by gravity. The volume of secretion was measured and recorded. The concentration of acid in the secretion was subsequently determined with an automatic titrator (Radiometer, Copenhagen, Denmark) to a pH of 7.0. Acid output was calculated from the volume and the acid concentration of the secretion. Either theophylline or papaverine solution was injected at the end of the control period into the femoral vein as a bolus. Biopsies were taken at 5, 15, 30 min, and 1 hr following the injection. Intravenous infusion of histamine in a dose of 30 μg/kg-hr was started immediately after taking 1-hr biopsy by using a constant flow infusion pump (Harvard Apparatus Co., Dover, Mass.). During the 1-1/2-hr histamine infusion period, 4 additional biopsies were obtained at 15, 30, 60 and 90 min following the start of infusion. Blood pressure changes were closely monitored throughout the experiment, since histamine, theophylline and papaverine are depressor drugs. The quantities of these substances to be used were largely limited by their hypotensive effects, especially when theophylline or papaverine was used in combination with histamine. The dose of 0.016 gm of theophylline per kg caused a 10 to 15% decrease in blood pressure before the infusion of histamine began, and a 20 to 30% decrease by the end of the histamine infusion period. Papaverine in a dose of 6 mg/kg has somewhat less effect on arterial blood pressure.

Intraarterial administration of phosphodiesterase inhibitors. In order to minimize the effect on blood pressure and to increase the local concentration in the mucosal tissue, either theophylline or papaverine was infused into the gastroepiploic arteries. For each experiment, either 3 doses of theophylline (0.2, 0.4 and 0.8 mg/kg-min) or 2 doses of

papaverine (0.15 and 0.3 mg/kg-min) were employed. Increase in dosage was effected by increasing the infusion rate of the pump. Each dose of the inhibitor was continued for 20 min. After termination of inhibitor infusion, no histamine was given. One biopsy sample was obtained at the end of every 20-min period. The remainder of the procedures were the same as those in the previous set of experiments.

The effect of both inhibitors in the highest doses on arterial blood pressure was similar to the effect with intravenous (I.V.) injection, since the total quantity of each drug used in intraarterial (I.A.) infusion was considerably greater than in I.V. injection.

In Vitro Study of Adenyl Cyclase Activity

Gastric adenyl cyclase activities *in vitro* were examined in both canine and guinea pig mucosal tissues. Two types of experiments were done with canine enzymic preparation: (1) Adenyl cyclase activities were assayed in the presence of various concentrations of histamine with different incubation times. Either solution #1 (0.5 μci/0.1 μmole) or solution #2 (1 μci/0.1 μmole) of C^{14}-ATP was used in the assay system. The effect of NaF on the enzyme was also examined in every experiment. The response of the enzyme to NaF was taken as an indication that enzymic preparation was sound. (2) Another type of experiments was dealing with the combined effect of histamine and NaF on gastric adenyl cyclase. The amount of NaF used in the combination was known to produce a submaximal stimulation by itself. The enzymic activities assayed in the presence of both histamine and NaF were compared to those obtained with the same amount of NaF alone.

With guinea pig mucosal tissue, only the first type of design

was performed. NaF was used for the same purpose.

In Vivo Study of Histamine Effect on Adenyl Cyclase Activity and Acid Secretion

Two periods were included in each experiment. Following the first hour control period, histamine was infused intravenously at a dose of 30 μg/kg-hr for two hours. One tissue biopsy was taken at the end of the control period and two others at 90 min and at 2 hr following the beginning of the histamine infusion. At the end of every 30 min period, blood pressure and secretory volume were recorded. The acid concentration of the secretion was determined as stated above.

In Vivo Study with Dibutyryl Cyclic AMP

Experiments were designed to include 3 sequential hour-long periods. After the first hour of control an intraarterial infusion of dibutyryl cyclic AMP was initiated. Two doses of dibutyryl cyclic AMP were used, 0.1 mg/kg-min for the first 30 min and 0.2 mg/kg-min for the second half hour. After this a 1-hour control period with intraarterial saline infusion followed. Blood flow to the stomach flap was measured every 30 min in two control periods and every 15 min during the infusion of dibutyryl cyclic AMP. An electromagnetic blood flow transducer (Micron, Los Angeles, Calif.) of either 2.0 mm or 1.0 mm size was used on the blood vessel for measurement of flow. The flow probe was connected to a 610 pulse logic flowmeter amplifier (Biotronex Laboratory, Inc., Silver Spring, Md.). The transducer had been previously calibrated *in vitro* with whole blood. The zero flow determination was obtained by occluding the vessel with a hemostat at a locus immediately distal to the probe. Blood flow values were then determined by measuring the distance

between the undisturbed mean flow and the subsequent zero on the graphic recording and measuring this deflection with the calibration for the flow probe. Beside the blood flow measurements, arterial blood pressure and secretory rates were determined as previously described, except that the blood pressure was measured every 15 min during the dibutyryl cyclic AMP infusion.

Method of Statistical Analysis

Since the majority of the experimental results in this investigation were considered based upon comparisons among the treatments and the controls, Duncan's new multiple-range test was, therefore, employed to examine the pair-wise comparisons of the means. The experiments were regarded as completely random in design (Steel and Torrie, 1960). The 5% level of significance was chosen for analysis. The missing data were approximated by the usual missing data formula for randomized complete block analysis. The actual calculations were performed on an IBM 1800 data acquisition and control computer system.

CHAPTER III

EXPERIMENTAL RESULTS

In Vitro Effect of Theophylline on Gastric Mucosal Phosphodiesterase Activity

Phosphodiesterase was prepared with mucosal tissue taken from 6 different dogs. Each preparation was assayed with and without theophylline. The final concentrations of the inhibitor in the reaction mixtures were 0.39, 0.78, 1.56, 3.13, 6.25 and 12.5 mM. Phosphodiesterase activities under the influence of theophylline were expressed as the percentage change from the controls (without theophylline) by regarding the activities of the controls as 100%. The results are shown in Fig. 2. Mean values ± standard errors of percentage inhibition of phosphodiesterase activities from 6 enzymic preparations were plotted against the final concentrations of the inhibitor. A hyperbolic curve describes the relationship. The inhibitory effect of theophylline was greatly enhanced (from 0 to about 30%) when concentration of the drug was increased from 0 to 1.56 mM. Further increase in drug concentration up to 12.5 mM produced much smaller increments in inhibitory effect. For the statistical analysis, actual enzymic activities were used instead of the percentage inhibition. It was found that each doubling of the drug concentration caused a significant decrease in enzymic activity, except the doubling between 3.13 and 6.25 mM. These results demonstrated that theophylline

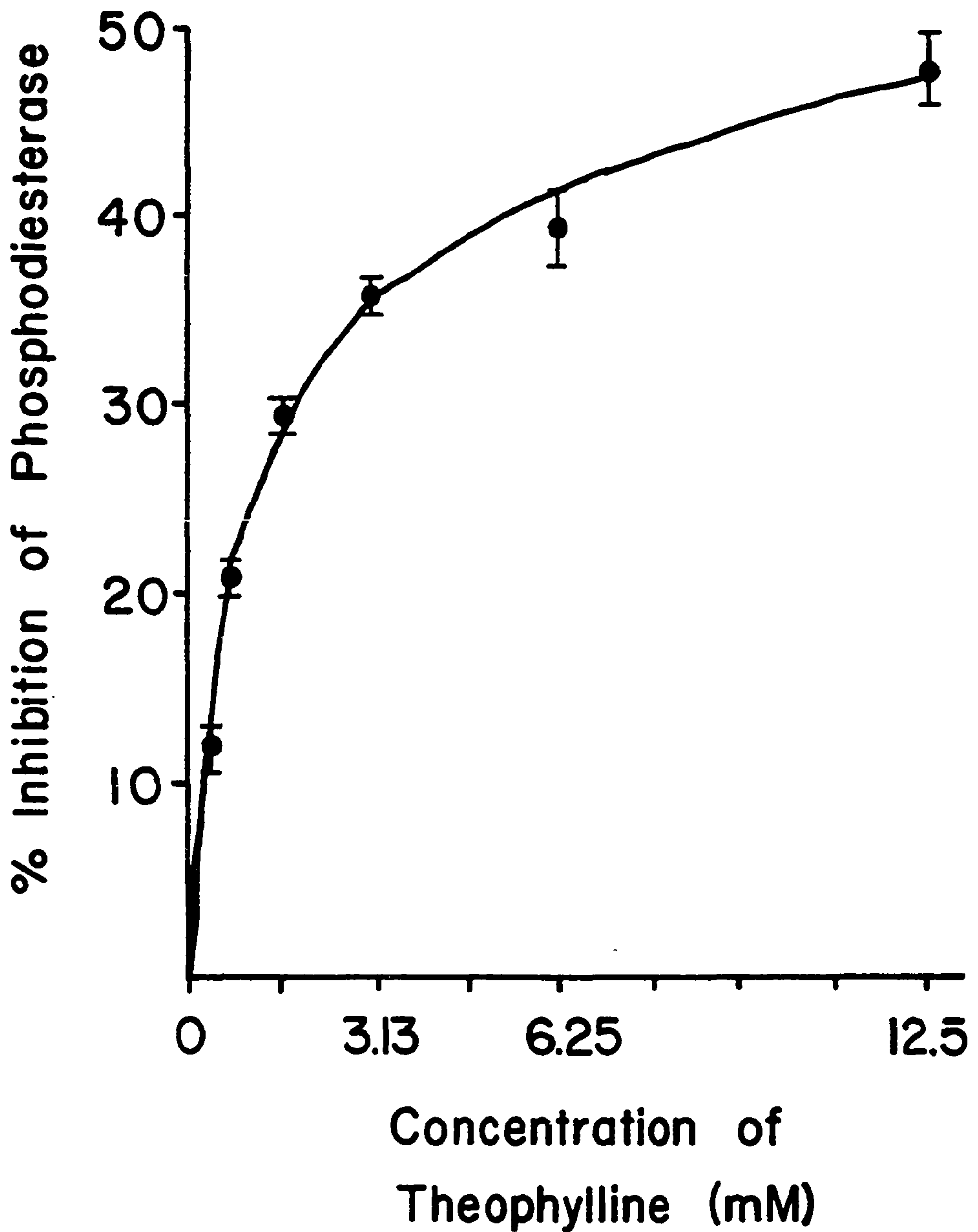

Figure 2. *In vitro* effect of theophylline on gastric phosphodiesterase activity. Vertical bars represent standard errors of 6 experiments.

is an inhibitor of phosphodiesterase activity in the gastric mucosa of the dog.

In Vitro Effect of Papaverine on Gastric Phosphodiesterase Activity

A study similar to the preceding one was conducted with a different enzymic inhibitor, papaverine. The results of 6 experiments are presented in Fig. 3. A hyperbolic curve was also obtained with papaverine when a plot was made of the percentage inhibition of phosphodiesterase activity as a function of the concentration of papaverine in the assay system. The molar concentrations of papaverine used were 1/10 the molar concentrations of theophylline employed in the preceding experiments. However, the dose response curve for papaverine showed a steeper slope than the plot for theophylline. A 50% inhibition was found with a concentration of papaverine less than 0.313 mM. 1.25 mM papaverine produced a 90% inhibition of the enzyme. These observations indicate that papaverine is about 20-fold more potent than theophylline as an inhibitor of gastric mucosal phosphodiesterase *in vitro*. Statistical analysis of the differences in inhibition with various concentrations of papaverine was performed using the actual phosphodiesterase activities. It was found that the mean enzymic activities with the concentrations of papaverine ranging from 0 to 1.25 mM were significantly different from one another except at the two lowest concentrations of the inhibitor (0.039 and 0.078 mM).

Acid Secretory Response of the Canine Stomach to the Intravenous Administration of Phosphodiesterase Inhibitors

Thirteen dogs were used in these experiments: 6 dogs served as

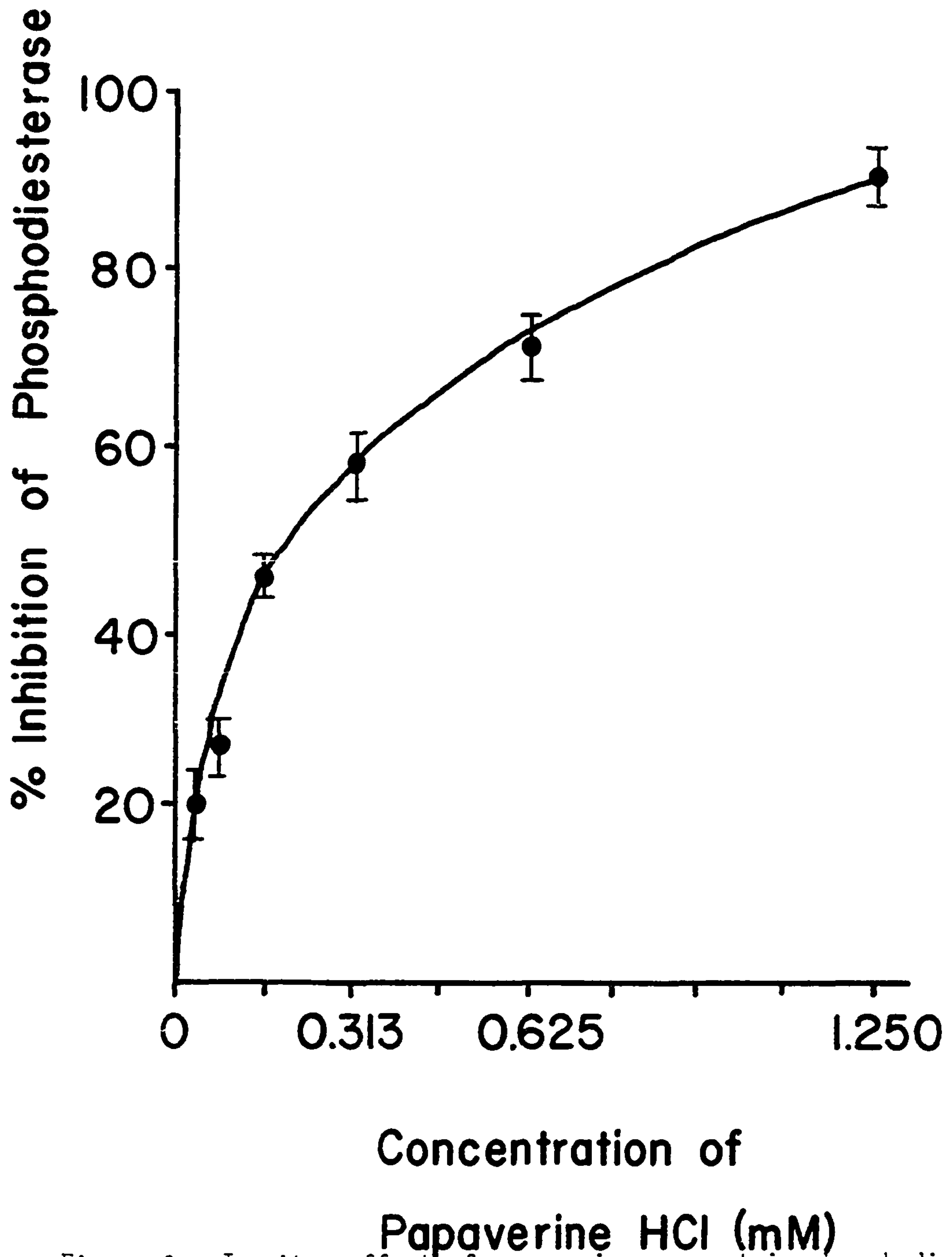

Figure 3. *In vitro* effect of papaverine on gastric phosphodiesterase activity. Each mean with plus and minus standard error represent 6 experiments.

control animals; 5 dogs were used to test theophylline, and 2 dogs were used to test papaverine. All animals were prepared with a chambered segment of the stomach (Fig. 1). Observations of acid secretion in both control experiments and in experiments with theophylline have been summarized in Figure 4. There was no spontaneous gastric secretion in any of the animals. Following two 1-hour control periods, the control animals were administered histamine by intravenous infusion at a dose of 30 μg/kg-hr. This treatment evoked a secretory response. The mean acid output from 6 dogs increased to 0.29 mEq/30 min by the end of the first hour after the onset of histamine infusion and to 0.38 mEq/30 min at 90 min.

In the 5 dogs used to test theophylline, a dose of this drug (0.016 g/kg) was injected intravenously. In none of these animals did gastric secretion increase from basal levels over the next hour. However, acid secretory rates did increase in response to the subsequent infusion of histamine and these rates were significantly higher than those observed in the control animals (histamine alone), at least at 60 min and 90 min following the infusion of histamine. The mean acid output measured at these two collection periods were 0.8 and 0.9 mEq/30 min, respectively. These results indicate that theophylline does not initiate gastric secretion in the resting stomach of the dog, although it potentiates the response to histamine.

Figure 5 shows a comparison of acid secretory response between control animals and 2 animals treated with papaverine (0.6 mg/kg). No acid secretion was observed after the intravenous administration of papaverine for 1 hour. The average acid output from 2 experiments in which

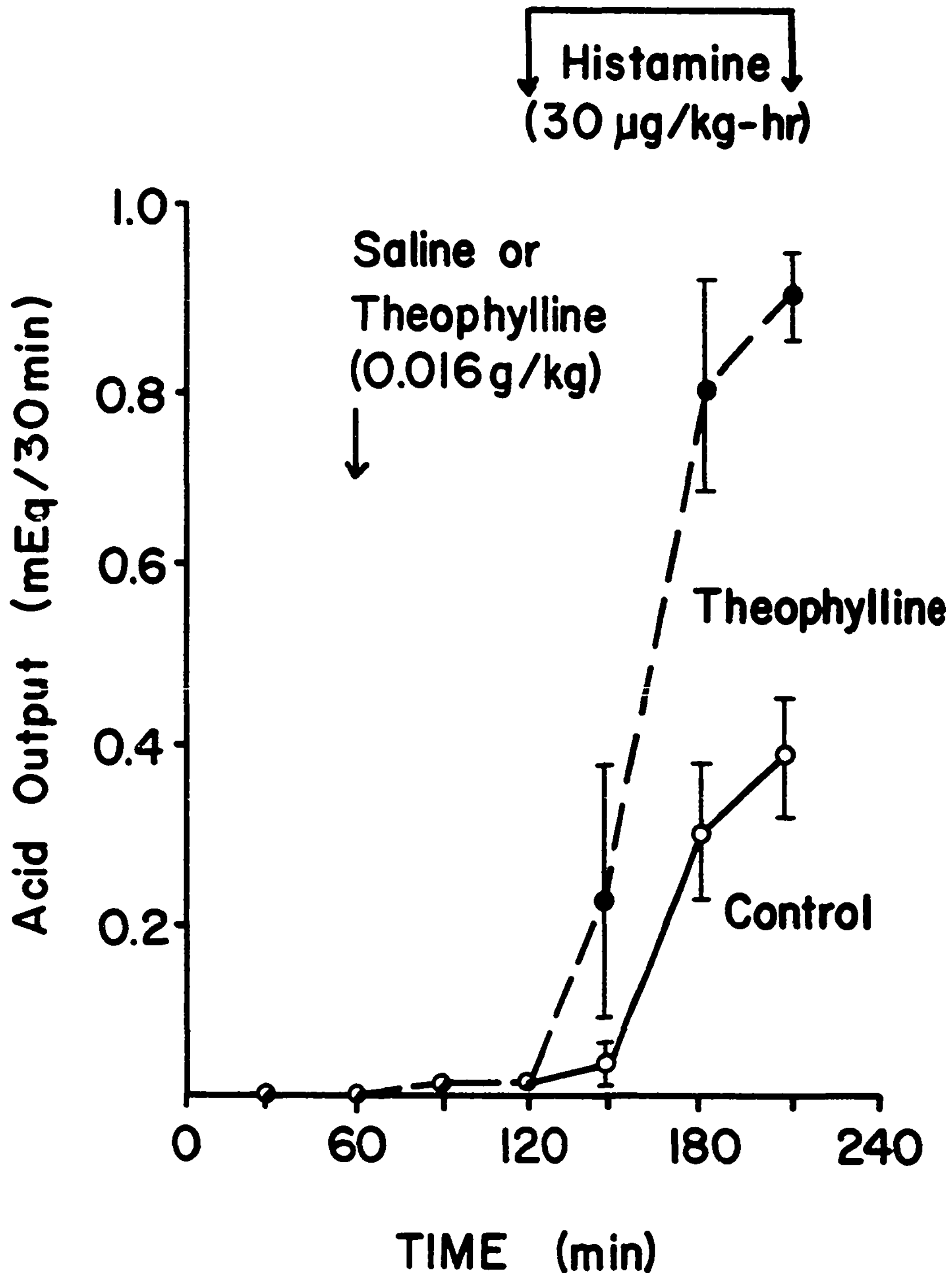

Figure 4. Acid secretory response of canine gastric segment to the intravenous administration of theophylline. The means, plus and minus standard errors represent 6 dogs in control curve and 5 dogs in theophylline curve. Only 4 animals are represented by the mean and standard errors at 210-min on theophylline curve.

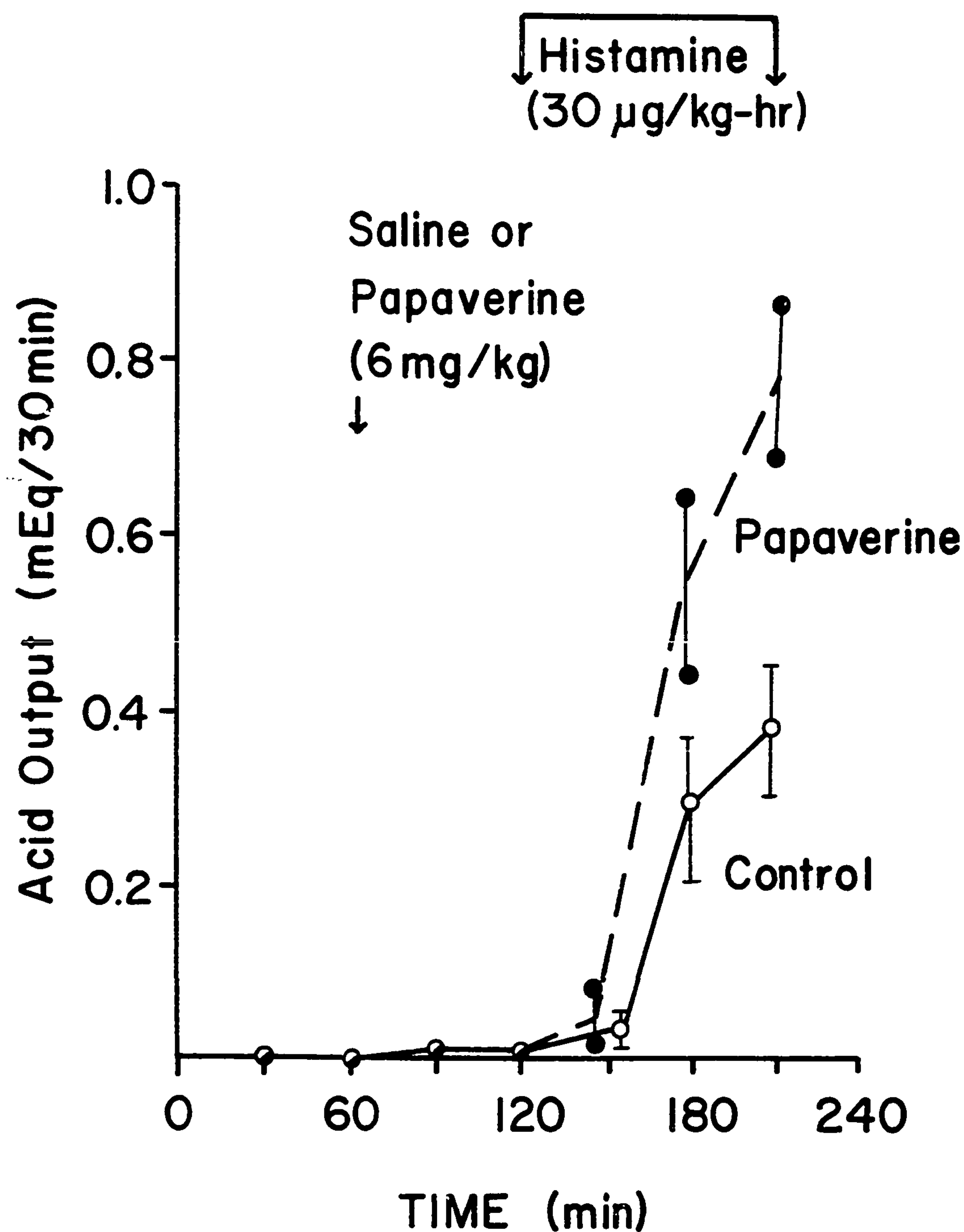

Figure 5. Acid secretory response of canine gastric segment to the intravenous administration of papaverine. The actual values of two experiments with papaverine are shown by solid circles. The control curve is the same shown in Figure 4.

both histamine and papaverine were given appeared greater than the output from control dogs in which histamine alone was administered. The number of experiments was too small for a meaningful statistical analysis, although these results support the preceding findings with theophylline.

Control Study of Gastric Phosphodiesterase in Vivo

Mucosal biopsies were taken at various times from the control group of animals in the preceding experiments. Tissues were assayed for phosphodiesterase activity. Table 1 shows all specific activities of the enzyme in the 6 control dogs. No significant differences were found among the 5 mean values (enzyme activity vs time) shown in the table. These results indicate that phosphodiesterase activity in the animals without treatment was not influenced by a time factor.

Gastric Phosphodiesterase Activity in Response to Intravenous Administration of Theophylline and Papaverine

Tissue phosphodiesterase was prepared from the biopsies obtained at various times in the animals treated with 0.016 gm/kg of theophylline. The results of enzymic determination appear in Table 2. There was no significant change in phosphodiesterase activity as a result of administering theophylline or histamine. These findings indicate either: 1) the *in vivo* dog stomach is not a suitable preparation to measure phosphodiesterase inhibition, or 2) phosphodiesterase is not inhibited *in vivo*.

The phosphodiesterase activities assayed from the biopsies obtained in 2 experiments with papaverine are shown in Table 3. No consistent change in enzymic activity was observed in either animal after

TABLE 1

CONTROL STUDY OF GASTRIC PHOSPHODIESTERASE *IN VIVO*

No. of Experiment \ Min	Gastric Phosphodiesterase Activity (moles of inorganic phosphate/mg protein-min x 10^{-9})				
	-30	0 (Saline ↓)	+15	+30	+60
1	2.9	3.0	3.2	3.8	3.1
2	5.0	4.2	4.2	5.3	-
3	3.3	4.8	-	4.2	3.3
4	3.9	4.0	3.6	3.1	-
5	-	4.9	5.1	4.3	4.6
6	6.8	5.0	6.3	6.0	6.2
$\bar{X} \pm$ S.E.	4.4 ± 0.7	4.3 ± 0.3	4.5 ± 0.6	4.4 ± 0.4	4.3 ± 0.7

TABLE 2

GASTRIC PHOSPHODIESTERASE ACTIVITY IN RESPONSE TO INTRAVENOUS ADMINISTRATION OF THEOPHYLLINE AND HISTAMINE

No. of Experiment \ Min	Gastric Phosphodiesterase Activity (moles of inorganic phosphate/mg protein-min x 10^{-9})									
		Theophylline (0.016 gm/kg) ↓					Histamine (30 µg/kg-hr) ↓			↓
	-30	0	+5	+15	+30	+60	+75	+90	+120	+150
1	4.4	4.6	3.8	4.2	4.6	4.7	4.2	5.0	4.2	5.2
2	3.9	3.3	6.2	3.9	4.4	3.2	4.5	4.3	2.5	3.2
3	3.4	2.2	3.3	4.4	2.0	3.0	3.4	-	2.1	3.2
4	4.4	3.4	3.7	3.2	5.1	2.4	3.7	3.6	3.8	3.2
5	1.9	-	2.5	2.3	1.9	2.2	1.9	3.0	2.9	2.2
$\bar{X} \pm$ S.E.	3.6±0.5	3.4±0.5	3.9±0.6	3.6±0.4	3.6±0.7	3.1±0.4	3.5±0.5	3.9±0.4	3.1±0.4	3.4±0.5

TABLE 3

GASTRIC PHOSPHODIESTERASE ACTIVITY IN RESPONSE TO INTRAVENOUS ADMINISTRATION OF PAPAVERINE AND HISTAMINE

No. of Experiment \ Min	Gastric Phosphodiesterase Activity (moles of inorganic phosphate/mg protein-min x 10^{-9})								
		Papaverine (6 mg/kg) ↓				Histamine (30 µg/kg-hr) ↓			↓
	-30	0	+5	+15	+30	+60	+75	+90	+120
1	4.2	3.2	3.7	4.5	4.1	4.2	4.4	3.5	3.9
2	4.2	4.0	4.1	3.8	3.9	4.7	3.9	4.1	4.3
$\bar{X}$	4.2	3.6	3.9	4.1	4.0	4.5	4.2	3.8	4.1

administration of papaverine or histamine. These results support the preceding findings with theophylline that no significant change in the phosphodiesterase activity as a result of intravenous administration of the phosphodiesterase inhibitor and histamine.

Gastric Phosphodiesterase Activity and Acid Secretory Response to Intraarterial Administration of Papaverine and Theophylline

Nine dogs were used for this study, 6 for the experiments with papaverine and 3 for those with theophylline. Two doses of papaverine were employed, 1.5 and 3.0 mg/kg-10 min infused for 20 min. In 3 experiments with papaverine the second dose was continued for 40 min. No secretion was initiated by papaverine in any experiment. The phosphodiesterase activities assayed from the biopsies taken in each experiment are shown in Table 4. There were no significant changes in enzymic activity as a result of infusing papaverine, except at 60 min where sample size was too small to be meaningful. These results indicate that increasing local concentrations of the phosphodiesterase inhibitor, papaverine, by intraarterial infusion fails to initiate gastric secretion in the dog.

In 3 animals, theophylline was infused intraarterially into the stomach segment in 3 graded doses (2.0, 4.0 and 8.0 mg/kg-10 min). Despite a considerable increase in drug concentration in the local area, neither was acid secretion initiated nor was phosphodiesterase activity affected (Table 5). These results support previous findings with papaverine.

TABLE 4

GASTRIC PHOSPHODIESTERASE ACTIVITY IN RESPONSE TO INTRAARTERIAL ADMINISTRATION OF PAPAVERINE

No. of Experiment \ Min	Gastric Phosphodiesterase Activity (moles of inorganic phosphate/mg protein-min x 10^{-9})				
	Rates of papaverine infusion (mg/kg-10 min): 1.5 ↓ (after 0), 3.0 ↓ (after +20), 3.0 ↓ (after +40)				
	-30	0	+20	+40	+60
1	3.3	3.9	3.8	3.7	-
2	-	3.5	3.3	2.9	-
3	3.6	4.4	4.5	3.8	3.4
4	4.9	4.9	5.2	4.3	2.9
5	3.7	4.0	4.4	4.2	4.2
6	3.4	3.8	3.8	3.5	-
$\bar{X} \pm$ S.E.	3.8 ± 0.3	4.1 ± 0.2	4.2 ± 0.3	3.7 ± 0.2	3.5 ± 0.4

TABLE 5

GASTRIC PHOSPHODIESTERASE ACTIVITY IN RESPONSE TO INTRAARTERIAL ADMINISTRATION OF THEOPHYLLINE

No. of Experiment \ Min	Gastric Phosphodiesterase Activity (moles of inorganic phosphate/mg protein-min x 10^{-9})				
	Rates of theophylline infusion (mg/kg-10 min)				
			2.0 ↓	4.0 ↓	8.0 ↓
	-30	0	+20	+40	+60
1	3.0	3.6	3.2	3.9	-
2	3.5	3.7	4.3	3.6	3.4
3	4.8	5.3	-	5.0	5.4
$\bar{X} \pm$ S.E.	3.8 ± 0.5	4.2 ± 0.6	3.8 ± 0.6	4.2 ± 0.4	4.4 ± 1.0

In Vivo Effect of Histamine on Canine Gastric Mucosal Adenyl Cyclase Activity and Acid Secretion

Results of experiments in 4 dogs are shown in Figure 6. During the control period (before infusing histamine) no spontaneous acid secretion was observed. The adenyl cyclase activity assayed from the control biopsies gave a mean value of $7.2 \pm 1.9 \times 10^{-12}$ moles of cyclic AMP per mg of dry weight of muscosal tissue (expressed in the graph as p moles/ mg). Infusion of histamine (30 μg/kg-hr) stimulated gastric secretion which reached a peak between 120 and 150 min with a secretory rate of 0.16 ± 0.03 mEq/30 min. Enzymic activities obtained from 150-min biopsies gave a value of 6.1 ± 1.1 p moles/mg. Acid secretion showed a slight decline in the last collection interval. Biopsies taken immediately prior to the termination of histamine infusion yielded similar activities to preceding biopsies with a mean value of 6.7 ± 0.8 p moles/mg. No significant difference was found among enzyme activities in biopsies taken at the 3 time periods. When the enzymic preparations from 3 of 4 control biopsies were assayed in the presence of 10^{-2} M NaF, a large increase in all activities was measured (44.7 ± 2.5 p moles/mg). These findings indicate that canine mucosal adenyl cyclase was activated by fluoride but not by doses of histamine which stimulated secretion.

In Vitro Effect of Histamine on Canine Gastric Adenyl Cyclase Activity

Figure 7 presents an _in vitro_ experiment in which tissue was incubated for 40 min with a wide range of histamine concentrations and one concentration of NaF in a buffer medium containing 0.5 mM C^{14}-ATP with a specific activity of 1.0 μci/0.1 μmole. The activities of the enzyme were

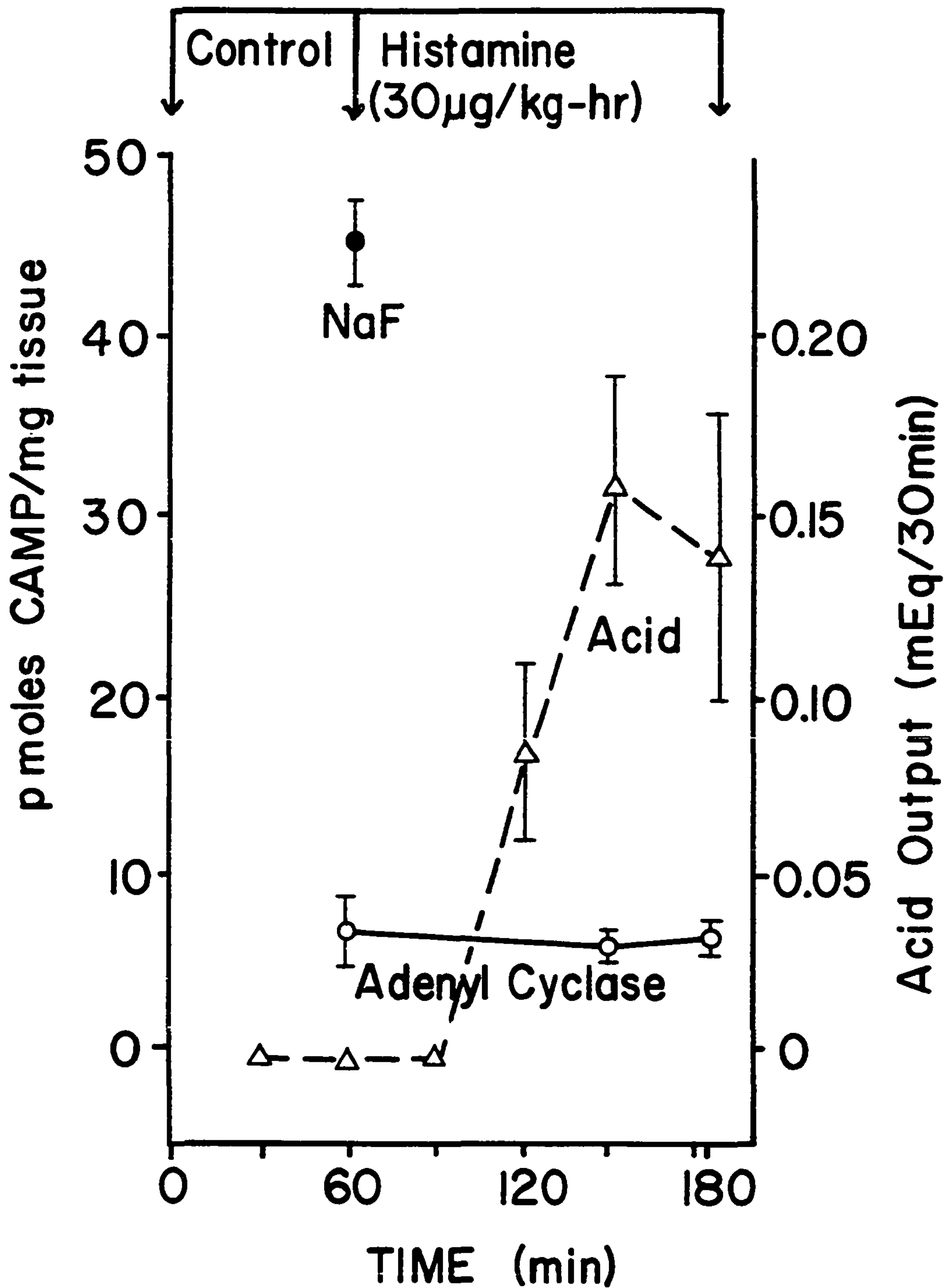

Figure 6. *In vivo* effect of histamine on canine gastric adenyl cyclase activity and acid secretion. The broken line denotes secretory response. The solid line shows the adenyl cyclase activity. The vertical bars represent standard errors of 4 experiments except that with NaF which represents 3 experiments.

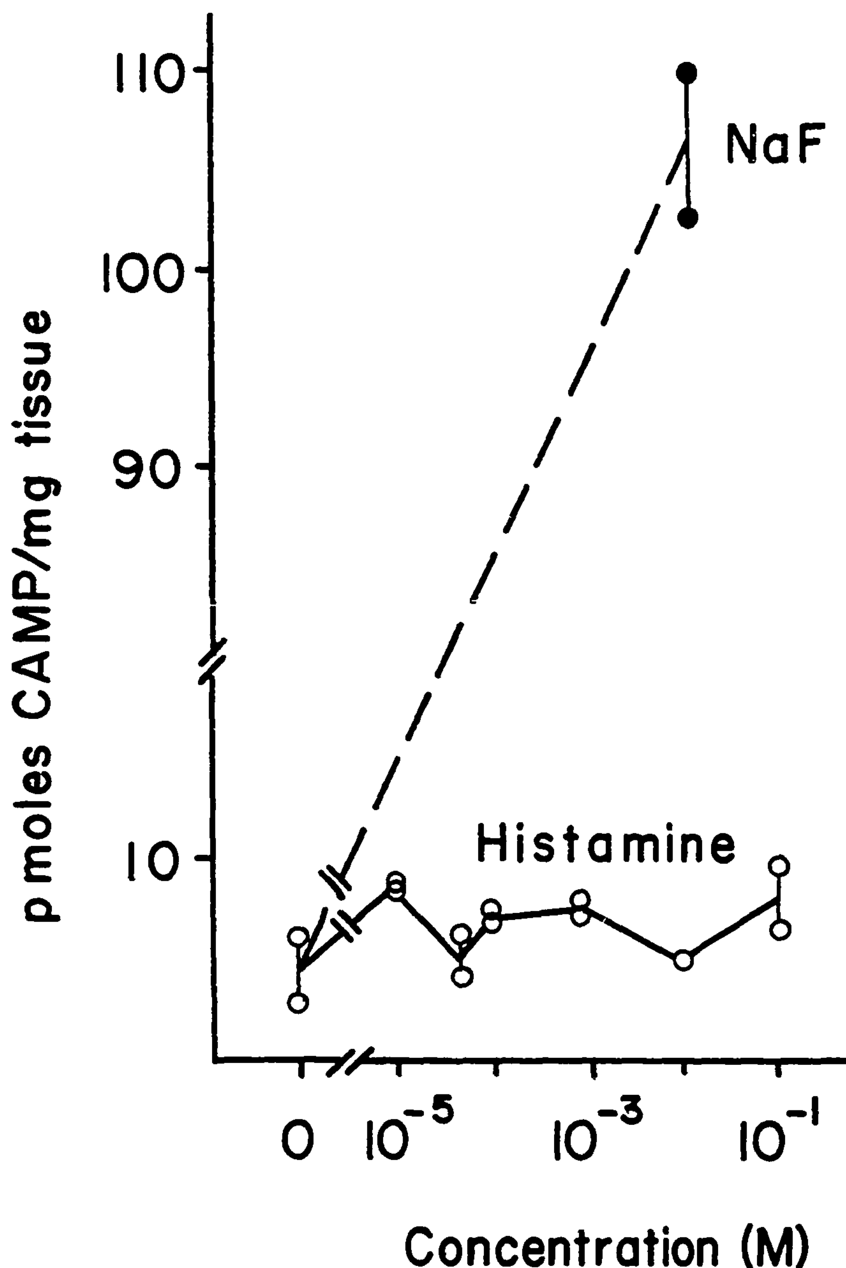

Figure 7. In vitro effect of histamine at various concentrations and sodium fluoride on canine gastric adenyl cyclase activity. Two identical symbols at each concentration except 10^{-2} M histamine represent the duplicated activities. All samples were incubated for 40 min.

plotted against the concentrations of the test substances on semi-logarithmic paper. All samples were duplicated except the one at 10^{-2} M histamine. Two identical symbols at each concentration represent the duplicated values. In all concentrations studied (10^{-5}, 5×10^{-5}, 10^{-4}, 5×10^{-4}, 10^{-2} and 10^{-1} M), histamine caused no appreciable change in adenyl cyclase activity from control values (at 0 concentration). By contrast, NaF at 10^{-2} M caused an approximate 20-fold increase in adenyl cyclase activity from the basal level. The results of this experiment support the preceding observations _in vivo_ that histamine did not stimulate dog gastric adenyl cyclase.

When mucosal adenyl cyclase was investigated at 2 different incubation times, 10 and 20 min, in 5 identical experiments, a time-response curve of enzymic activity was found with NaF stimulation, whereas adenyl cyclase appeared unresponsive to histamine stimulation at two concentrations studied, 10^{-3} and 10^{-4} M. These results are shown in Figure 8. In the presence of fluoride, the amount of cyclic AMP produced (92.3 ± 19.9 p moles/mg) was 10-fold greater at 20 min compared with its control (without any testing substance) at the corresponding time (8.7 ± 1.2 p moles/mg). At 10 min, the amount of cyclic AMP formed with fluoride was about 5 times greater than the control value (56.7 ± 9.9 vs 8.8 ± 1.5 p moles/mg). In contrast to the striking effect of NaF, histamine at 10^{-4} and 10^{-3} M resulted in activities similar to the controls. With histamine at 10^{-4} and 10^{-3} M, the enzyme produced 7.7 ± 1.2 and 7.0 ± 1.9 p mole/mg respectively at 10 min, and 9.3 ± 1.7 and 7.8 ± 1.0 p mole/mg at 20 min. These values were not significantly different from control values at 10 and 20 min, whereas the effects of fluoride were significant at both

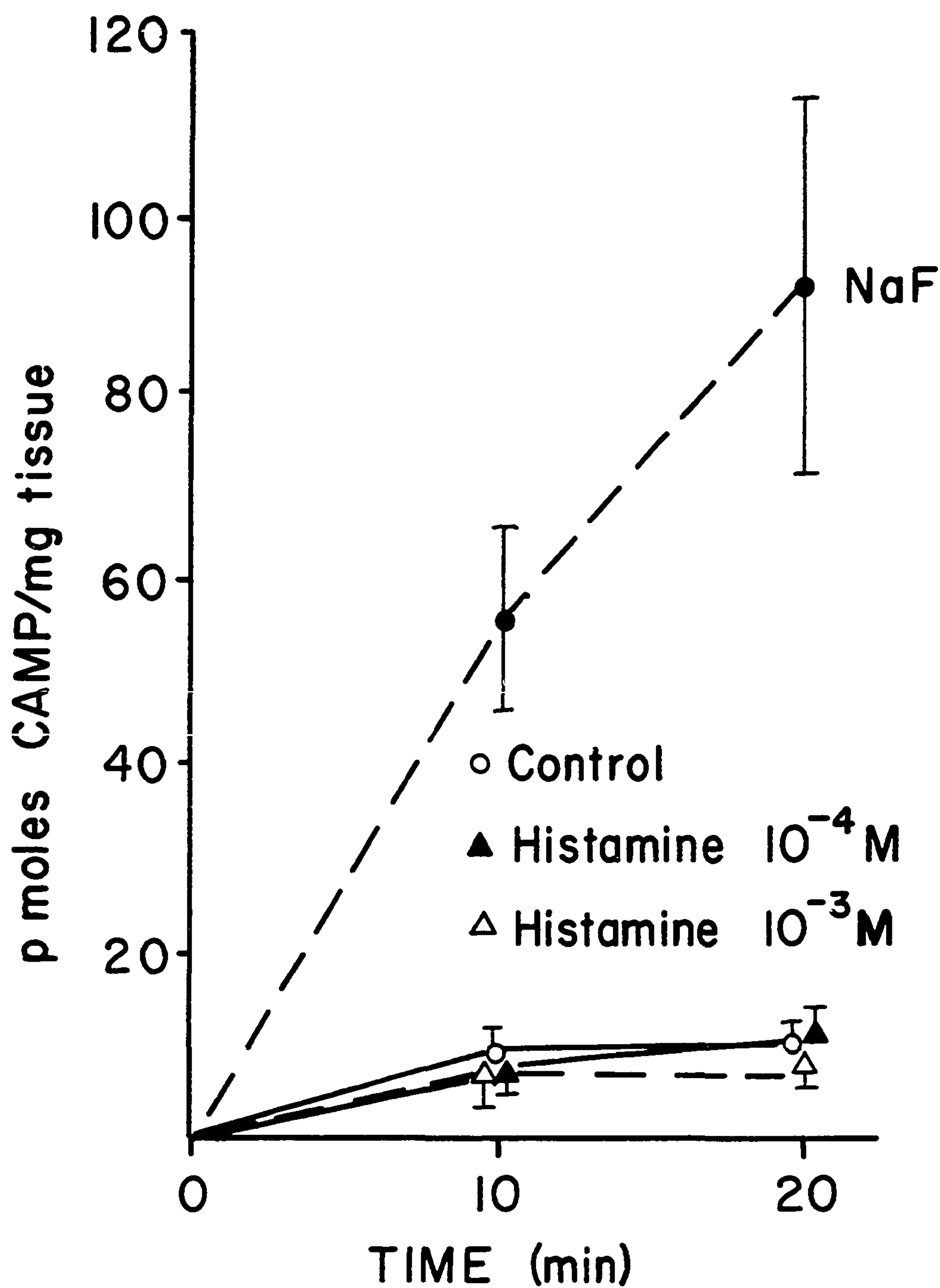

Figure 8. Effect of histamine and sodium fluoride on dog gastric adenyl cyclase activity _in vitro_. The means and standard errors represent 5 experiments.

times. These findings indicate that histamine does not activate canine gastric mucosal adenyl cyclase *in vitro*.

The effect of histamine and fluoride on gastric adenyl cyclase activity was investigated further in an assay system with twice the amount of substrate as used previously. Results from 5 experiments appear in Table 6. Fluoride consistently stimulated adenyl cyclase with a mean value of 103.0 $\pm$ 5.8 p moles/mg, which was more than 4-fold greater than the control. 10^{-3} M histamine failed to stimulate the enzyme (in fact, all values with histamine were slightly lower than their corresponding controls). These findings indicate that histamine did not increase adenyl cyclase activity of the dog stomach *in vitro*.

In the final set of experiments examining the *in vitro* effect of histamine on canine gastric adenyl cyclase, enzymic activity was compared in 2 situations: histamine plus NaF versus NaF alone. Results appear in Table 7. Fluoride alone at a concentration of 5 x 10^{-3} M activated adenyl cyclase to a mean activity of 81.7 $\pm$ 5.4 p moles/mg (more than 3 times control values). This was not the maximal capacity of the enzyme because in the same experiments, NaF at 10^{-2} M activated the enzyme to a higher level (110 $\pm$ 4.99 p moles/mg). When the enzyme was assayed in the presence of the combination of 5 x 10^{-3} M NaF and 10^{-3} M histamine, mean activity was not significantly different from that with 5 x 10^{-3} M NaF alone. These findings support the previously described *in vitro* experiments on the effect of histamine on adenyl cyclase in the stomach.

TABLE 6

IN VITRO EFFECT OF HISTAMINE AND SODIUM FLUORIDE ON GASTRIC ADENYL CYCLASE ACTIVITY IN CANINE MUCOSA*

Experiment \ Treatment	Adenyl Cyclase Activity (p moles cyclic AMP formed/mg tissue)		
	Control	Histamine (10^{-3} M)	Sodium Fluoride (10^{-2} M)
1	[21.43] [28.38] 24.91	[16.95] [20.24] 18.60	[98.88] [90.93] 94.91
2	[20.68] [18.34] 19.51	[19.80] [17.28] 18.54	[94.56] [79.72] 87.14
3	[16.98] [12.84] 14.91	[14.22] [11.62] 12.92	[112.62] [90.38] 101.50
4	[30.90] [36.06] 33.48	[37.60] [26.70] 32.15	[117.10] [119.56] 118.33
5	[26.88] [22.96] 24.90	[18.88] [18.08] 18.48	[120.08] [106.60] 113.34
$\bar{X} \pm$ S.E.	23.54 ± 3.11	20.13 ± 3.19	103.00 ± 5.75

*C^{14}-ATP concentration in the assay system was 1 mM with a specific activity of 0.5 µc/0.1 µmole. Incubation time for all samples was 10 min.

Duplicates of each sample are shown in the brackets. The average of the duplicates is outside the brackets.

TABLE 7

EFFECT OF HISTAMINE, SODIUM FLUORIDE AND THE COMBINATION ON GASTRIC ADENYL CYCLASE ACTIVITY OF CANINE MUCOSA *IN VITRO*

Experiment \ Treatment	Adenyl Cyclase Activity (p moles cyclic AMP formed/mg tissue)									
	Control		Histamine (10^{-3} M)		NaF ($5x10^{-3}$ M)		NaF ($5x10^{-3}$ M) + Histamine (10^{-3} M)		NaF (10^{-2} M)	
1	[16.98] [12.84]	14.91	[14.22] [11.62]	12.98	[70.06] [72.68]	71.27	[92.20] [63.62]	77.91	[112.62] [90.38]	101.50
2	[30.90] [36.06]	33.48	[37.60] [26.70]	32.15	[81.18] [90.28]	85.73	[72.50] [82.52]	78.01	[117.10] [119.56]	118.33
3	[26.88] [22.96]	24.92	[18.88] [18.08]	18.48	[87.60] [89.64]	88.62	[75.88] [58.36]	67.12	[120.08] [106.60]	113.34
X ± S.E.	24.43 ± 5.37		21.20 ± 5.70		81.87 ± 5.37		74.34 ± 3.61		111.00 ± 4.99	

Duplicates are shown in the brackets. The average of the duplicates is outside the brackets. Incubation time for all samples was 10 min.

Effect of Histamine and NaF on Gastric Adenyl Cyclase Activity of Guinea Pig Stomach in Vitro

The enzymic activity was determined with the same analytic method used in the canine enzyme analyses. The results appear in Table 8. Fluoride stimulated the adenyl cyclase activity about 4-fold compared with controls. In addition, guinea pig gastric adenyl cyclase responded to histamine. The activities of the enzyme with either concentration of histamine were consistently higher than corresponding control activities by about 20 to 40%. The activities resulting from the 2 concentrations of histamine were not clearly different from one another. These findings correspond to a report in the literature (Perrier and Laster, 1970) and indicate that the assay used in here is capable of detecting changes induced by histamine in another species.

Secretory and Hemodynamic Responses of Canine Stomach to Arterial Infusion of Dibutyryl Cyclic AMP

In 3 experiments in different dogs gastric secretory rate and arterial inflow were measured in response to infusion of dibutyryl cyclic AMP intraarterially. Blood flow was measured with an electromagnetic blood flow meter. Dibutyryl cyclic AMP was infused following the control period at a dose of 0.1 mg/kg-min. At the end of 30 min after the beginning of the infusion, the dose was doubled by doubling the infusion rate. Since the higher infusion rate was only 0.068 ml/min and arterial blood flow to the stomach flap was at least 1.5 ml/min, the infused volume *per se* was unlikely to cause any significant effect on the blood flow. Table 9 shows the responses during each experimental period. The numbers in the parentheses represent the experiments performed. The

TABLE 8

IN VITRO EFFECT OF HISTAMINE AND SODIUM FLUORIDE ON GASTRIC ADENYL CYCLASE ACTIVITY OF GUINEA PIG

Treatments \ Incubation Time (min)	Adenyl Cyclase Activity					
	0		5		10	
	Experiment 3		Experiment 3		Experiment 3	
Control	[0.33] [0.58]	0.46	[9.51] [8.53]	9.02	[10.90] [10.66]	10.78
Histamine ($5x10^{-5}$ M)	[0.23] [0.53]	0.38	[11.27] [11.92]	11.60	[14.16] [14.06]	14.11
Histamine (10^{-4} M)	[0.46] [0.55]	0.51	[11.39] [10.72]	11.06	[13.55] [13.44]	13.50
Sodium Fluoride (10^{-2} M)	[0.23] [0.83]	0.53	-----	-----	[33.55] [46.36]	39.96

Values in the brackets are duplicates of each sample. The average of the duplicates is shown outside the brackets.

TABLE 8--Continued

(p moles of cyclic AMP formed/mg tissue)							
20				40			
Experiment 3		Experiment 2		Experiment 1		Experiment 2	
[8.81] [10.37]	9.60	[35.10] [36.72]	35.91	[14.10] [18.42]	16.26	[33.30] [30.12]	31.71
[13.14] [15.17]	14.16	[49.45] [43.70]	46.58	[20.50] [18.08]	19.29	[43.15] [42.40]	42.78
[12.24] [10.93]	11.59	[46.42] [39.54]	42.98	[19.31] [21.40]	20.36	[40.09] [39.68]	39.89
-----	-----	-----	-----	[74.79] [59.84]	67.32	[128.19] [125.30]	126.75

TABLE 9

EFFECT OF INTRAARTERIAL INFUSION OF DIBUTYRYL CYCLIC AMP ON SECRETORY AND HEMODYNAMIC RESPONSES OF CANINE STOMACH

Condition	Acid Secretory Rate	Blood Flow (% Change from first control period)		Arterial blood pressure (mm Hg)
		Exp.1	Exp.2	
First Control	0 (3) 0 (3)	0 0	0 0	128 ± 7 (3) 128 ± 7 (3)
Dibutyryl Cyclic AMP (0.1 mg/kg-min)	0 (3)	---- +53.3	+25.9 +77.8	133 ± 4 (3) 131 ± 3 (3)
Dibutyryl Cyclic AMP (0.2 mg/kg-min)	0 (3)	+73.3 +73.3	+88.9 +88.9	135 ± 3 (3) 133 ± 2 (3)
Final Control	0 (3) 0 (3)	0 0	+44.4 +18.5	130 ± 3 (3) 130 ± 3 (3)

The number of experiments are shown in parentheses.

arterial blood pressures are expressed as the means ± standard errors of 3 experiments. Arterial blood flows in two experiments are shown in the middle column in terms of percentage changes from the values measured at the end of the first control period. The flow measurements were obtained every 30 min in two control periods and every 15 min during the infusion of dibutyryl cyclic AMP.

No acid secretion was found in either control period or during the 2 dibutyryl cyclic AMP infusion periods. The infusion of the cyclic AMP derivative did not cause any significant change in arterial blood pressure. However, the drug did induce large increases in arterial blood flow to the stomach segment. The increases appeared in 15 min following the start of infusion of dibutyryl cyclic AMP. The flow began to level off at 15 min after the start of the second dose. The largest magnitude of the increases was 73.3% in one experiment and 88.9% in the other. Following the termination of the infusion, blood flow returned toward the original level found in the first control period. These findings indicate that direct intraarterial infusion of the membrane permeable derivative of cyclic AMP does not initiate gastric secretion, although gastric blood flow is increased.

CHAPTER IV

DISCUSSION

Since Sutherland and Rall (1958) discovered the cyclic nucleotide phosphodiesterase in beef heart, it has been subsequently shown that almost all animal tissues studied contain this enzyme. Methylxanthines have been recognized as standard inhibitors of the enzyme. Adding these drugs to various tissue *in vitro* inhibited the phosphodiesterase activity (Sutherland and Rall, 1958; Butcher and Sutherland, 1962; Hynie *et al*., 1966; Cheung, 1966) and potentiated or mimicked the cyclic AMP mediated effects of various hormones (Hynie *et al*., 1966; Orloff and Handler, 1962; Handler *et al*., 1965; Rall and West, 1963; Butcher *et al*., 1965). Papaverine was recently found also to be a potent inhibitor of phosphodiesterase in a number of tissues *in vitro* (Triner *et al*., 1970; Kukovetz *et al*., 1969; O'Dea *et al*., 1970). It has been well established that theophylline inhibits phosphodiesterase competitively (Butcher and Sutherland, 1962). The kinetics of papaverine inhibition of the enzyme has not yet been delineated. According to Triner *et al*. (1970) the higher inhibitory effect of papaverine on phosphodiesterase from rat uterine muscle was associated with a lower concentration of the substrate (cyclic AMP) and *vice versa*; thus, papaverine seems also to be a competitive inhibitor of phosphodiesterase.

Although theophylline and papaverine inhibited phosphodiesterase

from various tissues, their effect on gastric mucosal phosphodiesterase has not yet been reported. Therefore, the first experiments of the present investigation were directed toward testing the effect of these drugs on cyclic phosphodiesterase in canine gastric mucosa. The results showed that both theophylline and papaverine inhibited the enzyme. Based upon the dose response curve (Fig. 2), concentrations of theophylline exceeding 1.56 mM caused a much flatter slope than those below this concentration. The flat portion of the curve indicates a lesser susceptibility of gastric phosphodiesterase to theophylline as compared with the observations which Butcher and Sutherland (1962) reported in other tissues of the dog. The difference in the concentration of substrate (cyclic AMP) used in the 2 assay systems also explained partially the smaller effect of the drug on the gastric enzyme. In the case of papaverine, a dose response curve (Fig. 3) with a much steeper slope was found as compared with theophylline. 1.25 mM papaverine decreased the gastric phosphodiesterase activity by 90%. On a molar basis, this inhibition is about 30% greater than that of theophylline on phosphodiesterase of beef heart, despite a much higher substrate concentration in the present assay.

For in vivo studies with intravenous administration, the doses of the phosphodiesterase inhibitors were chosen based on a series of 7 pilot experiments, 4 for theophylline and 3 for papaverine. The highest dose which did not cause more than a 30% decrease in blood pressure was used in the experiments described in this thesis. The doses of 16 mg/kg and 6 mg/kg for theophylline and papaverine respectively was finally selected. With these 2 doses, both theophylline and papaverine failed to cause the gastric segment to secrete, although they potentiated hista-

mine-stimulated acid secretion (Figs. 4 and 5). This observation essentially confirmed the findings by other investigators (Robertson *et al.*, 1950). From the standpoint of Harris's cyclic AMP hypothesis, there could be several explanations for these findings: 1) The local concentration of the phosphodiesterase inhibitors may only inhibit the gastric phosphodiesterase activity to such an extent that cyclic AMP accumulation would not be great enough to initiate the secretory process, but would be sufficient to potentiate histamine-stimulated acid secretion; 2) The phosphodiesterase activity in gastric secretory cells was inherently low or existed *in vivo* in an inactive state, as speculated by Cheung (1970). Further inhibition of the enzyme by an exogenous inhibitor would, therefore, at best contribute more cyclic AMP to produce a potentiating effect on acid secretion; 3) Canine parietal cell membrane might have a particularly poor permeability for methylxanthines and papaverine. This low permeability would largely limit the quantity of the drugs penetrating cell membrane; and 4) Increased concentrations of intracellular cyclic AMP are not essential for initiation of secretion by the stomach.

Although the permeability factor could explain the species difference to methylxanthines, it is difficult to be tested. Whether phosphodiesterase in the canine mucosa exists in an inhibited state is likewise untestable, because were it true, the *in vivo* inhibiting condition would not be maintained in the enzyme assay system *in vitro*. In other words, the enzyme would be released from its normal unfavorable condition when the enzyme was assayed. A comparison of phosphodiesterase activities among various tissues assayed by different investigators is difficult because of the differences in assay systems used. Butcher and

Sutherland (1962) have reported a survey of phosphodiesterase activity in a variety of canine tissues except gastric mucosa. In a rough comparison between the activity found in gastric tissue with the findings of Butcher and Sutherland, gastric mucosal phosphodiesterase activity might be classified as one of the lowest. Nevertheless this does not permit the conclusion that the low activity of phosphodiesterase found in dog gastric mucosa accounts for the failure of theophylline and papaverine to stimulate secretion, especially in the absence of information about the phosphodiesterase activity of gastric mucosa in those species which respond to methylxanthines by secreting acid.

In order to test the first possible explanation, another approach to the problem was utilized. By virtue of the stomach flap preparation, it was feasible to infuse drugs continuously into an artery which leads to the stomach segment. Upon considering that the volume rate of the infusion to the artery and the solubility of the drugs in saline were both limited, 2 doses of papaverine (1.5 and 3.0 mg/kg-10 min) and 3 doses of theophylline (2, 4 and 8 mg/kg-10 min) were chosen for the selective intraarterial infusion. Each dose was infused 20 min. The quantity of each drug reaching the mucosal tissue was much greater than that which could be achieved by intravenous injection. The results showed that in spite of this large increase in the local concentration of the drugs delivered to the stomach, neither theophylline nor papaverine stimulated acid output. This finding rendered the first explanation unlikely.

Phosphodiesterase activities assayed from the tissue biopsies taken from _in vivo_ experiments with either intraarterial or intravenous

administration of the drugs did not reveal enzyme inhibition (Tables 2-5). This failure to detect inhibition of phosphodiesterase *in vivo* is not totally unusual and has been reported in other tissues by other investigators. Verniko-Darrellis and Harris (1968) showed that phosphodiesterase from rat heart and brain was significantly inhibited in the presence of methylxanthine in an *in vitro* assay system, but pretreating the animals with caffeine or theophylline did not cause reduction of *in vivo* enzymic activity in these tissues. One of the possible reasons for this occurrence would be the competitive inhibition of methylxanthines and papaverine which then permits competition for the catalytic site(s) with the substrate (cyclic AMP). In the *in vivo* system, an extremely low level of cyclic AMP in the cell allows a better chance for inhibitors to occupy the binding site(s) on the enzyme, thereby allowing inhibition to occur easily. When phosphodiesterase was assayed from biopsy samples, the condition was different, because the inhibitors in the tissue sample were diluted and the high concentration of exogenous cyclic AMP which was used as the substrate restricted the opportunity for inhibitors to gain access to the critical site(s) on the enzyme. Furthermore, the binding force between the inhibitors and the enzyme does not seem to be strong; this weak binding was demonstrated in one of the preliminary experiments in which the enzyme was dialyzed against the buffer solution after being incubated with a high concentration of theophylline *in vitro*. The activity of the dialyzed enzyme was found to return to the control level (without incubation with theophylline, but with dialysis). This observation indicated that the bound inhibitors on the enzyme *in vivo* could be easily released from the binding site(s) during the *in vitro* assay pro-

cedure and replaced with the substrate. In the light of the preceding considerations the failure to detect inhibition of phosphodiesterase from the mucosal tissue pretreated with enzymic inhibitors *in vivo* does not necessarily mean that no inhibition occurred with the *in vivo* system.

The failure of phosphodiesterase inhibitors, theophylline and papaverine to initiate gastric secretion and the potentiation by these drugs of histamine-stimulated acid secretion were clearly observed in this investigation. Should the potentiating effect of the drugs derive from their ability to inhibit phosphodiesterase activity in gastric mucosa (thereby increasing cyclic AMP accumulation in the parietal cells) there must be another more powerful mechanism in the parietal cells to generate sufficient amounts of cyclic AMP to initiate the acid secretory process (as with histamine alone). Alternatively, one could conclude that accumulation of cyclic AMP is not a necessary requisite for secretion. A reasonable speculation would be that secretagogues, like histamine, activate the cyclic AMP producing system (adenyl cyclase) on the parietal cell membrane. To test this possibility, the effect of histamine on gastric adenyl cyclase activity was measured.

The gastric adenyl cyclase study was mainly composed of two portions: *in vivo* and *in vitro* effects of histamine on the enzymic activity. The results of the *in vivo* experiments showed that histamine caused the gastric mucosa to secrete acid but did not affect the adenyl cyclase system (Fig. 6). The adenyl cyclase activity in the gastric mucosa was, therefore, independent of the rate of acid secretion stimulated by histamine. The effect of histamine on canine gastric adenyl cyclase was examined directly in a variety of *in vitro* assay systems. All results ob-

tained (Figs. 7 and 8; Tables 6 and 7) supported the _in vivo_ finding. Therefore, based upon both _in vivo_ and _in vitro_ studies of adenyl cyclase, it was concluded that histamine did not stimulate canine gastric mucosal adenyl cyclase activity. Adenyl cyclase in the guinea pig stomach appeared different from that of the dog. Guinea pig mucosa responded to histamine of either 5 x 10^{-5} M or 10^{-4} M (Table 8). This observation was in agreement with the findings reported by Perrier and Laster (1970) and Nakajima _et al_. (1971). It thus indicated that a species difference exists in the response to histamine by gastric adenyl cyclase, although histamine stimulates the stomachs of both species to secrete acid (Shoemaker _et al_., 1964). Based upon these findings it seems untenable to hold that gastric adenyl cyclase is a major source of cyclic AMP required by gastric secretagogues to stimulate secretion.

Beside the adenyl cyclase system, another possible mechanism within the contact with the cyclic AMP hypothesis for initiating acid secretion would be the inhibition of phosphodiesterase in parietal cells by the secretagogues. In canine stomach, either methylxanthines or papaverine might inhibit phosphodiesterase to a small extent. The major inhibition on the enzyme might be accomplished by histamine or other physiological acid stimulants. However, this did not appear to be true. In 2 experiments, canine gastric phosphodiesterase preparation was assayed in the presence of histamine at 10^{-3}, 10^{-4} and 10^{-5} M; the enzymic activity was not affected by histamine at any concentration studied. Besides, it was reported by Amer and McKinney (1970) that both cholecystokinin (CCK), a weak acid stimulant and pentagastrin, a strong acid secretagogue, stimulated phosphodiesterase selectively in several tissues,

including gastric mucosa in vitro. If this stimulation of phosphodiesterase by acid secretagogues also happens in vivo, it would result in a decline in cyclic AMP concentration in gastric tissue concomitantly with an increase in acid secretion. The observation of phosphodiesterase stimulation by the secretagogues led the authors to put forward a hypothesis that acid secretion was mediated by a decrease of intracellular cyclic AMP concentration in parietal cells.

Levine and his coworkers reported that intravenous infusion or injection of cyclic AMP inhibited histamine-stimulated acid secretion with a concomitant decrease in mucosal blood flow. They concluded that the hemodynamic effect of exogenous cyclic AMP in vivo may be responsible for acid inhibition (Levine et al., 1967). This explanation would imply that cyclic AMP may still play an intermediary role in parietal cells for acid secretory function, but the hemodynamic effect derived from large amount of exogenous cyclic AMP in the circulation overshadowed the normal stimulating effect on acid secretion. In addition to their speculation, cyclic AMP is known as a poorly permeable compound for cell membranes. It was reported that a high concentration of exogenous cyclic AMP failed to mimic the physiological effect of lipolytic hormones in adipose tissue in vitro (Vaughan, 1960; Butcher et al., 1965). Whether infused cyclic AMP in the experiements of Levine et al. could pass through the cell membrane to reach the critical site(s) in the parietal cells was obviously questionable. In order to clarify these problems, the acid secretory response and arterial blood flow to the stomach flap were observed with intraarterial infusion of dibutyryl cyclic AMP. It was found that dibutyryl cyclic AMP did not cause acid secretion under a circum-

stance that arterial blood flow to the tissue was increased (Table 9). This suggested that cyclic AMP is a vasodilator and that the failure of dibutyryl cyclic AMP to evoke acid secretion from resting stomach tissue and inhibition of acid output from a secreting stomach by cyclic AMP can not be attributed to a decrease in arterial blood flow. The decrease in mucosal blood flow observed by Levine _et al_. during the infusion of cyclic AMP is probably secondary to the decline in acid secretion. The inhibitory effect of cyclic AMP on histamine-stimulated acid secretion is not understood.

In view of the experimental results obtained in this investigation, gastric secretion in the dog was not initiated by administering gastric phosphodiesterase inhibitors nor by infusing the membrane-permeable derivative of cyclic AMP. Furthermore the gastric secretagogue, histamine, neither stimulated adenyl cyclase nor did it inhibit phosphodiesterase. Thus the hypothesis of Harris and his collaborators that cyclic AMP is the intracellular mediator of acid secretion in the stomach was not supported.

CHAPTER V

SUMMARY

This research was addressed to evaluating the hypothesis of Harris and his associates that cyclic AMP is the intracellular mediator of gastric acid secretion. Experimental approaches were designed to correlate acid secretory function and enzymic activities which directly determine the cyclic AMP concentration in the gastric mucosa. Both *in vivo* and *in vitro* experiments were performed in this investigation. All *in vivo* experiments were conducted on anesthetized dogs equipped with a gastric chambered segment preparation.

Theophylline and papaverine were demonstrated to be effective inhibitors of gastric phosphodiesterase activity *in vitro*. Neither of these agents initiated acid secretion from the stomach flap when they were administered into the animals either intravenously or intraarterially, although both drugs potentiated histamine-stimulated acid secretion.

Effect of histamine on gastric adenyl cyclase activity was studied both *in vitro* and *in vivo*. When the enzyme was assayed from biopsies taken during the experiments with histamine infusion, it was found that the enzymic activity was not affected by histamine. *In vitro*, the effect of histamine on gastric adenyl cyclase was studied under the following

conditions: 1) a wide range of histamine concentrations; 2) two different concentrations of the substrate (ATP) for the assay; 3) various incubation times; and 4) with or without background stimulation by NaF. Under all conditions, histamine failed to stimulate adenyl cyclase.

The possibility that histamine might inhibit gastric phosphodiesterase was tested by incubating the enzymic preparation with several concentrations of histamine. The results showed no inhibition of the enzymic activity by histamine.

The final set of experiments were conducted to examine the effect of exogenous cyclic AMP on acid secretion. In an attempt to assure an increase in the concentration of the cyclic compound in the parietal cells, membrane permeable dibutyryl cyclic AMP was infused intraarterially into the gastric mucosal tissue. No acid secretion was observed during or following the infusion. Since the total arterial blood flow to the stomach tissue was markedly increased, it is unlikely that the failure of acid secretion with exogenous cyclic AMP was attributable to ischemia.

In consideration of the findings presented in this dissertational investigation, the conclusion appears warranted that the cyclic AMP hypothesis of Harris and his associates is untenable. Rather, the initiation of gastric secretion in the canine stomach does not seem to depend upon alterations in the activities of enzymes responsible for accumulation of cyclic AMP.

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