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QUANTIFICATION OF NEUROCHEMICAL RECEPTORS ON RAT INTESTINAL EPITHELIAL CELLS

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QUANTIFICATION OF NEUROCHEMICAL RECEPTORS ON RAT INTESTINAL EPITHELIAL CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

by

Thomas Joseph Rimele, B.S. Pharmacy

The Ohio State University
1981

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TO MADONNA AND CHRISTOPHER
ACKNOWLEDGEMENTS

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PUBLICATIONS


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LIST OF TERMS AND ABBREVIATIONS

\( cAMP \) = Cyclic adenosine 3',5'-monophosphate
\( B_{max} \) = Maximum receptor density
Crypt cell = Secretory intestinal epithelial cell
DMPP = 1,1-dimethyl-4-phenylpiperazinium iodide
EDTA = Ethylenediaminetetraacetate
Enterocyte = General term for intestinal epithelial cell
Goblet cell = Mucus secreting intestinal epithelial cell
\( n_H \) = Hill coefficient
5-HT = 5-Hydroxytryptamine (serotonin)
IC50 = Molar concentration of drug which inhibits specific binding by 50%
IC50* = Molar concentration of drug which inhibits total binding to tissue by 50%
\( I_{sc} \) = Short-circuit current
KD = Apparent dissociation constant
Microvilli = Fingerlike projections on individual epithelial cells (brush border)
\( \text{Na}^+\text{-K}^+\text{-ATPase} \) = Sodium-potassium adenosine triphosphatase
PD. = Transmural potential difference
QNB = Quinuclidinyl benzilate
S.E. = Standard error of the mean
Villous cell = Absorptive intestinal epithelial cell
VIP = Vasoactive intestinal polypeptide
WB-4101 = 2,6-dimethoxyphenoxyethylaminomethyl-1,4-benzodioxane
CHAPTER I

INTRODUCTION

There has been a renewed interest in the neural regulation of intestinal absorption and secretion over the last decade. Evidence will be cited to support a role for neurochemical receptors in the absorptive and secretory processes of intestinal water and electrolyte transport, but first a brief orientation as to the structure and innervation of the intestine will be presented.

GENERAL MORPHOLOGY OF THE GASTROINTESTINAL TRACT

The different portions of the gastrointestinal tract can be divided into four principal layers: the serous layer, the muscle layer (muscularis externa), the submucosa, and the mucosa (Trier, 1968; Junqueira, 1977; Reith and Ross, 1977). A description of the structure of each layer is summarized below, with the most important features illustrated in Figure 1.

The serosa is the outermost layer of the intestinal wall and consists of a sheet of simple squamous epithelium, and a small amount of connective tissue, rich in blood and lymph vessels.

The muscularis externa consists of smooth muscle cells, spirally oriented and divided into two sublayers. In the internal sublayer (closest to the lumen), the orientation is generally circular; in the external sublayer, mostly longitudinal. The myenteric (Auerbach's) nerve plexus, lies between the longitudinal and circular layers of the muscularis externa.

The submucosa consists mostly of connective tissue. It serves as the major route for the larger blood and lymph vessels that are within the intestinal wall. The submucosa also contains the submucosal (Meissner's) nerve plexus.
Figure 1. Characteristics of the intestinal wall. A diagram of a section of small intestine which has been partly separated into layers, showing the distribution of neural elements in the various layers of the intestine. Other important features are also illustrated. (Modified from Furness and Costa, 1980).
Figure 1
The mucosa of the intestine is divided into three sublayers. These include the muscularis mucosae, the lamina propria, and the epithelial cell layer (refer to Figure 1).

The muscularis mucosae is a continuous thin layer of smooth muscle separating the mucosa from the submucosa. As in the muscularis externa, it consists of inner circular and outer longitudinal muscle fibers. The muscularis mucosae promotes the movement of the mucosal layer independently of other intestinal movements, increasing mucosal contact with material in the lumen, while the contractions of the muscularis externa propel and mix the luminal contents.

The middle layer of the mucosa, the lamina propria, is a continuous subepithelial connective tissue space bounded by the muscularis mucosae and the intestinal epithelium. The lamina propria is heterogeneous in its composition and normally contains a variety of cell types including macrophages, lymphocytes, and mast cells, as well as noncellular connective tissue elements including collagen. Blood and lymph vessels, unmyelinated nerve fibers, and strands of smooth muscle (extending from the muscularis mucosae) are also present.

The innermost layer of the intestinal mucosa consists of a continuous sheet of columnar epithelial cells (enterocytes), directly associated with the underlying lamina propria.

ORGANIZATION OF THE INTESTINE AND FINE STRUCTURE OF INTESTINAL EPITHELIAL CELL TYPES

The small intestine consists of the duodenum, jejunum, and ileum. The segments have many characteristics in common, thus they will be discussed together. The villi, which are easily seen under low power magnification, are outgrowths of the mucosa (epithelium plus lamina propria) which project into the lumen of the small intestine. Between the villi are openings, the crypts, or glands of Lieberkühn (Plate I). The columnar epithelial cells that comprise the mucosal cell mass, are formed in the crypts, migrate continuously onto villi and are subsequently shed into the lumen at villous tips. As these cells move up from the
crypts towards the villi, they differentiate (Cheng and Leblond, 1974) and acquire the capacity to absorb nutrients and electrolytes. The enterocytes in the crypts, on the other hand, have been proposed to function in secretion (Hendrix and Bayless, 1970; Browning et al. 1978). Absorptive, goblet, and a few enteroendocrine cells comprise the villous epithelium, whereas undifferentiated, goblet, enteroendocrine, and Paneth (lysozyme containing) cells line the crypts.

Mammalian absorptive cells are tall, columnar cells with basally located nuclei. The striated (brush) border consists of numerous, slender, fingerlike projections, called microvilli (see Figure 2 and Plate II). The microvilli increase the surface area for absorption of nutrients, water and electrolytes. The brush border is also the site of activity for the disaccharidases which hydrolyze disaccharides into monosaccharides, which are then easily absorbed.

Goblet cells are so named because their shape resembles a brandy goblet. They are present in both the crypt and villous epithelium (Plate I), and are distinguished from other types of epithelial cells by the mucus globules which often fill the cytoplasm between the basally located nucleus and apical brush border of the cell (see Figure 2). They are less abundant in the duodenum and increase aborally. Goblet cells produce high molecular weight acid glycoproteins (mucins) whose main function is to lubricate the lining of the intestine.

Enteroendocrine cells are characterized by the presence of abundant dense secretory granules located near the basal membrane and therefore in close proximity to the blood supply. These cells belong to the APUD (amine precursor uptake and decarboxylation) series of endocrine cells (Pearse and Polak, 1978). APUD cells possess a common set of cytochemical characteristics, with the most striking and constant characteristics being the production of biogenic amines (norepinephrine, dopamine, 5-hydroxytryptamine) and the ability to produce and secrete polypeptide hormones (e.g. secretin, glucagon, gastric inhibitory peptide, gastrin, vasoactive intestinal polypeptide, and cholecystokinin).
The microvilli of the APUD cells may subserve a sensory function, responding to changes in the luminal contents and subsequently releasing stored amines and/or polypeptide hormones into the blood supply of the intestine. Furthermore, some APUD cells are in close proximity to nerve fibers so that nervous stimuli may also influence their functions (see section on innervation of the epithelium for further details).

The large intestine (colon) consists of a smooth mucosal surface; villi are absent (Plate I). The crypts are long and characterized by a great abundance of goblet cells. A primary function of the colon is to absorb water and electrolytes. The large number of goblet cells in the colon produce mucus which lubricates the mucosal surface.

THE EXTRINSIC INNERVATION OF THE INTESTINE

Several nerve plexuses consisting of interconnecting nerve fibers and ganglia are found in the various layers of the intestinal wall (Younans, 1968 and Schofield, 1968).

The connection between mesenteric nerve fiber bundles and the myenteric plexus is through a transitional subserous plexus. As in the mesentery, the nerve fibers contained in the subserous plexus, accompany blood vessels entering the wall of the intestine.

The myenteric plexus lies between the circular and longitudinal smooth muscle layers of the muscularis externa and consists of three plexiform meshworks (see Figure 1). The primary plexus is composed of large bundles of unmyelinated fibers linking ganglia of variable size and shape. The secondary plexus forms a closer mesh within and continuous with the primary plexus and consists of smaller bundles of unmyelinated fibers in which only a few neurons are seen. The secondary plexus in turn is continuous with the tertiary plexus, and both contribute to the bundles of fine unmyelinated nerve fibers which ramify within the muscle coat. A deep myenteric plexus situated within the circular muscle is also present. The submucosal plexus is linked with the myenteric plexus through the deep myenteric plexus.
The submucosal plexus consists of several networks of nerve fibers and ganglia arranged in a tiered formation in the submucosa. The ganglia are generally smaller than those in the myenteric plexus and contain fewer neurons (see Figure 1).

The mucosal plexus is continuous with and represents an extension of the submucosal plexus into the mucosa. Nerve fibers are present in the villi and surrounding the crypts (Furness and Costa, 1980; Gershon and Erde, 1981).

The intestinal wall is innervated extrinsically by the parasympathetic and sympathetic divisions of the autonomic nervous system. The parasympathetic supply is furnished by the vagus nerve (cranial outflow) to the small intestine and first portion of the colon, and by the pelvic nerve (sacral outflow) to the rest of the colon. The efferent fibers of the vagus and pelvic nerves are preganglionic and form synapses with neurons in the myenteric and submucosal ganglionated plexuses. Postganglionic fibers from these plexuses innervate the smooth muscle and secretory cells. The preganglionic sympathetic outflow (from the thoracic and lumbar regions of the spinal cord) is carried mainly by the splanchnic nerves. The cell bodies of the second neuron in the sympathetic pathway are contained in the prevertebral ganglia (celiac, superior mesenteric, and inferior mesenteric).

The intestinal wall also has a rich afferent innervation. These fibers are found in the same nerves that contain sympathetic and parasympathetic fibers.

THE ENTERIC NERVOUS SYSTEM: THE INTRINSIC INNERVATION OF THE INTESTINE

Bayliss and Starling (1899) recognized, at the end of the nineteenth century, that peristaltic contractions were true coordinated reflexes, initiated by mechanical stimulation of the intestine, and controlled by a local nervous mechanism. They also noted that the local nervous apparatus or the enteric nervous system could function independently of the central nervous system. The establishment of the independence of the enteric nervous system came 18 years later when Trendelenburg (1917) showed that the peristaltic reflex (a proplusive, analy moving wave of oral contraction and anal relaxation) was still present in vitro. This reflex was similar to the activity observed in vivo by Bayliss and
Starling (1899) suggested that all of the components (sensory neurons, interneurons, and both excitatory and inhibitory motor neurons) of the reflex were intrinsic to the enteric nervous system.

Langley (1921) added further evidence to the concept that the enteric nervous system could act independently of the central nervous system. He classified the enteric nervous system as a third division of the autonomic nervous system. Although Langley's classification of the autonomic nervous system into three divisions lost favor in the eyes of many investigators, recently the independence and complexity of the enteric nervous system has again been appreciated (Furness and Costa, 1980; Gershon and Erde, 1981).

The majority of the nerve cell bodies of the enteric nervous system are in the ganglia of the myenteric plexus. Axons of both parasympathetic and sympathetic origin and the peripheral processes of sensory neurons enter the various plexuses of the enteric nervous system, contribute to the fiber network and form connections with intrinsic neurons. However, the majority of the axons in the intestine are of intrinsic origin. Therefore, neurons whose cell bodies are within the enteric plexuses (i.e., myenteric, submucosal and mucosal) are referred to as enteric neurons and those whose cell bodies are outside the intestine, but whose axons contribute to the enteric plexuses, are extrinsic neurons.

The enteric nervous system contains a variety of nerve types which can be distinguished by their different electrical, pharmacological, functional, histochemical, biochemical and ultrastructural characteristics (see reviews by Furness and Costa, 1980; Gershon and Erde, 1981; Schultzberg et al., 1980). On the basis of these types of studies, a number of substances have been proposed to act as neurotransmitters or neuromodulators (an agent that affects transmitter release from other nerves) in the intestine. In addition to the classical neurotransmitters (norepinephrine and acetylcholine) the list, to date, contains the following candidates: 5-hydroxytryptamine, adenosine triphosphate, vasoactive intestinal
polypeptide (VIP), substance P, somatostatin, enkephalin, gastrin/cholecystokinin tetrapeptide, bombesin, and neurotensin. Although all of these agents have not yet met the strict criteria (Bumstock et al., 1979) for consideration as neurotransmitters or neuromodulators, these agents alter intestinal function in a fashion similar to that of the classical autonomic nervous system. Only those neurons and transmitters that pertain to the present study will be discussed further. These include: cholinergic neurons (acetylcholine), noradrenergic neurons (norepinephrine), serotonergic neurons (5-hydroxytryptamine, 5-HT), and the peptidergic neurons (enkephalin and VIP). The remaining portion of this section will focus on the distribution of these agents in the intestinal wall. The evidence for the innervation of the mucosa (epithelium) by some of these nerve types will be presented in the next section.

Acetylcholine is the transmitter of the preganglionic vagal and sacral fibers that reach the gut. These fibers form synapses within the enteric nervous system, primarily in the stomach and distal colon. Excitatory connections are formed with both intrinsic cholinergic neurons and enteric inhibitory neurons. Intrinsic ganglion cells that excite the intestinal smooth muscle are also cholinergic. Furthermore, most ganglion cells are cholinergic as revealed by acetylcholinesterase staining (Jacobowitz, 1965) and by ultrastructural characteristics (Gabella, 1972). There is also good evidence for cholinergic interneurons in the intestine (Furness and Costa, 1980).

The presence of noradrenergic axons in the intestine has been shown with the fluorescence histochemical technique (Norberg, 1964; Jacobowitz, 1965; Furness and Costa, 1974) and by dopamine-ß-hydroxylase-like immunoreactivity (Schultzberg et al., 1980). Noradrenergic axons originate from cell bodies in extrinsic, prevertebral, sympathetic ganglia. Within the gut wall these axons ramify extensively among the nerve cell bodies of the myenteric and submucosal plexuses. Noradrenergic fibers supply the circular smooth muscle of the sphincters with very few fibers found in non-sphincter muscle.
It has been known for many years that the gut contains 5-HT (Erspamer, 1966). In fact, the gut was the first organ in which this amine was found, and it is by far the largest 5-HT storage depot in the body. However, the bulk of the 5-HT of the gut is contained within mucosal enteroendocrine cells. This vast store of 5-HT, whose function remains uncertain, tends to overshadow the 5-HT that is found in the gut wall after the mucosa has been removed. There are several lines of evidence that suggest neurons containing 5-HT (or a related indoleamine) may be present in the intestine (for details see Gershon and Erde, 1981). 5-HT activates neurons that release acetylcholine and that are excitatory to the intestinal smooth muscle. In addition 5-HT also activates nonadrenergic, noncholinergic intrinsic inhibitory neurons.

Vasoactive intestinal polypeptide is present in gastrointestinal nerves which may be intrinsic to the gut wall (see Furness and Costa, 1980; Gershon and Erde, 1981). The distribution of VIP nerves in the intestine, as mapped by immunohistochemical methods, indicates a rich innervation in the myenteric and submucosal plexuses and throughout the circular muscle layer. Dense networks of VIP-positive fibers are observed in the villi, extending in a regular fashion into their tips. Recently, Jessen et al. (1980) showed reciprocal projections between the myenteric and submucosal plexuses by nerve fibers containing VIP and substance P, with the VIP nerve cell bodies in the submucosal plexus.

Immunoreactive Leu- and Met-enkephalin are also present in the myenteric plexus, the circular and deep muscular plexuses, with few fibers in the submucosa and mucosa (Schultzberg et al., 1980).

It is evident from the above discussion that these putative neurotransmitters, by way of their rich distribution in the myenteric plexus, probably play an important role in the regulation and modulation of intestinal motility. However, since this work is primarily concerned with neurochemical receptors on epithelial cells, the role of the enteric nervous system in motility will not be discussed further. For details concerning this matter the reader is referred to the following
reviews; Furness and Costa (1980), Gershon and Erde (1981). The remaining
discussion of intestinal innervation will focus on the innervation of the epithelium.

EVIDENCE FOR INNERVATION OF THE INTESTINAL EPITHELIUM

Innervation of surface-lining epithelial cells has been described for the
gallbladder and stomach. The gallbladder epithelium, which has a secretory
function, is densely innervated by autonomic nerve fibers with the ultrastructural
characteristics of cholinergic nerve endings (Wahlin et al., 1977). However,
synaptic arrangements were not observed by electron microscopy. On the other
hand, a functional effect of cholinergic agents on the surface epithelial cells was
demonstrated, suggesting the presence of muscarinic receptors on these epithelial
cells and thus putative nervous control (Wahlin et al., 1977). The parietal cells
of the stomach are also innervated by cholinergic fibers (Hanker et al., 1977).

In the intestine, Schofield (1960) identified nerve fibers between intestinal
glands (crypts) of the jejunal mucosa of the cat. Similar fibers, lying subjacent to
the surface epithelium of the mucosa, were found in the colon of the mouse with
some fibers appearing to be in virtual contact with the basal aspect of the
epithelial cells. Although neurons were not observed in the lamina propria of
mouse villi, Palay and Karlin (1959) did find axonal processes, some of which were
in close apposition to the basement membrane of the epithelium. More recent
studies have confirmed that axons can terminate as close as 50 nm to the base of
epithelial cells (Stach, 1973). The human jejunum exhibits a similar pattern of
innervation (Isaacs et al., 1976); cholinergic fibers surround the crypts and some
fibers extend into the villi. Many of the fibers described above appear to be
cholinergic based on the thiocholine technique for staining cholinesterase (Isaacs
et al., 1976). Many of the adrenergic fibers (identified in the submucosa and
mucosa by the histofluorometric method) are associated with the vasculature
(Jacobowitz, 1965; Norberg, 1964). More specifically, Paneth cells have been
shown to be innervated by both adrenergic (Gabella and Costa, 1968) and
cholinergic (Ahonen, 1973) nerve fibers. Enteroendocrine cells also appear to be
innervated by both adrenergic and cholinergic efferent fibers (Pettersson, 1979; see below). Since most of the evidence for the innervation of the intestinal epithelium comes from studies relating to enteroendocrine cells (Pettersson et al., 1978; Lundberg et al., 1978; Newson et al., 1979; Pettersson, 1979; Pettersson et al., 1980), these cells will serve as the model for further discussion of this subject.

The 5-HT-containing enteroendocrine cell (enterochromaffin cell) is the most common of the APUD cells in the intestine. Luminal stimuli such as hypertonic glucose, acid pH, and increased pressure are known to cause a degranulation of these cells and to release 5-HT (Bulbring and Crema, 1959; Drapanas et al., 1962; Rensnick and Gray, 1962). However, several studies have also demonstrated 5-HT release after vagal or splanchnic nerve stimulation, suggesting neural control may also be an important regulatory factor for the release of 5-HT from these epithelial cells (Ahlman et al., 1976; Burks and Long, 1966; Tansy et al., 1971). More specifically, fluorescence microscopic studies demonstrated adrenergic fibers running close to the enteroendocrine cells. By electron microscopy bundles of unmyelinated nerve fibers were observed near the basal membrane of the cells with the minimal distance between the nerve fibers and the enteroendocrine cells being 150-250 nm.1

1 It must be pointed out that the dimensions of autonomic neuroeffector junctional clefts vary considerably in different organs (Burnstock and Costa, 1975). In the densely innervated vas deferens, the minimum junctional cleft is 15-20 nm. In the longitudinal muscle coat of the intestine the separation is less than 100 nm (Bennett and Rogers, 1967). In the circular muscle coat of the intestine the distance between nerve and muscle is about 20 nm (Rogers and Burnstock, 1966). However, in large elastic arteries, the neuromuscular distances may be as great as 1000 to 2000 nm (Burnstock et al., 1979). Therefore, the distances that have been observed between nerve fibers and intestinal epithelial cells are well within the limits of the known autonomic junctional cleft dimensions. Furthermore, for neuroeffector junctions where membrane separation is greater than 20 nm, no postjunctional specialization is apparent (Burnstock et al., 1979). Thus, it cannot be assumed that the absence of postjunctional specialization on epithelial cells indicates the absence of a neural influence.
Recently (Newson et al., 1979), true synaptic specializations have been identified at the ultrastructural level in the rat ileal mucosa between varicose nerve terminals and intestinal endocrine cells. Furthermore, epinephrine, norepinephrine and isoproterenol decreased the 5-HT content of enterochromaffin cells in a concentration-dependent manner which was blocked by propranolol but not phentolamine or phenoxybenzamine, suggesting a \( \beta \)-adrenoceptor mechanism. Acetylcholine (in the presence of physostigmine) also decreased the 5-HT content of these cells, but was less potent than the adrenergic agents. Finally, transmural field stimulation decreased the 5-HT content of these cells. This effect was antagonized by both tetrodotoxin and propranolol. Thus, there is clear ultrastructural, physiological and pharmacological evidence for the neural control of 5-HT release from intestinal enteroendocrine cells. However, the close proximity of neural elements to the other epithelial cell types in both villi and crypts, suggests that neural activity may also serve to modulate the absorptive and/or secretory activity of these epithelial cells.

EVIDENCE FOR NEUROCHEMICAL RECEPTORS ON INTESTINAL EPITHELIAL CELLS: ROLE IN ION TRANSPORT

The intestinal mucosa is a complex and dynamic structure capable of simultaneously absorbing (lumen-to-blood flux) and secreting (blood-to-lumen flux) water and electrolytes (see Hendrix and Bayless, 1970; Field, 1974; Schultz et al., 1974; Schultz, 1979). Absorption is thought to take place primarily in the villous epithelial cells of the small intestine and in either the surface epithelium of the colon or the upper aspects of colonic crypts. Sodium (the major ion to be discussed) absorption can be considered a two-step process. The first step is passive entry of \( \text{Na}^+ \) across the luminal border of the absorptive cell, driven by a concentration gradient (cell \( \text{Na}^+ \) is low) and a potential difference (cell interior is electronegative). There are at least three modes of passive \( \text{Na}^+ \) entry:

(a) diffusion via an aqueous channel or a cation carrier, (b) cotransport with organic solutes (sugars or neutral amino acids), and (c) cotransport with \( \text{Cl}^- \) and...
possibly other anions. The second step in Na\(^+\) absorption is the active extrusion across the basolateral membrane which is linked to active Na\(^+\)-K\(^+\)-stimulated ATPase. Active Na\(^+\) extrusion tends to increase the osmolality of the solution between adjacent epithelial cells, resulting in osmotic water flow from the intestinal lumen into the intercellular spaces. The solutes cotransported with Na\(^+\) at the luminal border presumably diffuse across the basolateral border.

Secretion of water and electrolytes in the intestine appears to involve the active transport of anion (Cl\(^-\) and/or HCO\(^-\)) and passive movements of Na\(^+\) and water through the secretory crypt cell. In this cell, as opposed to the villous absorptive cell, the cotransport of NaCl occurs at the basolateral membrane. By virtue of its coupling to Na\(^+\) entry into the cell, Cl\(^-\) accumulates intracellularly above its electrochemical equilibrium. Actual secretion of Cl\(^-\) can then be initiated through an increase in the Cl\(^-\) conductance of the luminal border of the secretory cell (this may develop in response to cAMP and/or Ca\(^{++}\)). The Na\(^+\) that entered with Cl\(^-\) recycles to the lateral intercellular space (through the action of the Na\(^+\)-pump). The difference in electrical potential generated in this manner provides the driving force for the movement of Na\(^+\) across the tight junctions into the lumen. Water follows osmotically and thus accumulates in the lumen.

It has become evident in the past two decades that abnormalities in the above mentioned processes of intestinal ion transport can play a role in diarrheal diseases (Binder, 1977; Field, 1971; Phillips, 1972; Phillips and Gagninella, 1977). This is particularly true for certain secretory diarrheas, such as cholera (see Carpenter, 1980; Fishman, 1980; Field et al., 1972; Moss and Vaughan, 1980) and the diarrhea associated with VIP (see below).

Choleragen (cholera toxin), a protein produced by Vibrio cholerae, is the causative agent of cholera. Choleragen elicits its biological effects on Cl\(^-\) and water secretion by binding to specific receptors on intestinal epithelial cells and subsequently activating plasma membrane-bound adenylate cyclase and elevating cAMP levels in the affected mucosal cells. Thus, the precedent has been set for the identification of agents that have direct effects (via specific cell
surface receptors) on intestinal ion transport. The remaining portion of this section will therefore focus on the evidence for neurochemical agents having a role in modulating intestinal ion transport in both health and disease.

**Vasoactive Intestinal Polypeptide (VIP)**

In 1958, Verner and Morrison described the "watery diarrhea syndrome", which is characterized by a non-beta islet cell pancreatic tumor, severe watery diarrhea, hypokalemia, and achlorhydria. A number of hormones secreted by such tumors have been implicated as the causative agent(s), however several lines of evidence have indicated that VIP is primarily responsible for the clinical manifestations of this syndrome (Gaginella and O'Dorisio, 1979).

VIP-like immunoreactivity has been demonstrated in intestinal nerves. In the lamina propria, VIP fibers form an extensive ramifying network (many associated with small blood vessels) with some fibers extending up to the epithelial surface (Schultzberg et al., 1980). The close proximity of VIP nerve fibers to the epithelium suggests a role for VIP in intestinal ion transport. Indeed, VIP increases cAMP levels in the small intestinal mucosa in association with a decrease in net sodium absorption, and a conversion of net chloride absorption to net chloride secretion (Schwartz et al., 1974).

Therefore, from both the pathological and physiological point of view, it seems probable that VIP causes secretion by a direct action on intestinal epithelial cells (Gaginella and O'Dorisio, 1979). Specific binding sites for VIP have been characterized in isolated small intestinal epithelial cells from the rat (Prieto et al., 1979) and guinea pig (Binder et al., 1980) using $^{125}$I-VIP. The binding was of high affinity, saturable ($K_D$ in the nanomolar range), temperature dependent, and reversible. There was also a close correlation between the relative potency for inhibition of binding of $^{125}$I-VIP and for increasing intracellular cAMP (Laburthe et al., 1979a; Binder et al., 1980). The guanine nucleotides, GTP and Gpp(NH)p markedly decreased $^{125}$I-VIP binding (Amiranoff et al., 1980a), and potentiated the action of VIP on adenylate cyclase (Amiranoff et al., 1980b).
in a plasma membrane preparation from rat small intestinal epithelial cells. Furthermore, VIP has been shown to activate cAMP-dependent protein kinase activity in rat intestinal epithelial cells (Laburthe et al., 1979b). Thus, there has accumulated substantial evidence that traces the action of VIP on ion transport to specific receptors on intestinal epithelial cells.

Cholinergic Modulation of Intestinal Ion Transport

As early as 1892, Reid showed that pilocarpine caused the rabbit ileum to secrete fluid in vitro. Early in vivo studies on the cholinergic modulation of intestinal ion transport, established the concept that the parasympathetic nervous system and cholinergic drugs stimulate intestinal secretion (Wright et al., 1940; Florey et al., 1941). The review by Florey et al. (1941) clearly delineated the understanding of the autonomic control of intestinal ion transport as it existed then. The following observations had been made by that time: (1) Vagal stimulation produced a mucoid flow of fluid from the first part of the cat duodenum.
(2) Although vagal stimulation did not consistently cause secretion from the dog and cat jejunum or ileum, cutting the preganglionic sympathetic fibers to the intestine produced secretion which was enhanced by physostigmine and inhibited by atropine. This suggested that there was a sympathetic inhibition of the parasympathetically controlled secretory process. Secretion could also be promoted by local mucosal stimulation of isolated intestinal loops and by cholinergic drugs. (3) Both stimulation of the pelvic parasympathetic innervation and cholinergic drugs produced colonic secretion; both stimulants were inhibited by atropine. Thus, prior to 1941, it was apparent that the autonomic nervous system and cholinergic agents could modulate intestinal ion and water transport.

Somewhat later, bethanechol was shown to induce net secretion of chloride and water in dog jejunal loops (Tidball, 1961), whereas atropine has an opposite effect; it increased water and chloride absorption (Blickenstaff and Lewis, 1952; Tidball and Tidball, 1958). More recently, Hubel (1976; 1977) published similar findings in the rat: inhibition of absorption (jejunum) and promotion of secretion
Caren et al. (1974) demonstrated an atropine and hexamethonium-inhibitable secretory process initiated by luminal distention and tactile stimulation of canine small intestinal loops. Furthermore, cholinergic fibers in cats and humans (Jacobowitz, 1965; Isaacs et al., 1976), have been identified surrounding the crypts and extending into the villi, with some fibers nearly making contact with epithelial cells. These histochemical studies strengthen the argument for cholinergic involvement in intestinal secretion.

In vitro techniques (using mucosal sheets with the external muscle layers removed) for measuring transmural potential difference, short-circuit current, and unidirectional flux of $^{24}$Na and $^{36}$Cl, suggest the involvement of epithelial cell muscarinic receptors in the cholinergically-induced intestinal secretory processes. These techniques allow investigators to divorce the effect of cholinergic drugs on blood supply from an action on intestinal cells, and permit transport studies in the absence of chemical or electrical gradients. Hardcastle and Eggenton (1973) demonstrated that neostigmine in vivo and in vitro enhanced, and atropine antagonized, the increases in the PD across rat jejunum brought about

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2 Both the small intestine and colon generate a transmural potential difference, the serosal side of the tissue being positive with respect to the mucosal side. The electrical activity generated by this tissue reflects net ion movement across the epithelial cells (Barry, 1967) and therefore any modification in ion transport caused by drug addition is observed as a change in the transmural potential difference, provided that the tissue resistance remains constant. An increase in PD indicates net secretion.

3 By utilizing the short-circuit current technique for studying ion transport, Ussing and Zerahn (1951) hypothesized that potential difference changes and electrolyte movement across epithelia were manifestations of the same process, namely, active electrolyte transport. In essence, the short-circuit current establishes a zero potential difference across a membrane that separates identical bathing solutions. Therefore, any ionic current measured is due to active transport (Ussing and Windhager, 1964). An increase in $I_{SC}$ indicates net secretion, while a decrease represents net absorption.
by acetylcholine. This observation was soon confirmed by measurements of electrolyte transport in the human ileum (Isaacs et al., 1976) and rat colon \textit{in vitro} (Browning et al., 1977). In the human ileum, the combination of acetylcholine and neostigmine increased the $I_{SC}$ and stimulated $Cl^-$ secretion. Similar effects were seen in the rat colon. A recent study by Morris and Turnberg (1980), demonstrated that neostigmine provoked secretion of sodium chloride and water \textit{in vivo} in the human jejunum and atropine enhanced absorption in the jejunum and ileum.

Furthermore, by selectively damaging either the villi of the rat jejunum and the surface epithelium of the colon with hypertonic Na$_2$SO$_4$, or the crypts with cycloheximide, Browning et al. (1978) proposed that the crypts may be the site primarily involved in the secretory response to acetylcholine. The fact that the intestinal crypts may represent the locus of the secretory response to acetylcholine supports the work of Trier (1964) in human jejunum. He found that pilocarpine stimulation caused changes in the morphology of undifferentiated crypt cells.

Finally, there appears to be a dual effect of carbachol on electrolyte transport in the rabbit ileum. It has produced both cholinergic (increase in $I_{SC}$) and adrenergic (decrease $I_{SC}$) responses \textit{in vitro} depending upon the concentration used (Tapper et al., 1978; Powell and Tapper, 1979). These authors suggested that their low-dose carbachol effect resulted from activation of muscarinic receptors on the epithelial cells. Although an intense effort has been made to pinpoint the muscarinic response to the epithelial cell, direct evidence that muscarinic receptors exist on epithelial cells has been lacking.

\textbf{Adrenergic Modulation of Intestinal Ion Transport}

In 1859, Claude Bernard observed that removal of the solar ganglia \textit{(i.e. celiac, containing sympathetic nerve cell bodies)} induced the production of fluid in the intestine. Similar observations had been made repeatedly by a number of investigators (see review by Florey et al., 1941), but this effect was not ascribed to sympathetic denervation of the gut until the work by Wright et al.
(1940, see previous section). Thus, early investigations established the concept that the sympathetic nervous system promoted intestinal absorption, or at least inhibited secretion. The early suggestion that sympathetic activity may inhibit secretion is interesting in light of the more recent anatomical studies (Norberg, 1964; Jacobowitz, 1965; Silva et al., 1971, Ahlman et al., 1973) that have revealed only a few sympathetic fibers close to the villous epithelium (the site proposed for absorption), with dense adrenergic nerve endings in the submucosa, especially in the crypt region (the site proposed for secretion). Most of the more recent work on the sympathetic control of intestinal water and electrolyte transport has been done using adrenergic drugs.

In vitro studies with adrenergic agents have upheld the concept that these agents stimulate absorption. This was first shown by Aulsebrook (1965a; 1965b) in rat gut sacs, where acute pretreatment with reserpine (which releases catecholamines from endogenous stores) stimulated Na$^+$ and glucose absorption. Field and McColl (1973) studied the effect of catecholamines on isolated rabbit ileum with the Ussing chamber technique to measure $I_{sc}$ (Ussing and Zerahn, 1951). They demonstrated a marked reduction in the PD and $I_{sc}$, abolition of apparent HCO$_3^-$ secretion, and stimulation of Na$^+$ and Cl$^-$ absorption with epinephrine and norepinephrine. These changes did not occur with isoproterenol, were unaffected by propranolol, and were partially reversed by phentolamine, suggesting that they were alpha-adrenergic in nature. Hubel (1976) studied the effects of norepinephrine in vivo in the rat, and found that it augmented jejunal and ileal absorption of Na$^+$, Cl$^-$ and water. Similar effects of adrenergic agents on intestinal absorption have also been demonstrated in the rat (Racusen and Binder, 1979) and rabbit (Lennane et al., 1975; Albin and Gutman, 1980) colon. However, it is less clear that the effect is purely alpha-adrenergic in the colon. For example, in the rat colon (Racusen and Binder, 1979), epinephrine, norepinephrine and isoproterenol all decreased $I_{sc}$ and PD. The authors suggested that their studies with alpha- and beta-adrenergic antagonists, tetrodotoxin and
reserpine, indicated that the adrenergic agonists were interacting directly with mucosal epithelial cells and not via intermediate neurochemical agents. Both alpha- and beta- receptors influence rabbit colonic PD in vivo (Lennane et al., 1975). In the rabbit colon in vitro, addition of phenylephrine resulted in net Na⁺ absorption which was abolished by phentolamine (Albin and Gutman, 1980). Furthermore, Brunsson et al. (1979) observed that stimulating the sympathetic fibers to the cat small intestine increased net water absorption independently of blood flow. These studies suggest the possible involvement of adrenergic receptors on intestinal epithelial cells in the modulation of water and electrolyte absorption.

Finally, based on the effects of carbachol on electrolyte transport in vitro, the classical concept of the autonomic control of intestinal electrolyte transport may not be as simple as many of the initial studies would suggest (Tapper et al., 1978; Powell and Tapper, 1979; Tapper and Lewand, 1981). Carbachol at 1 x 10⁻⁸ M briefly increased the Lsc while stimulating net Cl⁻ secretion; this effect was inhibited by atropine (1 x 10⁻⁶ M), suggesting involvement of muscarinic receptors. High concentrations of carbachol (1 x 10⁻³ M) depressed Lsc and stimulated NaCl absorption. This effect was not blocked by 1 x 10⁻⁶ M atropine, but was blocked by hexamethonium (1 x 10⁻⁵ M). This nicotinic response is identical to that of alpha-adrenergic agents (see above) and was inhibited by phentolamine (1 x 10⁻⁷ M). Furthermore, DMPP at 1 x 10⁻⁵ M, depressed Lsc and increased NaCl absorption. This effect was antagonized by hexamethonium and phentolamine. Thus, Tapper and Powell's findings suggest that nicotinic receptor agonists may cause intestinal absorption by activating presynaptic nicotinic receptors on adrenergic nerves, thereby causing release of norepinephrine which then acts on alpha-adrenergic receptors on mucosal epithelial cells. Proof for this suggestion awaits the demonstration of adrenergic receptors on the epithelial cells.
Opiates and Ion Transport

Opiates have long been known for their powerful actions on the gastrointestinal tract. Opium has been used from antiquity for the relief of diarrhea and dysentery. The constipating actions of narcotic analgesics, such as morphine, have also been a subject of investigation for many years. In most animals, including man, morphine inhibits gastric emptying, increases smooth muscle tone, inhibits propulsion of intestinal contents, decreases gastric and pancreatic secretions, and inhibits bile flow (Jaffe and Martin, 1975). The antidiarrheal activity of the opiates is presently explained by increased contractile activity of the circular muscle and inhibition of the propulsive activity in the intestinal tract (Vaughan-Williams, 1954; Daniel et al., 1959; Bass, 1968; Konturek, 1978; Konturek, 1980).

However, it is now widely accepted that an important pathophysiological mechanism of diarrhea involves abnormalities of intestinal water and electrolyte transport (Phillips, 1972; Phillips and Gaginella, 1977; Phillips and Gaginella, 1979; Gaginella and O'Dorisio, 1979). Therefore, the remaining discussion will focus on the effects of opiates on ion transport.

Coupar (1978) demonstrated that morphine could inhibit prostaglandin E_1-stimulated fluid secretion by the rat jejunum. Karim and Adiakan (1977) found a similar effect with loperamide after administration of prostaglandin E_2 in the rat. If has also been shown in the rat that morphine, levorphanol (but not its stereoisomer, dextrophan), and loperamide inhibited prostaglandin E_1-, VIP-, and bisacodyl-induced secretion (Beubler and Lembeck, 1979; Beubler and Lembeck, 1980; Lee and Coupar, 1980). However, these in vivo studies did not differentiate between an opiate effect on motility, blood flow or electrolyte transport by enterocytes. In fact, Mailman (1980) showed that morphine increased net absorption in dogs by altering intestinal blood flow. The effects of morphine on ion transport have been studied in vitro using the short-circuit current technique (Dobbins et al., 1980; Kachur et al., 1980; McKay et al., 1981). As assessed by this technique, measured changes in electrolyte transport cannot occur through
alterations in smooth muscle activity (serosa and external muscle layers are removed, leaving only the mucosa and submucosa) or alterations in blood flow. In general, these authors found enhanced Na\(^+\) and Cl\(^-\) absorption with a decrease in \(I_{sc}\) and PD upon addition of morphine, codeine, stereospecific synthetic opiate analogues, and enkephalin analogues, both in rabbit (Dobbins et al., 1980; McKay et al., 1981) and guinea pig (Kachur et al., 1980) ileum. All opioid effects could be reversed by naloxone.

While these in vivo and in vitro studies suggest an opiate involvement in intestinal ion transport, it is not clear where in the mucosa the opiates might be acting. The effect could be directly on the enterocyte or, alternatively, on mucosal nerves. Lack of inhibition of the opiate effect by atropine, hexamethonium, propranolol, phentolamine, chemical sympathectomy with 6-hydroxydopamine, or by haloperidol, tend to rule out involvement of the cholinergic, adrenergic or dopaminergic systems (Dobbins et al., 1980; McKay et al., 1981). Dobbins et al. (1980) also demonstrated apparent inhibition of the opiate effect with tetrodotoxin, a response which might incriminate a neural component in the pharmacologic effect. However, studies by Hubel (1978) suggest that tetrodotoxin might have an effect on the enterocyte in addition to inhibiting neural activity. Therefore, the site of opiate action in the mucosa remains unclarified.
STATEMENT OF THE PROBLEM

The neuroregulation of mucosal transport of water and electrolytes is now recognized as an important factor in intestinal epithelial function. Many neurochemicals alter intestinal ion transport both in vivo and in vitro. From the preceding review, it should be evident that acetylcholine may play an important role in intestinal secretion. Although information regarding the precise site(s) of action of cholinergic agents is lacking, many studies have suggested that these agents act by interacting with muscarinic receptors on intestinal epithelial cells. Since the intestinal mucosa is heterogenous, being composed of connective tissue, blood and lymph vessels, nerves and epithelial (absorptive and secretory) cells, studies employing whole mucosa do not allow one to separate effects on a given component of this tissue. One can only be sure of the presence of muscarinic receptors on the epithelial cells by separating these cells from the other components of the mucosa.

Quinuclidinyl benzilate (QNB) is a potent ligand that has been widely used to identify muscarinic receptors in a variety of tissues. In the present study, intestinal epithelial cells will be isolated and used in binding experiments to determine if muscarinic receptors exist on these cells. In addition, since norepinephrine and opiates enhance intestinal absorption, receptors for these agents will also be explored.

Specific aims include: (1) the isolation of rat ileal and colonic intestinal epithelial cells, from the other components of the mucosa, by a vibration technique, (2) the preparation of a mucus-free plasma membrane fraction of isolated intestinal epithelial cells, (3) use of the potent muscarinic receptor antagonist, [3H]QNB in in vitro ligand binding studies to epithelial cell membrane fractions in an effort to quantitate muscarinic receptor affinity and density, (4) and the use of [3H]QNB in an in vivo ligand binding study to whole rat intestinal epithelial cells. Similar experiments will be performed in an attempt to quantitate opiate and alpha-adrenergic receptors in these cells.
CHAPTER II

IN VITRO AND IN VIVO IDENTIFICATION OF MUSCARINIC RECEPTORS ON RAT ILEAL AND COLONIC EPITHELIAL CELLS:
BINDING OF [$^{3}$H]-QUINUCLIDINYL BENZILATE

[$^{3}$H]-QNB has been widely used as a ligand to identify muscarinic receptors in various tissues including brain (Yamamura and Snyder, 1974), heart (Fields et al., 1978; Wei and Sulakhe, 1979), and smooth muscle (Rimele et al., 1979). However, contrary to binding in these tissues, crude suspensions of intestinal epithelial cells contain mucus. In a preliminary study (see STUDY 1) it was observed that mucus binds [$^{3}$H]-QNB in a displaceable fashion (atropine inhibited [$^{3}$H]-QNB binding). Because of the similarities in the binding characteristics of [$^{3}$H]-QNB to mucus and epithelial cells, we felt it necessary to first study mucus binding relative to that in a tissue known to contain muscarinic receptors and to be free of mucus. Information obtained from this study is described under STUDY 1 and was used to design the subsequent in vitro (STUDY 2) and in vivo (STUDY 3) binding experiments.

STUDY 1
BINDING OF [$^{3}$H]-QNB TO INTESTINAL MUCUS: AN ARTIFACT IN THE IDENTIFICATION OF EPITHELIAL CELL MUSCARINIC RECEPTORS

Materials and Methods

Preparation of Tissue for Binding Studies

The following procedure, modified from Gaginella et al. (1977), was followed for isolation of ileal and colonic epithelial cells by the vibration
technique (for additional details see Figure 2, Plate I and Materials and Methods of STUDY 2). Male Sprague-Dawley rats (4 per experiment), obtained from Laboratory Supply Co., Indianapolis, Indiana weighing between 200-350g were sacrificed by a sharp blow to the head. Immediately after sacrifice, 30 cm of distal ileum or the entire colon was resected, cleaned of fat and mesentery and luminal contents flushed three times with 10 ml of 0.9% (w/v) sodium chloride (normal saline) at room temperature. Segments were allowed to stand in normal saline at room temperature for 10 min and once again flushed before being everted over an aluminum vibrating coil (rod diameter 0.5 cm). Individual segments were secured on the coils by tying both ends with silk sutures. The rods with the tissues were placed in 200 ml of isolation buffer in a plastic container for 10 minutes. The isolation buffer (pH 7.4 at 22°C) had the following composition (millimolar): NaCl, 115.5; KCl, 4.6; NaH₂PO₄, 1.2; NaHCO₃, 21.9; MgSO₄, 1.2; glucose, 11.5; EDTA, 5. The everted segments were then vibrated at high frequency and low amplitude for 30 min (22°C) in 250 ml of fresh isolation buffer with a Vibro-Mixer, Model E-1 (Chemapac, Inc., Hoboken, N.J.) to harvest cells. The cell suspension was filtered through a nylon mesh (250 µm) to trap mucus and subsequently centrifuged at 1,500 xg for 5 min (22°C) to sediment the isolated cells. Cells were homogenized on ice for 20 sec in the isolation buffer with a Model PT-10 Brinkmann Polytron, (Westbury, N.Y.) at setting 7. This cellular homogenate was then used in the binding assay.

Intestinal mucus was isolated in the following manner. Male rats (200-350g; 4 per experiment), were killed by a sharp blow to the head and three intestinal segments from each rat (25 cm in length) were resected, cleaned of fat and mesentery, and the luminal contents flushed with normal saline as described above. Segments were everted using a glass rod (diameter, 0.5 cm) and both ends were tied with silk sutures (to prevent contamination by serosal tissue) and placed in 350 ml of normal saline at 37°C for 30 min. The segments were removed, discarded, and the mucus that was secreted into the medium was
Figure 2. Intestinal epithelial cell isolation by the vibration technique. Illustrated is an everted intestinal segment secured on a coil being vibrated at high frequency and low amplitude for 30 min at 22°C in an isolation buffer containing 5 mM EDTA (calcium ions are important in maintaining cell cohesion). Also shown is an electron micrograph of several isolated cells (MV, microvilli; G, goblet cell; magnification is approximately 8,000 X).
Figure 2
Plate I. Light micrographs of rat ileum and colon before and after epithelial cell isolation by the vibration technique. A. Ileal section before vibration. B. Ileal section after vibration. C. Colonic section before vibration. D. Colonic section after vibration (V, villi; CR, crypt; Ep, colonic surface epithelium; g, goblet cell). Note the removal of the epithelial cells from the small intestinal villi and colonic surface epithelium by the vibration technique.
collected by centrifugation at 3,000 xg for 5 min (22°C). Pellets were homogenized (in the appropriate buffer) for 20 sec (Polytron at setting 7) and used in the binding assay. Therefore, mucus is defined as the viscous secretion obtained from intestinal segments, which is collectable by centrifugation after a 30 min incubation in normal saline. These secretions of the small intestinal and colonic goblet cells, are composed principally of high molecular weight glycoproteins (mucins), ions and water (Forstner, et al., 1973). The pellet may also contain denuded epithelial cells, cellular debris, and bacteria (Forstner et al., 1973).

To obtain an estimate of the amount of mucus in the homogenates used for the binding assay, mucin content was determined by radioimmunoassay (Forstner et al., 1977) specific for rat goblet cell mucin (kindly performed by Janet Forstner). Binding was also measured to mucin, and rat heart which was used as a control tissue known to be mucus-free and to contain muscarinic receptors (Fields et al., 1978; Wei and Sulakhe, 1979).

Heart tissue from male rats (200-350g) was prepared for the binding assay in the following manner. The heart was removed, blotted free of blood, minced and homogenized in ice cold buffer (0.25 M sucrose, 50 mM tris (hydroxymethyl) aminomethane (Tris), pH 7.4 at 37°C) with a Polytron at setting 5. The homogenate was filtered through three layers of cheesecloth and centrifuged at 30,000 xg for 15 min at 4°C in a Sorvall centrifuge (Model RC 2-B). The pellet was collected and washed once in 50 mM Tris buffer (pH 7.4 at 37°C) and used for binding experiments.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

[^H]-Quinuclidinyl Benzilate Binding

In general, tissue was added to 2 ml polystyrene cups (Kew Scientific, Columbus, Ohio) and incubated at 37°C for 30-40 min in a Dubnoff incubator with constant shaking (100 cycles/min), with 0.5 to 1.5 nM[^H]-QNB (12.0 or 29.4 Ci/mmol) and specific drugs(where indicated) in a total volume
of 1.5 ml of 50 mM Tris buffer (pH 7.4 at 37°C). After incubation, samples were filtered under vacuum through Tris-prewetted Whatman GF/B glass fiber filters. The filters were washed two times with 5 ml of ice-cold buffer (total filtration time 10-20 sec), placed into scintillation vials and allowed to air dry. To each vial, 0.5 ml of tissue solubilizer (Protosol, New England Nuclear) was added and the vials were capped tightly and placed in a Dubnoff incubator at 50°C for 30 min. The samples were then allowed to cool to room temperature, at which time 50 μl of glacial acetic acid and 10 ml of Thrift Solve (Kew Scientific) were added. The vials were then mechanically shaken for at least 1 hour on an Eberbach shaker (240 cycles/min) and subsequently counted in a Beckman LS-345 liquid scintillation counter. Appropriate corrections were made for quench which was monitored by an automatic external standard. Counting efficiencies for tritium were routinely between 35 and 40%.

[^{3}H]-QNB bound in the presence of 1 X 10^-5 M atropine was defined as nonspecific binding. Nonspecific binding was subtracted from total binding to obtain "specific" binding. IC50* is defined as the molar concentration of drug which inhibited total [^3]H]-QNB binding to tissue by 50%. IC50* values were determined from a log-probit plot, using four to five different concentrations of the inhibitor. Binding to the filters ( ~ 1% of total radioactivity added) accounted for most of the nonspecific binding when [^3]H]-QNB was present at ~ 0.5 nM. There was no detectable binding to the plastic incubation cups.

Sources of Drugs

DL-[benzilic-3-[^3]H]quinuclidinyl benzilate (12.0 Ci/mmol) was obtained from Amersham and DL-[benzilic-4,4'-[^3]H] quinuclidinyl benzilate (29.4 Ci/mmol) was obtained from New England Nuclear. The radiochemical purity of [^3]H]-QNB was routinely checked by thin layer chromatography using silica gel-GF plates (New England Nuclear), and a solvent system of chloroform:acetone:diethylamine (60:30:10). The radiochemical purity was greater than 95%.
Other drugs were obtained as indicated: atropine sulfate, bovine albumin (Fraction V), hexamethonium bromide, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulfate, mucin (crude, type 11 from porcine stomach), and Trizma HC1, Trizma base (Sigma Chemical Company). Unlabeled (+)-QNB HC1 was kindly provided by Hoffman-LaRoche, Inc.

Results

Estimation of Mucus Content

Initial experiments were designed to obtain an estimate of the relative amount of mucus contained in the isolated epithelial cell and mucus pellets. When samples were assayed for goblet cell mucin by radioimmunoassay (Forstner et al., 1977) twice as much mucin (per μg protein) was found in the mucus as compared to the cellular pellet (13.4 vs. 7.2 ng mucin/μg protein). There was no measurable mucin in tissues (e.g., skeletal muscle) devoid of mucus.

Characteristics of [3H]-QNB Binding to Ileal and Colonic Epithelial Cells and Mucus

Preliminary experiments were designed to test the ability of atropine to inhibit [3H]-QNB binding to a homogenate of isolated colonic epithelial cells. Atropine inhibited the binding of [3H]-QNB in a concentration dependent manner (Figure 3), reaching maximum inhibition at 5 x 10^{-6} M to 1 x 10^{-5} M, with an IC50 of 1.4 x 10^{-6} M. Similar results were obtained for ileal cells (data not shown). Surprisingly, atropine also inhibited [3H]-QNB binding to mucus. However, the curve for binding to mucus was more shallow and was shifted to the right; the IC50 was 2.1 x 10^{-7} M, about 15-fold greater than for the cell homogenate (Figure 3).

We chose to compare binding of [3H]-QNB to mucus and heart muscle (as a "positive" control), in an attempt to elucidate the differences and/or similarities in the characteristics of the binding. Both heart and mucus bound [3H]-QNB, as a function of protein concentration, in a similar manner (Figure 4).
Figure 3. Inhibition of [3H]-QNB binding by atropine. Incubations were performed at 37°C for 35 min in the isolation buffer described in Materials and Methods. [3H]-QNB was present at 1.0nM with 400-500µg protein. A. Colonic epithelial cells isolated by vibration. Points represent total [3H]-QNB binding minus filter binding and are the mean ± S.E. from three experiments each in triplicate. An IC50 of 1.4 X 10^{-8}M for atropine was obtained for binding to isolated cells. B. Intestinal mucus. Data are from a representative experiment performed in duplicate. Control indicates incubations without atropine and filter refers to filter binding. An IC50 of 2.1 X 10^{-7}M for atropine was obtained for binding to mucus.
Figure 3
Figure 4. Effect of protein concentration on $[^3H]QNB$ binding. Incubations were performed at $37^\circ\text{C}$ for 35 min in Tris buffer as described in Materials and Methods. A. Total binding to heart. Data are from two experiments each in triplicate. B. Total binding to mucus. Data are from two experiments each in triplicate.
Figure 4
Unlabeled QNB inhibited the binding of [3H]-QNB to heart in a concentration-
derpendent manner (Figure 5), reaching maximum inhibition at about $1 \times 10^{-7}$ M with an IC50* of $1.2 \times 10^{-9}$ M. In contrast, a flatter curve was obtained for binding to mucus, with an apparent IC50* that was 300-fold higher at $4.0 \times 10^{-7}$ M (Figure 5).

"Specific" binding to mucus, operationally defined as the difference between total and nonspecific binding (in the presence of $1 \times 10^{-5}$ M atropine), although inhibitable by atropine was not saturable over the concentration range of [3H]-QNB tested (Figure 6). On the other hand, specific binding to heart approached saturation at a [3H]-QNB concentration of about 2 nM, with half-maximal saturation occurring at $\sim 0.5$ nM (Figure 6). The apparent dissociation constant ($K_D$) and maximum number of binding sites ($B_{max}$) obtained from a Scatchard plot of specifically bound [3H]-QNB for heart were 0.76 nM and 136 fmol/mg protein, respectively.

### Characteristics of [3H]-QNB Binding to Mucin

Mucus secretions of the intestine are composed principally of high molecular weight glycoproteins known as mucins. We therefore measured [3H]-QNB binding to isolated mucin in an effort to determine if binding to this component could account for the observed binding to mucus. [3H]-QNB bound to mucin in a linear fashion (Figure 7). However, much more mucin (per mg protein) was required to bind the same amount of ligand as compared to heart and mucus (see Figure 4). The [3H]-QNB binding to mucin was not inhibited by atropine (Figure 7). Furthermore, three non-muscarinic drugs had no effect on [3H]-QNB binding to mucin (Table 1). A similar pattern for non-muscarinic agents was seen for heart and mucus (Table 1).

### Effect of pH and Temperature on [3H]-QNB Binding

Binding was also measured as a function of pH and temperature. There was a broad pH optimum for binding to the heart (Figure 8), with specific binding remaining relatively stable between pH 5.5 and 7.5. In contrast, binding to mucus...
and mucin exhibited a pH optimum of 6.5 (Figure 8). At their pH optima specific binding to heart, mucus and mucin were 70%, 30%, and 0% of total binding, respectively.

Binding of [3H]-QNB to heart was temperature dependent; both total and specific binding increased when tissue was incubated at 0, 25, and 37°C. Specific binding increased from 33% at 0°C to 40% at 25°C and 78% at 37°C. Nonspecific binding stayed relatively constant. When tissue from heart was preincubated for 10 min at 90°C, specific binding was completely abolished and nonspecific binding increased. "Specific" binding to mucus remained at 40% of total binding, and both total and nonspecific binding decreased, from 0 to 37°C. When mucus was preincubated at 90°C "specific" binding was abolished but total binding was twice that at 37°C. Total and nonspecific binding to mucin were identical (no specific binding) and progressively decreased when incubated at 0, 25, 37 and 90°C, respectively.

STUDY 2

IN VITRO BINDING OF [3H]-QNB TO RAT INTESTINAL EPITHELIAL CELLS

Materials and Methods

Isolation of Epithelial Cells (Enterocytes) and Preparation of Membrane Fractions

Male Sprague-Dawley rats (4 per experiment) obtained from Laboratory Supply, Indianapolis, Ind., weighing between 200-300 g were killed by a sharp blow to the head. Immediately after sacrifice, a segment of proximal jejunum (20 cm), or distal ileum (30 cm) or the entire colon was resected, cleaned of fat and mesentery, and luminal contents flushed three times with 10 ml normal saline at room temperature. Intestinal segments were allowed to stand in normal saline at room temperature for 10 min and once again flushed before being everted over an aluminum vibrating coil (rod diameter 0.5 cm). Individual segments were
Figure 5. Inhibition of \(^3\text{H}\)-QNB binding by QNB. Incubations were performed at 37°C for 35 min in Tris buffer as described in Materials and Methods. \(^3\text{H}\)-QNB was present at \(\sim 1.0 \text{ nM with } \nu 400-600 \mu\text{g protein. Points represent total } \(^3\text{H}\)-QNB binding minus filter binding and are the mean ± S.E. from three experiments each in duplicate. For each experiment, heart and mucus were isolated from the same rat and binding assays performed simultaneously. IC50's of 1.2 \times 10^{-9}\text{M and } 4.0 \times 10^{-7}\text{M were obtained for QNB in heart and mucus, respectively.}
IC_{50}^{+} (M)

HEART: 1.2 \times 10^{-9}
MUCUS: 4.0 \times 10^{-7}

Figure 5
Figure 6. Binding as a function of increasing concentrations of [³H]-QNB. Tissue homogenates were incubated, as described in Materials and Methods, with various concentrations of [³H]-QNB. Nonspecific binding was measured in the presence of 1 X 10⁻⁵ M atropine. A. Binding to heart. Data are from a representative experiment performed in triplicate. Specific binding approached saturation at a [³H]-QNB concentration of about 2 nM; half maximal saturation occurred at about 0.5 nM. The apparent dissociation constant (K_D) and maximum number of binding sites (B_max) obtained from a Scatchard plot of specifically bound [³H]-QNB were 0.76 nM and 136 fmol/mg protein, respectively. B. Binding to mucus. "Specific" binding to mucus, operationally defined as the difference between total and nonspecific binding, although displaceable by atropine was not saturable.
Figure 6

\[ \text{Specific} \quad \text{Non-Specific} \quad \text{Total} \]

\[ [\text{H}_2\text{O}] \]

\[ [\text{H}_3\text{O}] \]

\[ \text{[H}^3\text{O}] - \text{QNB bound (dpm x 10^3)} \]
Figure 7. $^3$H-QNB binding to mucin. Incubations were performed at 37°C for 35 min in Tris buffer as described in Materials and Methods. $^3$H-QNB was present at ~1.0 nM. A. Effect of protein concentration on $^3$H-QNB binding. Points represent total minus filter binding and are the mean ± S.E. from three experiments each in duplicate.

B. $^3$H-QNB binding to mucin as a function of atropine concentration. Data represent the mean ± S.E. from three experiments each in triplicate or duplicate. Filter binding has been subtracted. Binding to the heart is presented for comparison.
Figure 7
Figure 8. Effect of pH on $[^3H]$-QNB binding to heart, mucus and mucin. Incubations were performed at 37°C for 35 min in Tris buffer. Data are from representative experiments each performed in triplicate.

A. Binding to heart. B. Binding to mucus. C. Binding to mucin. Nonspecific binding was measured in the presence of $1 \times 10^{-5}$M atropine. The difference between total and nonspecific binding is defined as "specific" binding.
Figure 8
TABLE 1

Effect of Drugs on Total Binding of $[^3\text{H}]$-QNB

<table>
<thead>
<tr>
<th>Drug</th>
<th>$[^3\text{H}]$-QNB Bound (% of Total Binding)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td>Atropine</td>
<td>27.9 ± 3.1</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
<td>98.4 ± 5.9</td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>89.2 ± 2.9</td>
</tr>
<tr>
<td>Histamine</td>
<td>94.1 ± 5.4</td>
</tr>
</tbody>
</table>

\[ ^a \text{ Incubations were performed for 35 min at 37°C with }[^3\text{H}]\text{-QNB present at } \approx 1.0 \text{ nM.} \]
\[ ^b \text{ Drugs were present at } 1 \times 10^{-5} \text{M.} \]
\[ ^c \text{ Values represent means } \pm \text{ S. E. for three experiments each in triplicate.} \]
secured on the coils by tying both ends with silk sutures, placed in 200 ml of isolation buffer in a plastic container, and allowed to soak for 10 min. The isolation buffer (pH 7.4 at 22°C) had the following composition (millimolar): NaCl, 150; K2HPO4, 3; EDTA, 5; sucrose, 10; and Tris (hydroxymethyl aminomethane (Tris), 5. The everted intestinal segments were then vibrated at high frequency and low amplitude for 30 min (22°C) in 250 ml of fresh isolation buffer using a Vibro Mixer, Model E-1 (Chemapest, Inc., Hoboken, N.J.) to harvest cells. The cell suspension was filtered through a nylon mesh (250 µm) to trap mucus and subsequently centrifuged at 1,500 xg for 5 min (22°C) to sediment the isolated cells. Figure 2 is a schematic of the vibrating coil and Plate I shows the appearance of segments of rat ileum and colon before and after epithelial cell isolation by the vibration technique.

A modification of the method of Murer et al. (1976) was used to prepare cellular fractions from the isolated cells. Cells were homogenized on ice for 20 sec in 50 ml of buffer (0.25 M sucrose, 0.01 M triethanolamine HC1, 0.5 mM EDTA, pH 7.5, at 22°C) with a Brinkmann Polytron, Model PT-10 (Westbury, N.Y.) at setting 7 and further disrupted in a Potter-Elvehjem glass-Teflon homogenizer (clearance 0.095-0.115 mm) attached to an overhead stirrer (Wheaton Instruments, Millville, N.J.) at setting 5 (∆1200 rev./min) for 25 strokes. Homogenates were centrifuged at 2,600 xg for 15 min with a Sorvall Centrifuge (Model RC 2-B) at 4°C. The pellet was discarded and the supernatant centrifuged at 30,000 xg for 20 min. The pellet from this spin was collected and washed once in 50 mM Tris buffer (pH 7.5 at 37°C) and used for all binding experiments except for those described below involving subcellular localization of binding. Goblet cell mucus contaminates crude homogenates and interferes with [3H]-QNB binding (see STUDY 1). However, the final pellet obtained as described above was mucus free (<1% contamination) as determined by radioimmunoassay specific for rat goblet cell mucin (kindly performed by Dr. Janet Forstner; Forstner et al., 1977).
The following procedure was followed for the subcellular localization experiment. An aliquot of isolated cells, harvested as described above, was homogenized once for 45 sec in 50 mM Tris, with a Polytron at setting 7. The suspension was then centrifuged at 30,000 xg for 20 min; this fraction was defined as the total particulate fraction. The remaining cells were homogenized in 50 ml of buffer (0.25 M sucrose, 0.01 M triethanolamine HCl, 0.5 mM EDTA) with a Polytron and glass-Teflon homogenizer (see above) and centrifuged at 2,600 xg for 15 min. The resultant pellet (Pellet 1) was defined as the nuclear fraction and was saved for subsequent binding experiments. The supernatant (Supernatant 1) was then centrifuged at 10,000 xg for 20 min. The white, fluffy top layer of Pellet 2 was resuspended in Supernatant 2, rehomogenized and centrifuged at 30,000 xg for 20 min. The lower layer of Pellet 2 (mitochondrial fraction) was saved. The white fluffy top layer of Pellet 3 was resuspended in 50 mM of Tris buffer, rehomogenized and centrifuged at 30,000 xg for 10 min. The resultant pellet was collected and defined as the plasma membrane fraction. All final pellets were washed at least once with 50 mM Tris buffer before being adjusted to volume with Tris for the binding assay. The incubation buffer (50 mM Tris) is hypotonic, therefore any membrane vesicles formed during the isolation were assumed to be lysed during the binding assay and [³H]-QNB measured was assumed to be tissue bound and not a reflection of vesicular uptake.

Binding was also measured in rat intestinal smooth muscle. The procedure for isolating intestinal muscle homogenates was as follows: Segments of jejunum, ileum, cecum and colon were removed, flushed of luminal contents, everted and the mucosa and serosa scraped off with a glass slide. The remaining muscle was blotted dry and homogenized with a Polytron in 50 mM Tris buffer at setting 7 for 1 min on ice. The homogenate was then centrifuged at 4°C for 15 min at 40,000 xg. The resulting pellet was resuspended in 50 mM Tris buffer and used in the binding assay.
Protein was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard in the protein assay.

[^3H]-Quinuclidinyl Benzilate Binding

In general, membrane fractions (500 μL, containing 100-400 μg protein) were added to 2 ml polystyrene cups (Kew Scientific, Columbus, Ohio) and incubated at 37°C for 45 min in a Dubnoff incubator with constant shaking (100 cycles/min), with 0.5 nM[^3H](-)QNB (40.2 Ci/mmol) and specific drugs (where indicated) in a total volume of 1.5 ml of 50 mM Tris buffer (pH 7.4 at 37°C). After incubation, samples were filtered under vacuum through Tris-prewetted Whatman GF/B glass fiber filters. The filters were washed two times with 5 ml of ice-cold Tris buffer (total filtration time 10-20 sec), placed into scintillation vials and allowed to air dry. To each vial, 0.5 ml of tissue solubilizer (Protosol, New England Nuclear) was added, and the vials were capped tightly and shaken (50 cycles/min) in a Dubnoff incubator at 50°C for 30 min. The samples were then allowed to cool to room temperature, at which time 50 μL of glacial acetic acid and 10 ml of Thrift-Solve (Kew Scientific) were added. The vials were then mechanically shaken for at least 1 hr on an Eberbach shaker (240 cycles/min) and subsequently counted in a Beckman LS-345 liquid scintillation counter. Appropriate corrections were made for quench which was monitored by an automatic external standard. Counting efficiencies for tritium were routinely between 35 and 40%.

Radioactivity bound to tissue and filters in the presence of 1 X 10^{-7} M (+)QNB was defined a nonspecific binding. Nonspecific binding was subtracted from total binding to tissue and filters to obtain specific binding. Typically, total binding of[^3H](-)QNB (0.2-0.5 nM) was about 2000 dpm, 65-75% of which was specific. At higher concentrations of[^3H](-)QNB the percentage of specific binding decreased (for example, at 1.0-1.5 nM[^3H](-)QNB specific binding was 50% of total binding). Binding to the filters (less than 1% of total radioactivity added) accounted for most of the nonspecific binding when[^3H](-)QNB was present at 0.5 nM. Conditions were used to limit total binding to less than 5% of the radioactivity added to each incubation so that the concentration of free ligand did not change appreciably during the binding assay. There was no detectable
binding to the plastic incubation cups.

To assess the stability of $[^3H](-)QNB$ throughout the experiments, binding was performed in the usual manner except only one tube with a final incubation volume of 10 ml was used. The membranes were then collected by centrifugation at 30,000 xg for 15 min at $4^\circ C$. Radioactivity from the supernatant and that extracted from the pellet (which was homogenized in 5 ml of 0.2% acetic acid in 95% ethanol, left at ambient temperature for 30 min and centrifuged at 30,000 xg for 15 min at $4^\circ C$) was chromatographed on thin layer plates (silica gel-GF, New England Nuclear) in the solvent system chloroform:acetone:diethylamine (60:30:10). Both the bound and free $[^3H](-)QNB$ displayed $R_f$'s identical to authentic $[^3H](-)QNB$, as well as unlabeled QNB.

Assays for Subcellular Makers

--- **Na**$^+\text{-K}^+$-ATPase.** ATPase activity was measured by the method of Quigley and Gottler (1969). Briefly, enzyme activity was measured in the presence of $Mg^{2+}$, $Na^+$ and $K^+$ (total ATPase), and in the presence of only $Mg^{2+}$($Mg^{2+}$-ATPase). The $Na^+\text{-K}^+$-stimulated ATPase was calculated by subtracting $Mg^{2+}$-ATPase from the total ATPase. For assay of total ATPase the media contained the following: 30 mM Tris (pH 7.1), 5 mM ATP, as the disodium salt (neutralized with Tris to pH 7.1), 7.5 mM MgCl$_2$, 120 mM NaCl, and 20 mM KCl. $Mg^{2+}$-ATPase was measured in a system containing 30 mM Tris (pH 7.1), 5 mM ATP (pH 7.1) and 7.5 mM MgCl$_2$. Incubations were carried out for 10 min at $37^\circ C$ in a total volume of 1.0 ml. The reaction was terminated by addition of 1.0 ml of 12% (w/v) trichloroacetic acid. After centrifugation (10,000 xg, $4^\circ C$) the amount of inorganic phosphate released was measured by the method of Taussky and Shorr (1953). After corrections using the appropriate blanks, specific activities were expressed as $\mu$moles of inorganic phosphate released/mg protein/hr.

--- **Adenylate Cyclase.** Adenylate cyclase activity was determined as previously described (O'Dorisio et al., 1979). Briefly, the reaction was started by addition of 20 $\mu$L of membrane homogenate (25-100 $\mu$g protein) to a medium
containing 1 mM ATP, 2 mM MgCl₂, 10 mM theophylline, 0.4% bovine albumin, 3 mM phosphocreatine, 3 U creatine phosphokinase, 0.1 mM dithiothreitol, and 100 mM Tris buffer (pH 7.7 at 25°C) in a total volume of 0.1 ml. Assays were conducted for 20 min at 37°C. The reaction was stopped by the addition of 0.1 ml of cold 0.1 M sodium acetate, pH 4, followed by heating for 3 min at 90°C. Cyclic AMP generated was succinylated prior to determination by radioimmunoassay. Antisera to succinyl cAMP was prepared in the laboratory of Dr. M. S. O'Dorisio. Generation of cAMP was linear over the range of 25-100 μg protein for time periods up to 30 min.

DNA. After digestion of RNA in 1.0 N KOH, DNA was measured by the ethidium bromide technique (Blackburn et al., 1973). Calf thymus DNA was used as the standard in the DNA assay.

Electron Microscopy.

Samples of the various subcellular fractions were fixed in 3% glutaraldehyde buffered with 0.2 M Sorensen's phosphate buffer (pH 7.2) at room temperature for 1 hr and then washed several times in phosphate buffer before being post-fixed in 1% osmium tetroxide. Samples were then embedded in 3% agar to form tissue blocks and dehydrated in a graded series of ethanol (30-100%) for 10 min each. Samples were then embedded in Epon, sectioned and mounted on uncoated copper grids, stained with uranyl acetate and lead citrate and examined with a Zeiss EM 95-2 electron microscope.

Sources of Drugs.

L-[benzilic-4,4'-3H] quinuclidinyl benzilate (mol. wt. 337.4, 40.2 Ci/mmol) was obtained from New England Nuclear. The radiochemical purity of [3H]QNB was routinely checked by thin layer chromatography using silica gel-GF plates (New England Nuclear), and a solvent system of chloroform: acetone: diethylamine (60:30:10). The percent radiochemical purity was found to be never less than 95%.
Other drugs were obtained and used in the forms indicated: acetylcholine chloride, neostigmine bromide, bovine albumin (Fraction V), yohimbine HCl, adenosine 5'-triphosphate (disodium salt), atropine sulfate, carbamylmethylcholine chloride (bethanechol), pilocarpine HCl, dichloroisoproterenol HCl, hexamethonium bromide, decamethonium bromide, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulfate, arecoline HCl, carbamylcholine chloride (carbachol), physostigmine salicylate, Trizma HCl, Trizma base, deoxyribonucleic acid (DNA), theophylline, dithiothreitol, creatine phosphokinase, phosphocreatine (Sigma Chemical Co.); 1,1-dimethyl-4 phenylpiperazinium iodide (DMPP), oxotremorine sesquifumarate (Aldrich Chemical Co.). The following drugs were kindly provided by the companies noted. Propranolol HCl (Ayerst Laboratories); phentolamine HCl (Ciba); haloperidol (McNeil Laboratories); racemic, (+)- and (-)-QNB HCl (Hoffman-LaRoche, Inc.); methscopolamine bromide, prostaglandin E1 (Upjohn); glycopyrrolate (A.H. Robins); dihydroalprenolol (Hässle); clonidine HCl (Boehringer Ingelheim); prazosin HCl (Pfizer); vasoactive intestinal peptide (VIP), Pennisula Labs. Dexetimide and levetimide were generously provided by Dr. Guy Poirier, University of Sherbrooke.

Statistics

Statistical analysis was done using an analysis of variance for a one-way randomized design. Statistically significant F values were tested using Dunnett’s t-statistic or Duncan’s New Multiple-Range Test (Steel and Torrie, 1960; Sokal and Rohlf, 1969).

Results

General Characteristics of Binding

Specific binding increased linearly with increasing tissue concentration over the range of 10–500 µg protein (Figure 9). Binding assays were, therefore, always conducted with less than 500 µg protein. Specific binding was also temperature dependent (Figure 9). Assays performed at 0, 22, or 37°C for 45 min
Figure 9. General characteristics of \[^{3}\text{H}](-)\text{QN8} binding to membranes from rat colonic epithelial cells. A. Specific binding as a function of protein concentration. B. Total binding as a function of incubation temperature. C. Total binding as a function of pH.
Figure 9
resulted in a temperature-dependent increase in specific binding, with temperature having little effect on nonspecific binding. Tissue preincubated for 10 min at 90°C resulted in a complete loss of specific binding. All further binding assays were therefore performed at 37°C. Binding of [3H](-)QNB to membranes from rat colonic enterocytes exhibited a broad pH optimum (Figure 9) with maximum total and specific binding occurring at pH 7.5.

**Kinetics of Binding**

At 37°C, specific [3H](-)QNB binding occurred rapidly. Binding reached a half-maximal value in about 10 min and attained a plateau by 30 min (Figure 10). In contrast, nonspecific binding was time-independent. Kinetic data from Figure 10A were used to calculate, according to the method of Williams and Lefkowitz (1978), a second order forward rate constant, $k_1$ of $8.6 \times 10^{-7} \text{M}^{-1} \text{min}^{-1}$ (Figure 10). Dissociation of [3H](-)QNB binding was very slow with a half-life of 97 min (Figure 11). Although control binding was not stable after 2 hr of incubation at 37°C and a very large excess of (+) QNB was required to prevent rebinding (see Figure 11), a first order kinetic rate constant for dissociation $k_2$ of $6.0 \times 10^{-3} \text{min}^{-1}$ was calculated from dissociation data where control binding remained stable (Figure 11). The ratio $k_2/k_1$, an estimate of the apparent equilibrium dissociation constant $K_D$, was $K_D = k_2/k_1 = 0.07 \text{nM}$.

**Saturability of [3H](-)QNB Binding**

Specific binding approached saturation at a [3H](-)QNB concentration of about 2 nM; half maximal saturation occurred at 0.2 nM (Figure 12). Nonspecific binding did not saturate and increased linearly with increasing [3H](-)QNB concentrations. Scatchard plot analysis (Scatchard, 1949) of specifically bound [3H](-)QNB revealed the presence of only one specific binding site over the ligand concentration range tested (Figure 12). The mean ± S.E. for the apparent dissociation constant ($K_D$) from three experiments was $0.11 ± 0.03 \text{nM}$. The calculated maximal number of binding sites ($B_{\text{max}}$) from three experiments was $103.6 ± 9.0 \text{fmol/mg protein}$. A plot of the binding data according to the Hill
Figure 10. Time course for association of specific $[^3H](-)QNB$ binding to membranes from rat colonic epithelial cells. A. Aliquots of membrane homogenate (200μg protein) were incubated with 0.8 nM $[^3H](-)QNB$ in 50 mM Tris buffer for various times at 37°C. Association was begun by addition of tissue and terminated by filtration. The data shown are from two experiments, each done in duplicate. B. Kinetic plot for the association of specific binding. Data from Figure 10A (except for points at 30 and 60 min) were used to determine $X$ (amount of specific $[^3H](-)QNB$ bound at time $t$) and $X_{eq}$ which is the amount of specific $[^3H](-)QNB$ bound at equilibrium, which was taken as 51.8 fmol/mg protein (point at 60 min in A). The line, fitted by linear regression ($r = 0.98$), has a slope of $k_{ob}$ which represents the pseudo-first order rate constant. The second order forward rate constant $k_1$, was calculated from $k_1 = (k_{ob} - k_2) / [QNB]$ where $k_2$ is the rate constant for dissociation (Figure 11B) and $[QNB]$ is the concentration of $[^3H](-)QNB$ (0.8 nM).
\[ \ln \left( \frac{X_{eq}}{X_{eq} - X} \right) \]

**Figure 10**

**A**

\[ ^{3}H \] (-)-ONB SPECIFICALLY BOUND (fmol/mg protein)

**B**

\[ k_1 = \frac{0.075 \text{ min}^{-1}}{1 + \frac{[I]}{K_i}} \]

\[ k_2 = 8.6 \times 10^5 \text{ M}^{-1} \text{ min}^{-1} \]
Figure 11. Time course for dissociation of $[^3]$H(−)QNB binding.

A. Tissue homogenates (400 μg protein/ml final incubation buffer) were incubated with 0.8 nM $[^3]$H(−)QNB, in a total volume of 10 ml, under three conditions at 37°C. After 30 min (to allow for association) a large excess of (±)QNB ($1 \times 10^{-4}$M) was added to one tube to prevent the rebinding of free radioligand. The other two tubes were incubated in the absence (control) and presence of $1 \times 10^{-4}$M (±)QNB throughout the experiment. The time of (±)QNB addition (after the 30 min association period) was defined as $t = 0$. At the indicated times, 1 ml aliquots were removed from each tube and $[^3]$H(−)QNB binding determined by filtration. Data are from a representative experiment. B. Kinetic plot for the dissociation of binding. Data from Figure 11A where control binding was stable were used (30, 60 and 90 min). The line was determined by linear regression ($r = 0.98$). $X_{eq}$ represents the amount of binding immediately before the addition of (±)QNB, and $X$ the amount of binding at each time after its addition. The slope of the line is equal to the first-order rate constant, $k_2$. 

59
Figure 11
Figure 12. $[^3\text{H}](-)\text{QNB}$ binding as a function of increasing concentrations of $[^3\text{H}](-)\text{QNB}$. Homogenates of membrane from rat colonic epithelial cells were incubated, as described in Materials and Methods, with various concentrations of $[^3\text{H}](-)\text{QNB}$. Nonspecific binding was measured in the presence of $1 \times 10^{-7} \text{M} (\pm)\text{QNB}$. These data are from a representative experiment performed in duplicate. A. Specific binding, determined by subtracting nonspecific from total binding at each concentration. B. Scatchard plot of specifically bound $[^3\text{H}](-)\text{QNB}$. C. Hill plot of binding data, with a Hill coefficient of $n_H = 0.99$. The mean ± S.E. for the apparent dissociation constant ($K_D$) from three experiments was $0.11 ± 0.03 \text{ nM}$; maximal number of binding sites ($B_{\text{max}}$) and the Hill coefficient were $103.6 ± 9.0 \text{ fmol/mg protein}$ and $n_H = 0.93 ± 0.04$, respectively.
equation (Hill, 1910) revealed a straight line with a Hill coefficient $n_H = 0.99$, indicating the absence of cooperative interactions (Figure 12). The Hill coefficient from the three experiments was $0.93 \pm 0.04$.

**Pharmacologic Specificity of $[^3H](-)QNB$ Binding**

The pharmacologic specificity of binding was measured by studying the effects of a variety of muscarinic antagonists, agonists and nonmuscarinic drugs on $[^3H](-)QNB$ binding. The muscarinic antagonists, QNB, methscopolamine, atropine and glycopyrrolate inhibited 50% of specific $[^3H](-)QNB$ binding to membranes from rat colonic enterocytes at nanomolar concentrations, whereas the agonists arecoline, pilocarpine and bethanechol inhibited specific binding by 50% in the micromolar range (Figure 13 and Table 2). In contrast, nonmuscarinic agents failed to alter specific binding, to a great extent, even at high concentrations (Table 2).

Specific binding was stereoselective, with the pharmacologically active isomer $(-)QNB$ being 180-fold more potent than $(+)$QNB at inhibiting specific binding (Figure 14 and Table 2). The stereoisomers of the muscarinic antagonist benzetimide were also tested for their ability to inhibit specific $[^3H](-)QNB$. Dexetimide was 10,000-fold more potent than levetimide at inhibiting specific binding (Figure 14 and Table 2).

Specific binding was also detectable in intestinal smooth muscle (Figure 15), with $[^3H](-)QNB$ having equal affinity in the jejunum, ileum, cecum and colon. Furthermore, the IC50 obtained for QNB in membrane homogenates from colonic epithelial cells was similar to the IC50 for QNB obtained in muscle homogenates (Figure 15 and Table 2). Control specific binding ($[^3H](-)QNB$ was present at a saturating concentration, 1.7 nM) expressed in fmoles/mg protein for the four regions was (means ± S.E., n = 3): jejunum, 245 ± 49; ileum, 184 ± 31; cecum, 162 ± 20; and colon 165 ± 18. When tested for significance using a one-way analysis of variance, control specific binding for the four regions was not significantly different (F0.05).
Figure 13. Inhibition of specific binding of $[^3\text{H}](-)\text{QNB}$ (present at 0.2–0.5 nM) from homogenates (200–400 µg protein) of rat colonic epithelial cells by cholinergic agents. Incubations were performed at 37°C for 45 min as described in Materials and Methods. Data for (±)QNB, arecoline, and pilocarpine are means ± S.E. from three experiments, each in duplicate. Data for the other agents are means from two experiments, each done in duplicate.
Figure 13
Figure 14. Stereoselective inhibition of specific $[^3\text{H}](\text{--})QNB$ binding to membrane homogenates (400 µg protein) from rat colonic epithelial cells. $[^3\text{H}](\text{--})QNB$ concentration was 0.5 nM. Data are the means ± S.E. from three experiments, each done in duplicate.
Figure 15. In vitro inhibition of \(^{3}\text{H}\)(−)QNB binding by (±)QNB in homogenates of intestinal smooth muscle. A. Binding to ileum and jejunum. B. Binding to cecum and colon. \(^{3}\text{H}\)(−)QNB was present at a saturating concentration, 1.7 nM. Data are the mean ± S.E. from three experiments each in duplicate. Values in parentheses are the 95% confidence limits for IC50.
TABLE 2

Relative Potencies of Drugs that Inhibited Specific [\(^3\)H\](-)QNB Binding to Membrane Homogenates from Rat Colonic Epithelial Cells\(^a\)

<table>
<thead>
<tr>
<th>DRUG</th>
<th>(\text{IC}_{50}(\text{M})^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)QNB</td>
<td>(2.6 \times 10^{-10})</td>
</tr>
<tr>
<td>Dexetimide</td>
<td>(8.2 \times 10^{-10})</td>
</tr>
<tr>
<td>((\pm))QNB</td>
<td>(8.5 \times 10^{-10})</td>
</tr>
<tr>
<td>Methscopolamine</td>
<td>(4.0 \times 10^{-9})</td>
</tr>
<tr>
<td>Glycopyrrolate</td>
<td>(6.4 \times 10^{-9})</td>
</tr>
<tr>
<td>Atropine</td>
<td>(9.0 \times 10^{-9})</td>
</tr>
<tr>
<td>((+)&gt;QNB</td>
<td>(4.8 \times 10^{-8})</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>(2.3 \times 10^{-6})</td>
</tr>
<tr>
<td>Acetylcholine(^c)</td>
<td>(7.3 \times 10^{-6})</td>
</tr>
<tr>
<td>Levetimide</td>
<td>(8.2 \times 10^{-6})</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>(2.6 \times 10^{-5})</td>
</tr>
<tr>
<td>Carbachol</td>
<td>(5.5 \times 10^{-5})</td>
</tr>
<tr>
<td>Arecoline</td>
<td>(7.1 \times 10^{-5})</td>
</tr>
<tr>
<td>Bethanechol</td>
<td>(3.5 \times 10^{-4})</td>
</tr>
</tbody>
</table>

No inhibition at 10\(\mu\text{M}\)

Histamine, 5-hydroxytryptamine, haloperidol, propranolol, hexamethonium, physostigmine, yohimbine, dihydroalprenolol

Less than 15% inhibition at 10\(\mu\text{M}\)

DMPP, phentolamine, decamethonium, neostigmine, dichloroisoproterenol, clonidine, prazosin

\(^a\)Membrane homogenates (100-300\(\mu\)g protein) were incubated with 0.2-0.5 nM [\(^3\)H\](-)QNB for 45 min at 37\(^\circ\)C.

\(^b\)Molar concentration of drug which inhibited specific [\(^3\)H\](-)QNB binding by 50%. \(\text{IC}_{50}\) values were determined from a log-probit plot using four to five different concentrations of the inhibitor. Values are the means of at least two experiments, each in duplicate or triplicate.

\(^c\)Incubations also contained 1\(\mu\text{M}\) physostigmine to prevent hydrolysis of acetylcholine.
Subcellular Localization of $[^3H](-)QNB$ Binding in Rat Colonic Epithelial Cells

Initial experiments were designed to test for the presence of the two plasma membrane markers, adenylate cyclase (Walling et al., 1978; Murer et al., 1976) and Na$^+$/K$^+$-ATPase (Quigley and Gotterer, 1969), in the subcellular fraction used for the binding studies. The known stimulants of adenylate cyclase, vasactive intestinal peptide (VIP, $1 \times 10^{-6}$ M) and prostaglandin E$_1$ (PGE$_1$, $1 \times 10^{-4}$ M) significantly ($P < 0.05$) increased adenylate cyclase activity by about 3 fold over basal (Table 3). ATPase activity was also present in this fraction with the Na$^+$/K$^+$-stimulated ATPase representing 25% of the total activity (Table 3). Thus, we were assured of the presence of basal-lateral membranes in the fraction(s) to which $[^3H](-)QNB$ was binding.

To determine the subcellular localization of specific binding, $[^3H](-)QNB$ (0.5 nM) was incubated at 37°C for 45 min with the different subcellular fractions from colonic enterocytes (Plate II). Specific binding (82.9 ± 17.7 fmol/mg protein; mean ± S.E., n = 3) and VIP-stimulated ($10^{-5}$ M) adenylate cyclase activity (5-fold over basal) were highest in a relatively mitochondrial-free fraction that appeared to be enriched in plasma membranes. The Na$^+$/K$^+$-stimulated ATPase activity in this fraction was 44% of the total ATPase activity. Specific binding (22.9 ± 0.9 fmol/mg protein) was lowest and the DNA content (120.3 ± 11.6 μg DNA/mg protein, mean ± S.E., n = 3) highest in the nuclear fraction, followed by the total particulate (32.7 ± 1.2 fmol/mg protein) and mitochondrial (47.6 ± 6.2 fmol mg/protein) fractions. There was considerable binding in the mitochondrial fraction, as well as VIP-stimulated adenylate cyclase activity, indicating a contamination of this fraction with plasma membranes. On the other hand, the plasma membrane fraction contained few mitochondria as assessed by electron microscopy, and specific binding was increased in comparison to all other fractions. If $[^3H](-)QNB$ was binding to mitochondria, one would have expected a decrease in binding upon removal of the
<table>
<thead>
<tr>
<th>Addition</th>
<th>Adenylate Cyclase Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ATPase Activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (basal)</td>
<td>2.5 ± 0.5 (3)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.96 ± 0.60 (3)</td>
</tr>
<tr>
<td>VIP (10&lt;sup&gt;-6&lt;/sup&gt;M)</td>
<td>8.5 ± 1.3 (3)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.60 ± 0.24 (3)</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;1&lt;/sub&gt; (10&lt;sup&gt;-4&lt;/sup&gt;M)</td>
<td>8.0 ± 1.1 (3)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Homogenates of isolated colonic enterocytes were centrifuged at 2,600 xg for 15 min, the resultant supernatant was centrifuged at 30,000 xg for 20 min, and the pellet washed in 50 mM Tris buffer.

<sup>b</sup>See Materials and Methods for assay conditions.

<sup>c</sup>Mean ± SE. of number of experiments in parentheses, each in triplicate or duplicate.

<sup>d</sup>Mean significantly different (P<0.05) from control (basal) using Dunnett's t statistic (one-tailed).
Plate II. Subcellular localization of \(^3\text{H}\)(-)-QNB binding in rat colonic epithelial cells. Electron micrographs of fractions of rat colonic epithelial cells isolated as described in Materials and Methods.
A. Normal colonic epithelial cell. B. Nuclear fraction. C. Mitochondrial fraction. D. Plasma membrane fraction. Calibration bars represent 1 µ. Magnifications: A, 8,000 X; B, 16,000 X; C and D, 40,000 X. Numbers represent the amount of specific binding of \(^3\text{H}\)(-)-QNB (fmol/mg protein) in each fraction. \(^3\text{H}\)(-)-QNB was present at 0.5 nM. Value shown in A is for the total cell particulate fraction.
Specific binding in the plasma membrane fraction D was significantly higher (p < 0.05) than all other fractions by an analysis of variance and Duncan's New Multiple-Range Test. Specific binding in the nuclear fraction was significantly lower (p < 0.05) than the mitochondrial fraction, but not the total cell particulate fraction.
mitochondria. Markers for the endoplasmic reticulum were not measured. Therefore, specific binding appears to be enriched in the microsomal fraction which contains plasma and internal reticular membranes.

Regional Distribution of Muscarinic Receptors in the Rat Intestine.

Preliminary experiments were done to test for differences (or similarities) in muscarinic receptor density in enterocytes from the jejunum, ileum and colon. When membranes for these regions were prepared as described in Materials and Methods from a single rat, specific binding of $[^3H](-)QNB$ (present at 0.3 nM) was highest in the colon (specific binding in fmol/mg protein: jejunum, 11.5; ileum, 8.6; colon 22.9). Although the colonic enterocytes appeared to have a greater receptor density than those in the jejunum and ileum, these data were not able to give an indication of the affinity of $[^3H](-)QNB$. Therefore, saturation experiments were performed in order to obtain an estimate of both the $K_D$ and the $B_{max}$ for these regions (see Table 4). Over the concentration range of 0.02-2.1 nM $[^3H](-)QNB$, Scatchard analysis revealed the presence of only one specific binding site with the same $K_D$ in the jejunum, ileum and colon. Receptor densities in the jejunum and ileum were not statistically different ($p > 0.05$), but the receptor density in the colon was about 5-fold higher (Table 4).

STUDY 3

IN VIVO BINDING OF $[^3H]$-QNB TO RAT INTESTINAL EPITHELIAL CELLS

Materials and Methods

$[^3H]$-Quinuclidinyl Benzilate Binding

Male Sprague-Dawley rats (200-400g, obtained from Laboratory Supply Indianapolis, Ind.), provided 5% glucose in their drinking water, were fasted for either 24 hours (ileal experiments) or 48 hours (colonic experiments) and housed in metabolic cages. The animals were lightly anesthetized with
TABLE 4

Regional Distribution of Muscarinic Receptors in a Membrane Fraction from Isolated Intestinal Epithelial Cells of the Rat.

<table>
<thead>
<tr>
<th>Region</th>
<th>Dissociation Constant (nM)</th>
<th>Receptor Density (fmol/mg protein)</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum</td>
<td>0.08 ± 0.04c</td>
<td>21.9 ± 1.5d</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.14 ± 0.01</td>
<td>32.1 ± 4.5d</td>
<td>0.83 ± 0.03</td>
</tr>
<tr>
<td>Colon</td>
<td>0.11 ± 0.03</td>
<td>103.6 ± 9.0e</td>
<td>0.93 ± 0.04</td>
</tr>
</tbody>
</table>

aMembrane homogenates (200-400 μg protein) were incubated with 5-9 different concentrations (0.02-2.1 nM) of [3H](-)QNB for 45 min at 37°C. When tested for significance using a one-way analysis of variance, mean K_D values and Hill coefficients were not significantly different (F0.05) but mean B_max values were different (F0.05). Significant values for the one-way analysis of variance were subsequently tested using Duncan's New Multiple Range Test.

bMembranes from jejunum and ileum were prepared as described for colon in Materials and Methods.

cRepresents the mean ± SE. from three experiments, each in duplicate or triplicate.

dB_max values not significantly different from each other (P > 0.05).

eB_max significantly different from the other B_max values (P < 0.05).
diethylether, a small abdominal incision made, and a 30 cm segment of distal ileum was ligated, being careful not to disrupt the mesenteric blood supply to the segment. In experiments using colon, the colonic segment was ligated only proximally. Ligations were made to prevent any access of $[^3]$H-QNB to the segment except through the mesenteric blood supply. Ethyl carbamate (30% w/v, 0.2 ml/100 g body weight) was then instilled into the peritoneal cavity and the incision closed with wound clips. Rats were then placed on dissecting boards and the left jugular cannulated for injections of $[^3]$H-QNB (7.0 $\mu$Ci/kg b.w.) and/or competing agents (0.001 to 1.0 mg/kg b.w.) in heparinized (20 U/ml)saline (maximum injection volume was 0.30 ml). Control animals were exposed to $[^3]$H-QNB for 45 min, whereas test animals were pre-exposed to competing agents for 30 min before injecting $[^3]$H-QNB. Upon completion of the experiment, the animals were sacrificed by opening the chest. A sample of blood was taken by cardiac puncture and the heart was removed, blotted free of blood, and a sample of left ventricular muscle obtained. The ligated segment of intestine was removed, stripped of serosal fat and mesentery and intestinal cells harvested in the following manner. Luminal contents were flushed three times with 10 ml normal saline at room temperature and saved for subsequent assay for radioactivity. Intestinal segments were everted over aluminum vibrating coils (rod diameter 0.5 cm) and secured by tying both ends with silk sutures, placed in 250 ml of isolation buffer in a plastic container, and allowed to soak for 10 min. The isolation buffer (pH 7.4 at 22°C) had the following composition (millimolar): NaCl, 150; K$_2$HPO$_4$, 3; EDTA, 5; sucrose, 10; and tris(hydroxymethyl)aminomethane (Tris), 5. The everted intestinal segments were then vibrated at high frequency and low amplitude for 30 min (22°C) using a Vibro Mixer, Model E-1 (Chemapex, Inc., Hoboken, N.J.) to harvest cells. The cell suspension was then centrifuged at 1,500 xg for 5 min (22°C) to sediment the isolated cells. A sample of intestinal muscle from the same segment was also obtained after removing the remaining mucosa with a glass slide.
Tissue was prepared for liquid scintillation counting as follows: Three samples of blood (0.1 ml) and solid tissues (blotted dry and weighed) were placed into scintillation vials and 0.5 ml of a Protosol:ethanol mixture (1:2 v/v) added and the vials gently swirled. The vials were then capped tightly and placed in a water bath until the tissue was solubilized (blood, 60°C for one hour; solid tissues, 37°C overnight). The vials were allowed to cool to room temperature and 0.5 ml of 30% hydrogen peroxide added dropwise, and the vials were capped loosely and incubated for an additional 30 min at 60°C. After cooling, 15 ml of Thrift-Solve (Kew Scientific, Columbus, Ohio) was added and the vials were shaken vigorously, 0.5 ml of 0.5 N HCl added, and the vials once again shaken. Samples were allowed to stand in the dark at 10°C for at least 10 hours before being counted in a Beckman LS-345 liquid scintillation counter. Appropriate corrections were made for quench which was monitored by the automatic external standard method. Using this procedure, counting efficiency for tritium was 20-30%.

Yamamura et al. (1974) have shown that [3H]-QNB is stable in vivo in the rat for at least two hours, therefore experiments were limited to 30 min exposure of the competing agent and 45 min exposure for [3H]-QNB.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

**Intestinal Loops**

In order to determine the ability of QNB to antagonize net secretion induced by cholinergic stimulation, the following experiments were done. Male rats (200-400 g) were anesthetized with diethylether and the ileum and/or colon were exposed through a midline incision and intestinal loops (approximately 10 cm in length) were isolated by loose ligatures. A small incision was made in the proximal and distal end of the loop and the contents flushed with at least 20 ml of normal saline. The ligatures were then tied and 1.5 ml of normal saline injected into the loop using a small needle. Pilocarpine (20 mg/kg b.w.) was injected subcutaneously at the time of preparation of the loops. Antagonism by QNB of the
pilocarpine effect was determined by injecting 1.5 mg/kg b.w. (±)QNB subcutaneously 30 minutes prior to injection of pilocarpine. Separate control animals received a subcutaneous injection of saline in place of either or both drugs. After the 30-minute exposure to pilocarpine, the loops were excised, weighed emptied and re-weighed. The difference in weight (grams) was taken as a measure of net fluid accumulation.

Sources of Drugs

DL-[benzilic-4,4'-3H]-quinuclidinyl benzilate, mol. wt. 337.4, (29.4 Ci/mmol, New England Nuclear) was used for experiments shown in Figure 16. In all other experiments, L-[benzilic-4,4'-3H]quinuclidinyl benzilate, with a higher specific activity (40.2 Ci/mmol, New England Nuclear), was used. The radiochemical purity of [3H]QNB was routinely checked by thin layer chromatography using silica gel-GF plates (New England Nuclear), and a solvent system of chloroform:acetone:diethylamine (60:30:10). The percent radiochemical purity was found to be never less than 95%.

Other drugs were obtained and used in the forms indicated: bovine albumin (Fraction V), atropine sulfate, pilocarpine HCl, Trizma HCl, Trizma base (Sigma Chemical Co.). Racemic QNB was kindly provided by Hoffman-LaRoche, Inc. and dexetimide and levetimide were generously provided by Dr. Guy Poirier, University of Sherbrooke, Quebec, Canada.

Results

[^H]-QNB Binding

Preliminary experiments were performed in the rat ileum with [3H](±)QNB (see Figure 16), before [3H](-)QNB became available. All other experiments were done using [3H](-)QNB. The binding to intestinal enterocytes was compared with that in tissues known to contain muscarinic receptors, as measured in vitro (rat cardiac muscle, Fields et al., 1978; rat ileal and colonic muscle, Figure 15).
Luminal washes, obtained before the enterocytes were isolated, contained only small amounts of $[^3H](-)$QNB. Typically, $<1.0$ fmol/ml of $[^3H](-)$QNB was measured in luminal washes in rats injected with 7.0 $\mu$Ci/kg $[^3H](-)$QNB. Pre-exposure to atropine or $(\pm)$QNB had no effect on the amount of $[^3H]$QNB measured in the luminal wash solution.

Atropine inhibited $[^3H](\pm)$QNB binding to cardiac and ileal muscle and ileal enterocytes (Figure 16) but did not affect $[^3H]_3(\pm)$QNB accumulated in the blood (Figure 16, inset). Similar effects were seen in the colon, with $(\pm)$QNB inhibiting $[^3H](-)$QNB binding in cardiac and colonic muscle and colonic enterocytes (Figure 17). $[^3H](\pm)$QNB accumulated equally in blood in the presence and absence of $(\pm)$QNB (Figure 17, inset). Only 2% of the initial amount of $[^3H](-)$QNB injected was detectable in the blood after 45 min.

Dexetimide, a potent muscarinic antagonist, inhibited in vivo binding of $[^3H](-)$QNB to cardiac muscle and colonic enterocytes (Figure 18) as well as colonic muscle (Figure 19) in a dose-dependent manner. Furthermore, dexetimide was more active than levetimide at inhibiting in vivo $[^3H](-)$QNB binding (Table 5).

Intestinal Loops

Pilocarpine produced a small but significant increase in colonic ($P < 0.05$) fluid accumulation (Table 6). The mean increase during the 30 minute period was 26%. Pretreatment with $(\pm)$QNB completely blocked ($P < 0.05$) the response to pilocarpine. A similar increase in fluid accumulation induced by pilocarpine was seen in the ileum; the effect was blocked by $(\pm)$QNB but was not statistically significant by Dunnett's $t$ test (Steel and Torrie, 1960; Sokal and Rohlf, 1969).
Figure 16. In vivo inhibition of $[^3\text{H}](\pm)\text{QNB}$ binding by atropine. Clear bars represent $[^3\text{H}]\text{QNB}$ bound after 45 min exposure to 7.0 $\mu\text{Ci}/\text{kg}$ $[^3\text{H}](\pm)\text{QNB}$. Solid bars represent the effect of atropine (2.5 mg/kg) pre-exposure (30 min) on $[^3\text{H}]\text{QNB}$ bound after 45 min exposure to 7.0 $\mu\text{Ci}/\text{kg}$ $[^3\text{H}]\pm\text{QNB}$. Inset, amount of $[^3\text{H}]\pm\text{QNB}$ accumulated in whole blood. Values are the means or means $\pm$ S.E. for the number of rats in parentheses. Binding to cardiac muscle is shown for comparison.
Figure 16
Figure 17. In vivo inhibition of $[^3\text{H}](-)\text{QNB}$ binding by $(\pm)\text{QNB}$.

Clear bars represent $[^3\text{H}]\text{QNB}$ bound after 45 min exposure to 7.0$\mu$Ci/kg $[^3\text{H}](-)\text{QNB}$. Solid bars represent the effect of $(\pm)\text{QNB}$ (1.5 mg/kg) pre-exposure (30 min) on $[^3\text{H}]\text{QNB}$ bound after 45 min exposure to 7.0$\mu$Ci/kg $[^3\text{H}](-)\text{QNB}$. Inset, amount of $[^3\text{H}](-)\text{QNB}$ accumulated in whole blood. Values are the means ± S.E. for the number of rats in parentheses. Binding to cardiac muscle is shown for comparison.
Figure 17

[Diagram indicating 
\[ ^{3}H \] ONB BOUND (pmol/g protein) vs. time (h)]

[Bars representing different conditions: one, two, and three (e.g., muscle, red blood cells, etc.)]
Figure 18. In vivo inhibition of $[^3\text{H}](-)\text{QNB}$ binding by dexetimide.

Various doses of dexetimide were injected (i.v.) 30 min prior to 7.0 $\mu$Ci/kg $[^3\text{H}](-)\text{QNB}$, and the bound radioactivity determined 45 min later as described in Methods. Values are the means or means $\pm$ S.E. for the number of rats in parentheses. Binding to cardiac muscle is shown for comparison.
Figure 18

Cardiac Muscle

Colonic Enterocytes

$[^3]H[^-]QNB$ bound (pmol/g protein)

Dexetimide (mg/kg)

- 0
- 0.001
- 0.010
- 1.000
Figure 19. In vivo inhibition of $[^3\text{H}](-)\text{QNB}$ binding by dexetimide in colonic muscle. Various doses of dexetimide were injected (i.v.) 30 min prior to 7.0 $\mu\text{Ci/kg} \ [^3\text{H}](-)\text{QNB}$, and the bound radioactivity determined 45 min later as described in Methods. Values are the means $\pm S.E.$ for the number of rats in parentheses.
Figure 19

[\textsuperscript{3}H] (-)-QNB BOUND TO COLON MUSCLE (pmol/g protein)

DEXETIMIDE (mg/kg)

0 0.001 0.01 0.10 1.00

(5) (3) (3) (3)
TABLE 5

<table>
<thead>
<tr>
<th>Isomer</th>
<th>Cardiac Muscle</th>
<th>Colonic Enterocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexetimide</td>
<td>5.1 ± 1.1 (3)</td>
<td>10.9 (2)</td>
</tr>
<tr>
<td>Levetimide</td>
<td>83.1 ± 3.7 (3)</td>
<td>105.0 (2)</td>
</tr>
</tbody>
</table>

*Binding of [*3H]*(-)QNB, in vivo, was determined as described in Materials and Methods.*

*Levetimide and dexetimide were present at 1.0 mg/kg and [*3H]*(-)QNB was present at 7.0 µCi/kg.*

*Control binding for cardiac muscle was 34.5 ± 2.9 pmol/g protein (mean ± S.E., n=6) and for colonic enterocytes, 4.5 ± 0.4 pmol/g protein (mean ± S.E., n=6). Values in the table represent means or means ± S.E. for the number of animals in parentheses.*
TABLE 6

Effect of Pilocarpine and (±)QNB on Fluid Accumulation in Rat Colonic Loops.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fluid Accumulated (g/30 min)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$1.65 \pm 0.11 \ (6)$</td>
</tr>
<tr>
<td>Pilocarpine $^b$</td>
<td>$2.09 \pm 0.09 \ (6)^d$</td>
</tr>
<tr>
<td>Pilocarpine and (±)QNB $^c$</td>
<td>$1.60 \pm 0.05 \ (6)^e$</td>
</tr>
</tbody>
</table>

$^a$Values represent means $\pm$ S.E. for the number of animals in parentheses. Accumulated fluid contains secreted water, ions and probably also mucus.

$^b$20 mg/kg, s.c.

$^c$ (±)QNB (1.5 mg/kg, s.c.) given 30 min prior to pilocarpine.

$^d$Mean significantly different ($P < 0.05$) from control using Dunnett's $t$ statistic (two-tailed).

$^e$Mean not different from control ($P > 0.05$).
MUCUS BINDING

Preliminary experiments demonstrated that rat intestinal mucus bound \(^3\text{H}\)-QNB in a displaceable fashion. The characteristics of this binding differ from binding to "true" muscarinic sites in heart. However, the binding feigns receptor-related binding to such a degree that care must be taken when \(^3\text{H}\)-QNB is used to identify muscarinic receptors in a mucus-producing tissue such as the intestinal mucosa.

Mucus bound \(^3\text{H}\)-QNB nonspecifically to a large extent, but surprisingly atropine and QNB also inhibited the binding. However, the characteristics of binding to mucus and its inhibition by atropine and QNB were not similar to binding in heart (see Figures 5, 6, and 8). Since binding to mucus could be inhibited by atropine and QNB, the possibility was tested that this binding was saturable, as receptor (specific) binding should be saturable. However, the binding to mucus cannot be considered specific since it did not saturate; it is best defined as nonspecific binding that can be inhibited by atropine and QNB. The only similarities observed between \(^3\text{H}\)-QNB binding to mucus and heart were the amount of binding as a function of protein concentration and the fact that non-muscarinic drugs had no effect on binding to either heart or mucus.

The goblet cells of the intestinal tract secrete mucus glycoproteins (mucins) which are the principal constituents of the mucus gel matrix that lines the surface of the intestinal mucosa (Trier, 1968; Allen and Smary, 1972; Allen, 1978). Binding of \(^3\text{H}\)-QNB was therefore measured to mucin, to test if binding to this component could account for the observed binding to mucus. Although mucin bound \(^3\text{H}\)-QNB, atropine was unable to inhibit this binding, thus binding to mucin can be defined as truly nonspecific. Therefore, mucin per se does not account for the observed
inhibition by atropine of $[^3\text{H}]-\text{QNB}$ binding to mucus. It must be pointed out that mucin isolated from porcine stomach was used for this study and it may bind QNB different from isolated rat intestinal mucin, which was not available for purchase. Furthermore, the isolation procedure to obtain the mucin may have altered its binding characteristics.

It must also be remembered that mucus consists of more than just the mucin glycoproteins. Although some of the displaceable binding of $[^3\text{H}]-\text{QNB}$ observed in the mucus preparation may have been to epithelial cells that are constantly being shed into the lumen at villous tips, the binding characteristics were not the same as those of isolated cells (see Figure 3), or binding to a mucus-free plasma membrane preparation (see STUDY 2). Binding could have also been to secreted mucin-by-products which were altered in structure by enzymes intrinsic to the intestine, or by bacterial enzymes (Ofosu et al., 1978). It is also possible that $[^3\text{H}]-\text{QNB}$ became trapped in pores in the cross-linked mucopolysaccharides in a similar fashion to that reported for small antibiotic molecules (Saggers and Lawson, 1966; Barry and Braybrooks, 1974; 1975).

Therefore, the findings of this preliminary study suggest that $[^3\text{H}]-\text{QNB}$ binds to mucus in a displaceable fashion but the characteristics of this binding are not those of true receptor-related binding. Knowing mucus could bind $[^3\text{H}]-\text{QNB}$, experiments were designed to remove the mucus before $[^3\text{H}]-\text{QNB}$ was added to the incubation. This was accomplished by preparing a membrane fraction by differential centrifugation. This preparation was shown to be mucus free by radioimmunoassay for goblet cell mucin.

**IN VITRO BINDING**

The binding of $[^3\text{H}]-\text{QNB}$ to a mucus free membrane homogenate from rat colonic enterocytes satisfied common criteria applied to receptor identification: it was temperature dependent, saturable, of high affinity, rapid, exhibited
pharmacologic specificity in favor of muscarinic agents, and was stereoselective.

In agreement with other studies, rat gastric mucosal parietal cells (Ecknauer et al., 1980) and parotid acinar cells (Putney and Van De Walle, 1980), $[^3]$H-QNB binding sites in rat colonic epithelial cells appear to be present as a single population of saturable antagonist sites that were not cooperative. The apparent $K_D$ (0.11 nM) from Scatchard plots, for colonocytes is compared with values of 0.78 nM for parietal (Ecknauer et al., 1980) and 0.56 nM for parotid cells (Putney and Van De Walle, 1980). In comparison, the maximum density of muscarinic receptors in a membrane fraction of colonic cells (103 fmoi/mg protein) was twice the number (55 fmoi/mg protein) obtained with a particulate fraction of isolated parietal cells (Ecknauer et al., 1980) and one-half the value of 197.5 fmoi/mg protein obtained for living parotid acinar cells (Putney and Van De Walle, 1980). Thus, these data indicate that $[^3]$H-QNB is binding with high affinity to a limited number of binding sites in rat colonic cells.

It has been previously emphasized (Williams and Lefkowitz, 1978) that the interaction of ligand with its receptor occurs rapidly. The half-life for association of $[^3]$H-QNB with colonocyte receptors was estimated to be 10 min. A kinetically derived estimate of the equilibrium dissociation constant (0.07 nM) was in approximate agreement with the apparent dissociation constant (0.11 nM) obtained in saturation experiments. Dissociation was very slow, with a half-life of 97 min. Slow dissociation of $[^3]$H-QNB has been observed in other binding (Fields et al., 1978; Wastek and Yamamura, 1978; Burgermeister et al., 1978; Rimele et al., 1979) and functional studies (Morisset et al., 1977) and appears to be related to the hydrophobic characteristics of QNB (Aronstam et al., 1977).

The pharmacologic specificity of $[^3]$H-QNB binding is apparent from a comparison of IC50 values (Table 2). Muscarinic antagonists were potent inhibitors of specific binding, while muscarinic agonists were far less effective. This pattern was also observed in studies on parietal (Ecknauer et al., 1980) and parotid (Putney and Van De Walle, 1980) cells.
Binding was also stereoselective, as evidenced by the different abilities of the stereoisomers of QNB and benzatimide to inhibit specific $[^3]H$QNB binding. The (-) enantiomer of QNB was 180-fold more potent than (+)QNB, at inhibiting specific binding, in rat colonic enterocytes. This is in comparison with the 37-fold difference reported for cat lower esophageal sphincter smooth muscle (Rimele et al., 1979) and cat pyloric sphincter smooth muscle (Gaginella et al., 1980) where (-)QNB was 67-fold more potent than (+)QNB at inhibiting specific binding of $[^3]H$QNB. In behavioral studies in dog, (-)QNB is at least 20 times more potent than its (+) isomer (Meyerhoffer, 1972). Dexetimide was 10,000 fold more potent than levetimide in rat colonic enterocytes. Dexetimide has been reported by others to be about 4,000 times more active than levetimide in rat brain (Soudijn, 1973), 2,000 times more effective in rabbit heart (Fields et al., 1978), and 1,600 times more effective in cat pylorus (Gaginella et al., 1980).

The distribution of specific $[^3]H$QNB binding in the different subcellular fractions of rat colonic enterocytes demonstrated that the $[^3]H$QNB binding sites were primarily located in a fraction that was relatively mitochondria-free and enriched in the basal-lateral plasma membrane markers, adenylate cyclase and Na$^+$-K$^+$-ATPase. The mitochondrial fraction also bound a considerable amount of ligand. However, this fraction was also high in adenylate cyclase and Na$^+$-K$^+$-ATPase activities indicating some contamination with basal-lateral plasma membranes. Specific binding was lowest in the nuclear fraction. Thus, it is tempting to speculate that the sites for $[^3]H$QNB binding are located on the basal-lateral plasma membrane. The exact localization of the binding sites cannot be determined with certainty by binding experiments since intestinal epithelial cells contain two types of plasma membranes that are both morphologically and enzymatically distinct (Quigley and Gotterer, 1969; Murer et al., 1976; Walling et al., 1978). The receptor sites for $[^3]H$QNB binding could be located in the luminal (brush-border) and/or contraluminal (basal-lateral) regions of the membrane. Furthermore, since markers for internal membranes (rough and smooth
endoplasmic reticulum) were not measured, there is no indication to what extent the plasma membrane fraction was contaminated with these membranes. [³H]QNB could have also been binding to these membranes.

A question that cannot be answered by binding studies is the physiologic significance of these muscarinic receptors on rat intestinal epithelial cells. However, acetylcholine (Browning _et al._, 1978; Hardcastle and Eggenton, 1973), pilocarpine (Hubel, 1976) and bethanechol (Hubel, 1977) have been shown to induce secretion in both the small intestine and colon of the rat. This effect appears to be muscarinic since it can be inhibited by atropine (Hubel, 1976). Furthermore, although muscarinic receptors of equal affinity and density were found in all regions of muscle tested (see Figure 15), it is evident that the muscarinic secretory response does not originate from this area, since removal of the muscle layers does not abolish the secretory effect (Hardcastle and Eggenton, 1973).

It is also important to point out that the epithelial layer of the intestine consists of several epithelial cell types: immature crypt cells (proposed to function in secretion), mature absorptive villous (small intestine) or surface epithelial cells (colon), goblet (mucus-secreting) cells, enteroendocrine and Paneth cells (Cheng and Leblond, 1974). Results from our study indicate that muscarinic receptors exist in the epithelium of the jejunum, ileum and colon, with the greatest density in the colon. However, no attempt was made to separate the various cell types. Browning _et al._ (1978) by selectively damaging either the villi of the rat jejunum and the surface epithelium of the colon with hypertonic Na₂SO₄, or the crypts with cycloheximide, proposed that the crypts may be the site primarily involved in the secretory response of the rat jejunum and colon to acetylcholine. Therefore, it would be of interest in further studies to selectively isolate villous and crypt cells and measure muscarinic receptor affinity and density in these two populations. Whether muscarinic receptors are also expressed in enteroendocrine or Paneth cells will remain unknown until methods are developed to selectively isolate these cells.
While the number of enteroendocrine and Paneth cells compared to the other cell types is very small, mucus secreting cells comprise a more numerous component of the intestinal epithelium, increasing in number from jejunum to colon. Bradbury et al. (1980) have shown that rat colonic mucus output could be stimulated by intravenous administration of carbachol and bethanechol and this effect was inhibited by atropine. It is very likely, therefore that muscarinic receptors not only exist on the absorptive/secretory cells, but also are probably present on goblet cells.

**IN VIVO BINDING**

[^3H]-QNB is a hydrophobic ligand (Aronstam et al., 1977) that binds in a slowly-reversible manner to muscarinic receptors in vitro (Fields et al., 1978; Rimele et al., 1979; see STUDY 2) and in vivo (Yamamura et al., 1974). This property of [^3H]-QNB permitted the demonstration of its presence on enterocytes after intravenous administration and subsequent cell isolation.

The in vivo characteristics of [^3H]-QNB binding to ileal and colonic cells were qualitatively similar to binding previously measured in vitro (STUDY 2). The muscarinic antagonists atropine and QNB were effective inhibitors of binding in vivo, as they were in vitro. Furthermore, binding in vivo was stereoselective as evidenced by the greater ability of dextetimide, the biologically active optical isomer of benzetimide (Soudijn, 1973), to inhibit [^3H]-QNB binding.

The much greater binding in cardiac muscle as compared to intestinal muscle and enterocytes may represent differences in blood supply and/or [^3H]-QNB distribution to the tissues studied. In in vitro binding studies with membrane homogenates, the muscarinic receptor affinities and densities in rat cardiac and intestinal muscle, as well as the receptor affinity and density in colonic enterocytes, are similar (Fields et al., 1978; see STUDY 2). It is reasonable to assume that the
lower amount of binding to the epithelial cells occurred because the heart and other tissues were exposed to the highest initial concentration of $[{\text{H}}]-QNB$ during its i.v. administration; a lower concentration in the submucosal circulation would diminish the amount of the ligand available for binding to the cells.

Atropine and QNB inhibited $[{\text{H}}]-QNB$ binding. This was not the result of displacement from plasma binding sites because there was no detectable displacement of the ligand in whole blood sampled at the end of each experiment. Small amounts (<1 fmol/ml) of $[{\text{H}}]-QNB$ were detected in luminal washes obtained before isolating the enterocytes. $[{\text{H}}]-QNB$ may have entered the lumen via an intercellular route or may have been actively secreted into the lumen in a fashion similar to that reported for N-methylscopolamine and other organic cations (Turnheim and Lauterbach, 1977; 1980).
CHAPTER III

LACK OF EVIDENCE FOR OPIATE AND ALPHA-ADRENERGIC RECEPTORS ON RAT ILEAL AND COLONIC EPITHELIAL CELLS

Opiate drugs enhance intestinal absorption via an action on the mucosa (Beubler and Lembeck, 1979; Dobbins et al., 1980; Kachur et al., 1980; Lee and Coupar, 1980; McKaye et al., 1981). Utilizing labeled narcotic agonists and antagonists of high specific radioactivity, at least two specific opiate binding sites (receptors) have been identified and characterized in the brain (Lord et al., 1977; Chang and Cuatrecasas, 1979). \[^{3}\text{H}]\text{Naloxone}\) binds with high affinity to the "morphine receptor", whereas \[^{3}\text{H}]\text{enkephalinamide}\) binds with high affinity to the "enkephalin receptor". Therefore, these two labeled ligands were used in STUDY 1 in an attempt to characterize putative opiate receptors on intestinal epithelial cells.

Alpha-adrenergic agents have an action like the opiates (enhance absorption) on the mucosa (Field and McColl, 1973; Racusen and Binder, 1979; Albin and Gutman, 1980). In the brain and peripheral tissues the alpha-adrenergic agonist \[^{3}\text{H}]\text{clonidine}\) and the antagonist \[^{3}\text{H}]\text{yohimbine}\) label sites which are distinct from those sites labeled by the antagonist \[^{3}\text{H}]\text{WB-4101}\) (Richard and Snyder, 1979; Hoffman and Lefkowitz, 1980). \[^{3}\text{H}]\text{dihydroergocryptine}\) binds with equal affinity to both sites (Hoffman et al., 1979). Therefore, these ligands were used in STUDY 2 in an attempt to characterize putative alpha-adrenergic receptors on intestinal epithelial cells.
STUDY 1

OPiATE RECEPTOR BINDING

Materials and Methods

Preparation of Tissue for Binding Studies

Tissue was obtained from male Sprague-Dawley rats (200-300 g), female New Zealand white rabbits (3-4 kg), and male English short hair guinea-pigs (400-500 g). The procedure for the isolation of intestinal epithelial cells and preparation of membrane fractions, has been previously described (see p. 38). In some experiments the epithelial cells were obtained by scraping the mucosa as follows: Segments of ileum and/or colon were resected, cleaned of fat and mesentery, and the luminal contents flushed with 50 mM Tris buffer (pH 7.4 at 37°C). Segments were then everted using a glass rod (diameter, 0.5 cm) and both ends tied with silk sutures to prevent contamination by serosal tissue. The everted segments were then placed on a glass plate and mucosa removed by scraping with a glass slide. Membrane fractions were obtained as described above.

Binding was also measured to intestinal smooth muscle. Segments of jejunum, ileum, cecum and colon were removed, flushed of luminal contents, everted, and the mucosa and serosa scraped off with a glass slide. The remaining muscle was blotted dry and homogenized with a Polytron (setting 7) in 50 mM Tris buffer for 1 min on ice. The homogenate was then centrifuged at 4°C for 15 min at 40,000 xg. The resulting pellet was washed once in 50 mM Tris buffer and subsequently used in the binding assay. Binding to brain was used as a "positive" control. Brain membranes were prepared as follows: Animals were killed by decapitation, and the brains quickly removed and the cerebellum discarded. The remaining brain tissue was homogenized in 50 mM Tris buffer with a Polytron (setting 5). The homogenate was then centrifuged at 4°C for 15 min at 40,000 xg. The resulting pellet was washed once in 50 mM Tris buffer and used for opiate binding.
[\textsuperscript{3}H]Naloxone and [\textsuperscript{3}H]Enkephalinamide Binding

In general, tissue was added to 2 ml polystyrene cups and incubated at 37°C for 35 min with 1.0 nM [\textsuperscript{3}H]naloxone, or at 25°C for 40 min with 1.0 nM [\textsuperscript{3}H]enkephalinamide and specific drugs (where indicated) in a total volume of 1.0 ml of 50 mM Tris buffer (pH 7.4 at 37°C). After incubation, samples were filtered and prepared for liquid scintillation counting as described previously (see p. 50). In preliminary studies, attempts were also made to separate bound and free [\textsuperscript{3}H]naloxone by the following centrifugation method: Tissue and drugs were added to 1.5 ml polyethylene centrifuge tubes and incubated as described above. The incubation was terminated by centrifuging the samples for 1.5 min in a Beckman Microfuge (Model B). Membranes were pelleted almost immediately. The supernatants were aspirated with a needle attached to a vacuum line and the tubes placed upside-down on paper towels for 5 min. The tips of the centrifuge tubes were then cut off into scintillation vials and the membranes solubilized (1.0 ml of Protosol) overnight at 37°C. The vials were cooled and prepared for counting as described previously (see p. 78). Counting efficiency for tritium was 30-40%. All assays were performed in duplicate or triplicate, with variability of the duplicates or triplicates being less than 10% of the mean. The protein concentration was determined by the method of Lowry et al. (1951). IC50 and IC50* were calculated as described in Chapter II.

Sources of Drugs
[N-allyl-2,3-\textsuperscript{3}H]-naloxone (mol. wt. 327.4, 50.2 Ci/mmol) and [Tyrosyl-ring-3,5-\textsuperscript{3}H]-(2-D-alanine-5-L-methionine)-enkephalinamide (mol. wt. 586.7, 45.6 Ci/mmol) were obtained from New England Nuclear. Other drugs were obtained as indicated: [D-ala\textsuperscript{2}]-methionine-enkephalinamide (Sigma Chemical Co.); naloxone HCl (Endo Labs.); ethylketocyclazocine methanesulfonate (Sterling-Winthrop Research Institute); levallorphan tartrate (Hoffman-LaRoche, Inc.); morphine sulfate (The New York Quinine and Chemical Works Inc.).
Results

Comparison of $[^3]$H Naloxone Binding to Rat Ileal and Colonic Epithelial Cells, Muscle and Brain

Preliminary experiments were designed to test the ability of naloxone to inhibit $[^3]$H naloxone binding to membrane fractions from isolated rat ileal and colonic epithelial cells. Binding was also measured simultaneously to homogenates of rat brain. Naloxone inhibited the binding of $[^3]$H naloxone in brain in a concentration dependent manner, reaching maximum inhibition at $1 \times 10^{-7}$ M. On the other hand, binding to ileal and colonic enterocyte membranes was very low (only a few percent over filter binding) and increasing concentrations of naloxone did not alter binding from control values (Figure 20). Similar results were obtained when bound and free naloxone were separated by the centrifugation method. There was a concentration dependent decrease in binding in brain, with naloxone having no effect on binding to ileal membranes. In brain an IC50 ($1 \times 10^{-7}$ M naloxone was used to define specific binding) of $2.2 \times 10^{-9}$ M was calculated for naloxone using the filtration method; the IC50 using the centrifugation method was $3 \times 10^{-9}$ M. IC50's could not be calculated for intestinal tissue. Further experiments were conducted using the filtration method since it gave a higher ratio of specific to nonspecific binding in the control tissue (brain). Using the centrifugation method, unbound $[^3]$H naloxone appeared to be trapped in the pellet causing an increase in apparent baseline binding (see Figure 20).

Binding was also measured in intestinal smooth muscle. However, this binding did not exhibit the same characteristics as binding in brain (Figure 21). For example, IC50's for naloxone in the brain were in the nanomolar range, where IC50's in rat intestinal muscle were in the micromolar range. The calculated IC50's for naloxone in the four regions of the intestine were similar (Figure 21).
Since naloxone did not produce displaceable binding under the experimental conditions used for the preliminary studies, several changes in the conditions were made. However, when incubations were done at 0°C for 2.5 hr (Figure 22), binding to the various tissues was essentially the same as that described previously (see Figure 20). Furthermore, altering the concentration of \(^3\text{H}\)naloxone (Figure 22) or binding to isolated whole intestinal cells (data not shown) also failed to produce binding that was displaceable. Finally, preincubating the tissue at 37°C for 20 min (to destroy endogenous opiate ligands) or in 100 mM NaCl in 50 mM Tris at 0°C for 1 hr (to facilitate dissociation of endogenous inhibitors of ligand binding, Simantov et al., 1976) also failed to produce displaceable binding. Thus, specific binding could not be defined for rat intestinal epithelial cells. Although displaceable binding under the conditions mentioned above could not be detected in the enterocytes, naloxone was able to inhibit (displace) \(^3\text{H}\)naloxone binding in the muscle. However, this binding did not exhibit the same characteristics as binding in the brain (Figure 21 and 23).

3\[^3\text{H}\]Naloxone Binding to Guinea-Pig and Rabbit Ileal Enterocytes

Since opiates enhance in vitro absorption of electrolytes by the rabbit (Dobbins et al., 1980; McKay et al., 1981) and guinea-pig intestine (Kachur et al., 1980), \(^3\text{H}\)naloxone binding was also attempted in these species. However, as in the rat, \(^3\text{H}\)naloxone binding to ileal scrapings was not affected by increasing concentrations of naloxone in either of these species (Figure 24; data not shown for rabbit). Similar results were also obtained in unbroken guinea-pig intestinal cells. In addition, increasing concentrations of \(^3\text{H}\)naloxone (from 1.0 to 7.0 nM) had no effect on the ability of naloxone to inhibit \(^3\text{H}\)naloxone binding.

3\[^3\text{H}\]Enkephalinamide Binding

Experiments were designed to test the ability of enkephalinamide to inhibit \(^3\text{H}\)enkephalinamide binding to intestinal scrapings from rat, rabbit, and guinea-pig. Binding was also measured in rat and guinea-pig brain (control) and intestinal
muscle. Enkephalinamide inhibited the binding of $[^3\text{H}]$enkephalinamide in brain in a concentration dependent manner (Figure 25). However, binding to ileal scrapings from rat, rabbit and guinea-pig was very low (only a few percent over filter binding). These results are similar to those obtained for $[^3\text{H}]$naloxone (compare Figure 20 and 24 with Figure 25). Unlike $[^3\text{H}]$naloxone binding to muscle, $[^3\text{H}]$enkephalinamide binding to rat and guinea-pig ileal muscle was not displaced by enkephalinamide and was similar to the binding observed for ileal scrapings. Therefore, specific binding could not be defined in intestinal tissue using $[^3\text{H}]$enkephalinamide.
Figure 20. Inhibition of $[^3]H$naloxone binding by naloxone in the rat: Comparison of filtration and centrifugation methods.

$[^3]H$naloxone was present at $\sim 1.0$ nM with $\sim 1.5$ mg protein for brain and $\sim 1.0$ mg protein for ileum and colon. Membranes for brain and isolated intestinal cells were prepared as described in Methods. A. Bound and free naloxone separated by filtration. Data for brain and ileum are means $\pm$ S.E. from three experiments each in triplicate. Data for colon are from a representative experiment performed in triplicate. B. Bound and free naloxone separated by centrifugation. Data are the means $\pm$ S.E. from four experiments each in duplicate. An IC50 of $2.2 \times 10^{-9}$ M for naloxone in brain was obtained in A, and an IC50 of $3.0 \times 10^{-9}$ M in B. IC50's could not be calculated for homogenates of intestinal epithelial cells. In A filter binding has been subtracted from points shown.
Figure 21. Inhibition of $[^3H]$naloxone binding by naloxone in rat intestinal smooth muscle. $[^3H]$Naloxone was present at ~1.5 nM with ~1.0 mg of muscle protein. Homogenates of intestinal muscle were prepared as described in Methods. Data are the means ± S.E. from three experiments each in duplicate.

A. Binding in jejunum and ileum. B. Binding in cecum and colon. Numbers in parentheses are the 95% confidence limits for IC50.
Figure 22. $[^3\text{H}]$Naloxone binding at $0^\circ\text{C}$ and effect of increasing concentrations of $[^3\text{H}]$naloxone on binding to rat brain, ileal muscle and ileal scrapings. A. $[^3\text{H}]$Naloxone was present at $\sim 1.5$ nM. Incubations were performed at $0^\circ\text{C}$ for 2.5 hr. Data are from a representative experiment in duplicate.

B. Effect of increasing concentrations of $[^3\text{H}]$naloxone on binding to ileal scrapings. Incubations were performed at $37^\circ\text{C}$ for 35 min. Data are from a representative experiment in duplicate.
Figure 22
Figure 23. Inhibition of $[^3H]$naloxone binding by various opioid drugs. $[^3H]$Naloxone was present at $1.0 \text{nM}$ with $1.0 \text{mg}$ of protein. A. Rat brain. Data are from a representative experiment performed in triplicate. B. Rat ileal muscle. Data are the means $\pm$ S.E. from three experiments each in duplicate.
Figure 23: [³H] Naloxone Bound (% of Control)

- Dnaloxone
- ENK (mM)
- LEVALLORPHAN
- ETHYLORPHAN

Figure 23: [³H] Naloxone Bound (% of Control)

- Dnaloxone
- ENK (mM)
- LEVALLORPHAN
- ETHYLORPHAN

Figure 23: [³H] Naloxone Bound (% of Control)

- Dnaloxone
- ENK (mM)
- LEVALLORPHAN
- ETHYLORPHAN

Figure 23: [³H] Naloxone Bound (% of Control)

- Dnaloxone
- ENK (mM)
- LEVALLORPHAN
- ETHYLORPHAN
Figure 24. Inhibition of [3H]naloxone binding by naloxone in the guinea-pig. [3H] Naloxone was present at ~1.5 nM with ~1.5 mg protein for brain and ~1.0 mg protein for the ileal scrapings. Membranes for brain and ileal scrapings were obtained as described in Methods. Data are the means ± S.E. from three experiments each in triplicate or duplicate. An IC50 of 3.5 x 10^{-9} M for naloxone was obtained in brain. An IC50 could not be calculated for the ileal scrapings. Note the decrease in filter binding with increasing concentrations of naloxone.
Figure 24
Figure 25. Inhibition of binding by enkephalinamide in tissue from rat and guinea-pig. [3H]enkephalinamide was present at ~1.5 nM with ~1.0 mg of protein for the various tissues. A. Rat. Data for brain are the means ± S.E. for four experiments each in duplicate. Data for muscle are the means from two experiments each in duplicate, and a representative experiment in duplicate is shown for colonic scrapings. B. Guinea-pig. Data are from a representative experiment performed in duplicate. Filter binding has been subtracted in both A and B.
Figure 25
STUDY 2

ALPHA-ADRENERGIC RECEPTOR BINDING

Materials and Methods

Preparation of Tissue for Binding Studies

Tissue was obtained from male Sprague-Dawley rats (200-300g), and female New Zealand white rabbits (3-4 kg). The procedure for the isolation of intestinal epithelial cells and preparation of membrane fractions, has been previously described (see p. 38). Intestinal scrapings from the rat and rabbit ileum and brain homogenates were prepared as described on page 99.

Conditions for Ligand Binding

In general, tissue was added to 2 ml polystyrene cups and incubated at 25°C for 25 min ([3H]WB-4101), 30 min ([3H]para-aminoclonidine), 45 min ([3H]dihydroergocryptine), or 60 min ([3H]yohimbine), with ~1.0 nM of the labeled compound and specific drugs (where indicated) in a total volume of 1.0 ml of 50 mM Tris buffer (pH 7.4 at 37°C). Samples were filtered and prepared for liquid scintillation counting as described previously (see p. 50).

Sources of Drugs

[Methyl-3H]-yohimbine (mol. wt. 354.4, 82.6 Ci/mmol); para-[3,5-3H]-aminoclonidine (mol. wt. 245.1, 53.4 Ci/mmol); 9,10-[9,10-3H(N)]-dihydro-(alpha)ergocryptine (mol. wt. 577.7, 23.0 Ci/mmol); and [phenoxy-3-3H(N)]-WB-4101 (mol. wt. 345.4, 24.7 Ci/mmol) were obtained from New England Nuclear. Others drugs were obtained as indicated: Yohimbine (Sigma Chemical Co.); clonidine HCl (Boehringer Ingelheim); prazosin HCl (Pfizer); phenotolamine HCl (Ciba); dihydroergocryptine (Sandoz); and 2,6-dimethoxyphenoxyethylaminomethyl-1,4-benzodioxane (WB4101) was a gift from Dr.P.N. Patil, The Ohio State University.
Results

[^H]WB-4101 Binding

Experiments were performed with membranes from rat colonic enterocytes and brain. Prazocin (1 x 10^-9 M to 1 x 10^-4 M) had no effect on[^H]WB-4101 binding (present at 0.6 nM) to membranes from rat colonic enterocytes. Binding to membranes (~600 μg protein) was only a few percent over filter binding, which was ~20% of total binding. Similar results were obtained for phentolamine over the same concentration range. However, in the brain, 1 x 10^-5 M phentolamine completely inhibited [^H]WB-4101 binding to tissue. Furthermore, WB-4101 (1 x 10^-10 M to 1 x 10^-5 M) failed to inhibit [^H]WB-4101 binding to membranes and whole cells isolated from the rat colon.


Phentolamine (1 x 10^-10 M to 1 x 10^-4 M) had no effect (less than 15% inhibition at 1 x 10^-4 M) on[^3]H]dihydroergocryptine binding to membranes isolated from both ileal and colonic epithelial cells obtained from the rat. However in rat brain, IC50's (1 x 10^-5 M phentolamine was used to define specific binding) of 8 x 10^-9 M for phentolamine and 1 x 10^-9 M for ergocryptine were obtained.


Clonidine (1 x 10^-10 M to 1 x 10^-4 M) failed to inhibit the small amount (less than 10% over filter binding) of[^3]H]para-aminoclonidine binding to ileal scrapings from the rat and rabbit. Similar results were obtained with yohimbine (1 x 10^-9 M to 1 x 10^-4 M) when[^3]H]yohimbine binding was attempted in the rat and rabbit.
Opiates have long been known to be effective in the treatment of diarrhea because of their constipating action. The antidiarrheal activity of morphine-like compounds is classically explained by an increase in intestinal circular smooth muscle tone and an inhibition of the propulsive activity of the intestinal tract (Vaughan-Williams, 1954; Daniel et al., 1959, Bass, 1968; Konturek, 1978; Konturek, 1980). Inhibition of the propulsive ability of the gut has been demonstrated with morphine (Trendelenburg, 1917), loperamide (Van Nueten et al., 1974) and enkephalin (Van Neuten et al., 1977).

Opiates also inhibit intestinal fluid accumulation induced by a variety of secretagogues including cholera toxin, prostaglandin E₁, VIP, carbachol, and bisacodyl (Valiulius and Long, 1973; Karim and Adaikan, 1977; Coupar, 1978; Beubler and Lembeck, 1979, Beubler and Lembeck, 1980; Lee and Coupar, 1980). Furthermore, it has been suggested that the effect of opiates on ion transport is the result of an interaction with specific opiate receptors in the mucosa (Dobbins et al., 1980; Kachur et al., 1980; McKay et al., 1981). However, it is not clear where in the mucosa the opiates are acting. Their effects could be directly on enterocyte membranes, or alternatively, on nerves or paracrine (APUD) cells in the mucosa.

Although the enkephalins and related peptides possess morphine-like activity, and have some similar structural characteristics (Figure 26), there appears to be more than one type of opiate receptor. By the in vitro ligand binding technique, two classes of opiate binding sites have been distinguished in brain (Lord et al., 1977; Simantov et al., 1978; Chang and Cuatrecasas, 1979). One of these sites, with selective affinity for morphine-like alkaloid opiates, appears to correspond to the pharmacological μ receptor of Martín et al. (1976); the other shows selectivity for enkephalins and has been designated δ by Lord et al. (1977).
Figure 26. Structural characteristics of opiate compounds. Top, proposed X-ray crystallographic structure of morphrine and the amino acid sequence of met-enkephalin. Bottom, chemical formulas of morphine and naloxone. Note the minor differences in the structures of morphine which is an opiate agonist, and naloxone which is a pure opiate antagonist. The "tyramine" moiety in all structures is in heavy outline (Modified from Horn and Rodgers, 1977).
Figure 26
In the present study, using $[^3\text{H}]$naloxone (high affinity for the $\mu$ receptor) and $[^3\text{H}]$enkephalinamide (high affinity for $\delta$ receptor) specific (displaceable) binding to intestinal epithelial cell membranes could not be demonstrated. Binding was measured simultaneously in rat brain, a tissue known to contain both $\mu$ and $\delta$ opiate receptors (Lord et al., 1977; Chang et al., 1979; Chang and Cuatrecasas, 1979) and a membrane fraction from enterocytes known to possess muscarinic receptors (see Chapter II). In agreement with other studies, naloxone was a potent inhibitor of $[^3\text{H}]$naloxone binding in brain, as was enkephalinamide a potent inhibitor of $[^3\text{H}]$enkephalinamide binding. Our IC50 ($2.2 \times 10^{-9} \text{M}$) for naloxone was similar to the IC50 ($1.0 \times 10^{-9} \text{M}$) reported by Chang et al. (1979). A similar IC50 ($3.5 \times 10^{-9} \text{M}$) was obtained for guinea-pig brain. Furthermore, the IC50's we obtained in rat ($3.0 \times 10^{-9} \text{M}$) and guinea-pig ($3.5 \times 10^{-9} \text{M}$) brain (see Figure 25) for enkephalinamide were similar to those reported previously (Chang et al., 1979; Chang and Cuatrecasas, 1979; Lord et al., 1977). Displaceable binding could not be demonstrated in membrane preparations from rat intestinal enterocytes for either $[^3\text{H}]$naloxone or $[^3\text{H}]$enkephalinamide. Since displaceable binding is required to define specific (saturable) binding, various modifications were made in the experimental protocol. However, under the conditions used, specific binding still could not be defined. It must also be pointed out, that total binding was only a few percent over filter binding even in the presence of high protein (tissue) concentrations. Exhaustive attempts to vary ionic strength, pH, temperature and other incubation conditions were not performed.

Binding was also attempted in mucosal tissue obtained from the rabbit and guinea-pig; species in which opiates have been shown to alter mucosal ion transport (Dobbins et al., 1980; Kachur et al., 1980; McKay et al., 1981). Results obtained using various cell preparations from these species were similar to those in rat. Therefore, our results suggest that the effects of opiates on mucosal electrolyte transport are not the result of interaction with receptors on intestinal enterocytes.
In fact, results obtained by Dobbins et al. (1980) are in agreement with our results. They found that tetrodotoxin completely blocked the decrease in \( I_{sc} \) induced by D-ala\(^2\)-met-enkephalinamide, inferring that enkephalins are preganglionic neurotransmitters. Although the short-circuit current technique yields information on the active transport of ions in the intestinal mucosa, this technique does not distinguish between an effect of opiate agents on epithelial cell receptors and an effect on the neural elements of the mucosa. Thus, it is conceivable that opiate agonists alter ion transport indirectly by stimulating (or inhibiting) the release of some other substance that then affects the epithelial cell. The results of the present study and those of Dobbins et al., (1980) support this concept.

Additional experiments were done in an attempt to identify opiate receptors in a nerve-muscle (mucosa scraped off) preparation. IC50's in this preparation were in the micromolar range, whereas in the brain, IC50's were in the nanomolar range, suggesting that opiate involvement in our preparation was not identical to that in the brain. However, Leslie et al. (1980) have identified an apparent specific binding site for \([^{3}\text{H}]\text{enkephalin}\) both in the rat and rabbit ileum using the longitudinal muscle-myenteric plexus preparation. Our preparation also contained the circular muscle layer which may have masked a specific binding site (by increasing nonspecific binding). On the other hand, Leslie et al. (1980) did not observe a high affinity binding site for \([^{3}\text{H}]\text{dihydromorphine}\), which is consistent with our results for \([^{3}\text{H}]\text{naloxone}\) binding. It must also be pointed out that these authors preincubated their membranes in a buffer containing 100 mM NaCl for 60 min at 0°C to facilitate dissociation of putative endogenous inhibitors (presumably these inhibitors are identical with endogenous morphine-like peptides) of ligand binding (see Simantov et al., 1976). When this procedure was attempted in the present study, no specific enkephalin binding site could be detected.

The apparent nonspecific displaceable binding we observed with \([^{3}\text{H}]\text{naloxone}\) in the nerve-muscle preparation cannot be explained (see Figures 21 and 23). However, evidence from iontophoretic, receptor binding and
convulsant studies in human, rat and mice, suggest that naloxone is a GABA antagonist (Dingledine et al., 1978). Naloxone, morphine, levorphanol and its non-analgesic enantiomer, dextrorphan, displaced \[^{3}H\]-GABA from GABA receptor sites in homogenates of human cerebellum with IC50's in the micromolar range. Furthermore, a small population of neurons in the myenteric plexus of the guinea pig has been shown to possess high-affinity uptake sites for GABA (Burnstock et al., 1979). Therefore, the binding we observed with \[^{3}H\]naloxone, although apparently not related to opiate receptors, may involve GABA. In summary, specific \[^{3}H\]naloxone and \[^{3}H\]enkephalinamide binding sites were observed in brain but not in preparations of intestinal epithelial cells or nerve-muscle preparations obtained from rat, rabbit and guinea pig.

**ALPHA-ADRENERGIC RECEPTOR BINDING**

Alpha-adrenergic agents enhance the net transfer of NaCl across ileal and colonic mucosa (Field and McColl, 1973; Tapper et al., 1978; Racusen and Binder, 1979; Brunsson et al., 1979; Albin and Gutman, 1980). However, as is the case with opiates, it is not known where in the mucosa alpha-adrenergic agents act. Attempts were therefore made to measure specific alpha-adrenergic receptor binding in membranes from isolated ileal and colonic epithelial cells.

In the present study, specific binding could not be detected in mucosal tissue from rat and rabbit using non-selective (\[^{3}H\]-dihydroergocryptine), alpha\(_1\) (\[^{3}H\]WB-4101) and alpha\(_2\) (\[^{3}H\]clonidine and \[^{3}H\]yohimbine) receptor ligands. Therefore, it was concluded that alpha-adrenergic receptors, under the present conditions, could not be detected by the ligand binding technique.

Since alpha-adrenergic receptors apparently do not exist on rat intestinal cell membranes, other mechanisms might be envisioned. These could conceivably involve effects of alpha-adrenergic agonists on mucosal blood flow, intestinal cell enzymes or modulation of the activity of enteric nerves. Effects on blood flow can be ignored because norepinephrine is capable of producing its characteristic effects on mucosal ion transport in vitro. Sodium absorption from the lumen depends on
the activity of the sodium pump (i.e. the activity of \( \text{Na}^{+}-\text{K}^{+}-\text{ATPase} \)), thus, a possible mechanism for alpha-adrenergic compounds, may be as indirect stimulants of ATPase activity (Albin and Gutman, 1980). Furthermore, it is possible that norepinephrine may modulate the release of other transmitters, which in turn act on the epithelial cells resulting in net absorption. Indeed, norepinephrine has been shown to inhibit acetylcholine (a stimulant of secretion) release in the guinea-pig myenteric plexus (Paton and Vizi, 1969; Manber and Gershon, 1979). Thus, sympathetic nerve fibers may decrease active secretion of solutes by the epithelial cells in the crypts. Finally, sympathetic nerves may, via a ganglionic site of action, inhibit the release of a neurotransmitter which inhibits water uptake. For example, VIP is a proposed neurotransmitter in the gastrointestinal tract (Fahrenkrug et al., 1978a; 1978b) and is known to produce secretion in the small intestine. The release of this peptide from the gastrointestinal tract is decreased by sympathetic nerve stimulation (Fahrenkrug et al., 1978a).
CHAPTER IV

CONCLUSION

The present study has provided the first direct evidence for the existence of muscarinic receptors on rat ileal and colonic epithelial cells. The binding of \[^3\text{H}QNB\] both in vitro and in vivo satisfied common criteria applied to receptor identification: it was temperature dependent, saturable, of high affinity, rapid, exhibited pharmacological specificity in favor of muscarinic agents, and was stereoselective. Thus, it seems reasonable to postulate that cholinergic nerves in the intestinal mucosa, release acetylcholine which diffuses over a short distance (Newson et al., 1979), binds to muscarinic receptors located on the epithelial cells (present study), and subsequently elicits electrolyte secretion (Wright et al., 1940; Florey et al., 1941; Tidball, 1961; Hubel, 1976, 1977; Hardcastle and Eggenton, 1973; Isaacs et al., 1976; Browning et al., 1977; Browning et al., 1978; Morris and Turnberg, 1980; Tapper et al., 1978).

Our experiments demonstrate that muscarinic ligand binding studies are an effective means of directly measuring parameters of receptor occupancy in isolated intestinal epithelial cells. The significance of these studies to intestinal ion transport resides in the potential to assess differences (or similarities) in muscarinic receptor affinity and density in healthy tissue and that obtained from individuals having neurally-mediated functional bowel disorders and diarrheal disease.

On the other hand, opiate and alpha-adrenergic receptors apparently are not present on intestinal epithelial cells. Although opiates and alpha-adrenergic agents have documented effects on mucosal ion transport (Dobbins et al., 1980; McKay et al., 1981; Kachur et al., 1980; Field and McColl, 1973; Hubel, 1976; Racusen and Binder, 1979; Albin and Gutman, 1980), these effects must be due to actions on other elements of the mucosa.


