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Cellular Basis of Chemical Messengers in the Digestive System

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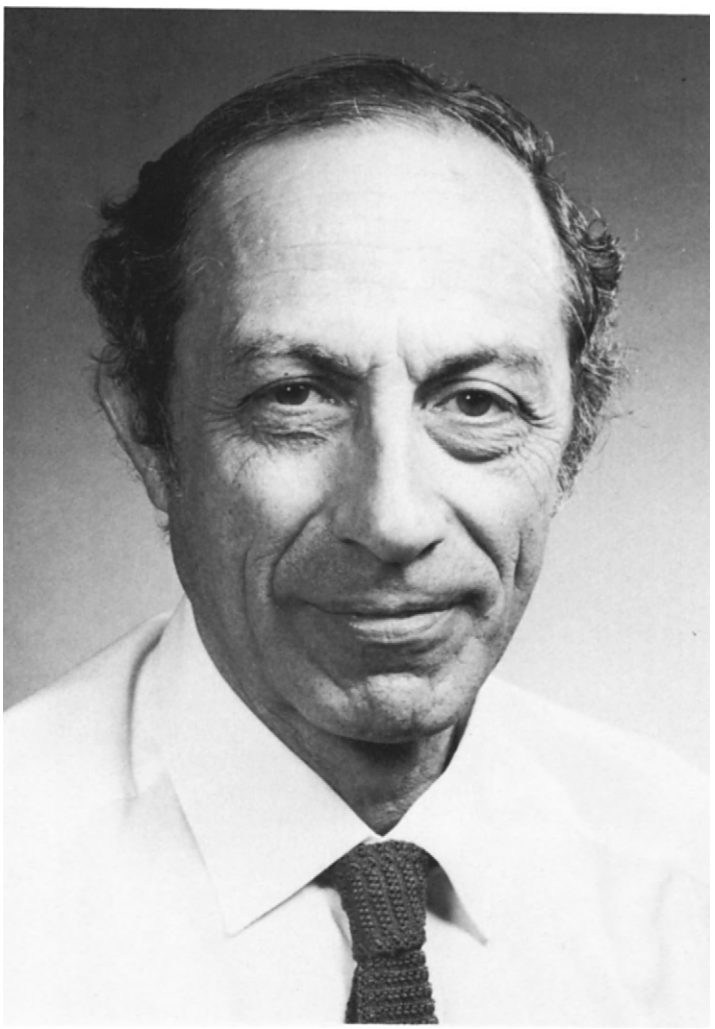

Volume dedicated to Dr. Morton I. Grossman

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Preface

The discovery that central and peripheral neurons contain a wide variety of peptides, many of which are either the same as or similar to hormones found in endocrine cells, has revolutionized regulatory biology. Nervous and endocrine systems, until recently thought to be quite separate control mechanisms, are suddenly revealed to be inextricably related. We now think in terms of a shared set of chemical messengers that are delivered to their targets by a variety of modes. These include three pathways: the endocrine (cell-to-distant-target, through circulation), paracrine (cell-to-neighboring-target, through interstitial spaces), and neurocrine (nerve-to-target, across a narrow synaptic gap). The novel and challenging concept that emerges is that the same chemical messenger may utilize all three of these modes of delivery.

Nowhere in the body is this duality of distribution of peptides in neurons and in endocrine–paracrine cells better represented than in the digestive system. For example, somatostatin is found in both endocrine–paracrine cells of the gut and pancreas and also in neurons of the gut, pancreas, and central and peripheral nervous systems. Accordingly, it was appropriate that an international symposium on the cellular basis of chemical messengers of the digestive system be held at this time. This volume is the proceedings of such a symposium held in Santa Monica, California on January 16, 17, and 18, 1980, under the sponsorship of UCLA, the National Institute of Arthritis, Metabolism and Digestive Diseases, and the Foundation CURE (Center for Ulcer Research and Education).

The chapters in this volume deal with the entire spectrum of problems related to the cellular aspects of chemical messengers in the digestive system. They consider which amines and peptides serve these functions; in which neurons and endocrine–paracrine cells each kind of chemical messenger is found; what the distribution of these cells is; what the evolutionary history of these substances is; what ontological features they show; what regulates their synthesis, storage, and release; what physiological roles they serve; and what changes occur in disease. Of course, the answers to many of these questions are just beginning to emerge. We hope that this volume will provide a reasonably comprehensive picture of the present status of this burgeoning, exciting, and promising field of study.

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Nature of Gut Peptides and Their Possible Functions

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Between the first part of this century and the past decade our knowledge of gut and pancreatic peptide hormones progressed at a steady pace with the chemical and physiological characterization of a few peptides having obvious physiological importance (35). Insulin and glucagon were the province of the endocrinologists interested in diabetes mellitus and glucose homeostasis, and gastrin, secretin, and cholecystokinin (CCK) were studied by gastrointestinal physiologists interested in regulation of gastric and pancreatic secretion and gut smooth muscle function. Each of these last three substances was localized to a relatively discrete region of the gut, could be extracted in biologically active form from the appropriate region, and was ultimately purified for determination of peptide structure (12,26). Strong physiological evidence was obtained to support the release of gastrin from the gastric antrum, and of secretin and CCK from the upper small intestine (13). Radioimmunoassays were developed first for insulin, then for the other peptides and ultimately confirmed many of the conclusions reached by previous physiological studies (2). Radioimmunoassay also revealed some previously unsuspected complexities. A larger form of insulin, later shown to be proinsulin, was detected in tissue extracts and blood, and the fragment removed from proinsulin during its conversion to insulin, C peptide, was also demonstrated (35). Gastrin in the blood was found to be heterogeneous, and the most abundant form, G34, was found to be a larger form of the most abundant form found in tissue extracts, G17 (8,38). CCK was found to exist in at least three different forms (7,26).

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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The molecular heterogeneity of gut and pancreatic hormones was a confounding factor in the analysis of their functions, but the problems raised by discovery of multiple forms appeared to be surmountable. Any complacency that might have existed at the end of the 1960s that the roles of the gut peptides soon would be sorted out was dispelled during the 1970s by the discovery of a dozen or more biologically active peptides in gut and pancreatic tissues in addition to those already known (14,37). One major source of these peptides was the laboratory of Viktor Mutt, who had prepared large quantities of porcine small intestine extracts in the process of purifying secretin and CCK with Eric Jorpes. He had the foresight to retain the side fractions from these purifications and made them available to John Brown, who isolated motilin (31) and gastric inhibitory peptide (GIP) (3), and to Sami Said, who isolated vasoactive intestinal polypeptide (VIP) (30). The same laboratory has continued this work and recently reported isolation of a gastrin-releasing peptide (GRP) (33) resembling bombesin and of other peptides with chemical structures and biological activities that have not yet been fully determined. These peptides generally have been isolated by monitoring a biological effect. Mammalian pancreatic polypeptide (PP) was isolated entirely by chemical methods and was initially identified only as a contaminant in the purification of insulin (21). Another group of peptides now known to be present in gut tissues includes those initially isolated from the brain. This group includes substance P (33), neurotensin (15), somatostatin (22,29), thyrotropin-releasing hormone (TRH) (25), and the enkephalins (17). In several cases the method of identification in the gut was by immunological cross-reactivity with antibodies raised against the brain peptides revealed by radioimmunoassay of gut tissue extracts and/or by immunocytochemistry. The known or suspected chemical structures of several of the gut peptides are shown in Table 1. The list is necessarily incomplete because new members are being added constantly. The insulin and glucagon peptides are not included in this table. Only partial amino acid sequences have been determined for several other peptides that are known to exist. These include chymodenin (1), glicentin (24), and porcine intestinal histidine (N-terminal) isoleucine amide (C-terminal) peptide (PIHIA) (27). Larger molecular forms have been found for several others by radioimmunoassay of chromatographic fractions, including somatostatin and GIP, but have not been fully characterized. In a few cases peptide sequences have been determined for several mammalian species, but in many cases the sequences are known only for porcine peptides (6).

Sequence homologies permit classification of some of these peptides into "families" of which the best known are the gastrin-CCK family and the secretin-glucagon-VIP-GIP family (6). Erspamer and co-workers (10) have isolated from amphibian skin a number of peptides that have counterparts in mammalian gut and brain. They include physalaemin and related peptides that are similar to substance P, caerulein and related peptides that resemble

TABLE 1
Structure of Some Gut Peptides

Peptide		Amino acid sequence[a]
Cholecystokinin	CCK39	YIQQARKAPSGRVSMIKNLQSLDPSHRISDRDŶMGWMDF#
	CCK33	----------
	CCK8	----------
Enkephalins	Leu	YGGFL
	Met	--------M
Gastric inhibitory peptide (GIP)		YAEGTFISDYSIAMDKIRQQDFVNWLLAQQKGKKSDWKHNITQ
Gastrin releasing peptide (GRP)		APVSVGGGTVLAKMYPRGNHWAVGHLM#
Bombesin		*QQRL-----Q----------
Gastrin	hG34	*QLGPQGPPHLVADPSKKQGPWLEEEEEAYGWMDF#
	hG17	----------
	hG14	----------
Glucagon		HSQGTFTSDYSKYLDSRRAQDFVOWLMDT
Motilin		FVPIFTYGELQRMQEKERNKGQ
Neurotensin (hNT)		*QLYENKPRRPYIL
Pancreatic Polypeptide	PP	ASLEPVYPGDDATPEQMAQYAAELRRYINMLTRPRY#
	hPP	--P----------BB ----------D----------
Somatostatin		AGCKNFFWKTFTSC
Secretin		HSDGTFTSELSRLRDSARLQRLLQFLV#
Substance P (equine)		RPKPQQFFGLM#
Thyrotropin-releasing Hormone (TRH)		*QHP#
Vasoactive Intestinal Peptide (VIP)		HSDAVFTDNYTRLRKQMAVKKYLNSILN#

[a]Key to symbols used: *Q, PCA (pyroglutamyl); #, carboxyl terminal amide; Ŷ, tyrosine sulfate; h, human (all others are porcine unless otherwise indicated); -, same as line above.

the biologically active region of CCK, bombesin and related peptides that resemble GRP. These peptides all are present in cutaneous glands but also can be found in gut tissues. Erspamer's prediction that it will be rewarding to examine systematically gut, brain, and skin for peptides analogous to those found in only one site in one species already has been borne out and almost certainly will be one avenue of investigation that will continue to provide new information. There are many more examples of the occurrence of peptides similar to those found in mammalian gut in lower vertebrates, suggesting that they may perform roles that favor conservation during evolution (6).

Certain other features of some of the gut peptides are interesting. At least eight peptides (CCK, gastrin, GRP, PP, secretin, substance P, TRH, and VIP) contain carboxyl-terminal amide groups that in several cases are necessary for full biological activity. The mechanism for amidation has not been established for any of these peptides, but production of the amide by enzymatic cleavage of a precursor extended at the C-terminus through a glycine residue has been suggested as the mechanism for another peptide, mellitin (34). Sulfation of a tyrosine side chain is essential for full biological activity of CCK, but similar sulfation does not enhance the acid-stimulating activity of gastrin (37). None of the well-characterized gut peptides has been shown to be a glycopeptide. In several cases (gastrin, CCK, bombesin, glucagon, insulin) cleavage of precursors to shorter forms apparently increases biological activity.

Immunocytochemistry has provided an important clue about the possible role of several gut peptides by the demonstration that they are located in nerve fibers and cell bodies (5,16,18,19). Mammalian bombesin-like peptides, enkephalins, TRH, and VIP may be found exclusively in nerves and are thus likely to function primarily as neuropeptides to influence structures adjacent to their sites of innervation, although a circulatory neuroendocrine role cannot be excluded. Other peptides have been found both in nerves and in well-defined endocrine cell populations. These include somatostatin and substance P. There is recent evidence that CCK peptides, gastrin, and motilin also may exist in nerves as well as endocrine cells and that some forms of VIP and the enkephalins may exist in endocrine cells as well as nerves. In the case of bombesin-like peptides, endocrine cells are easily demonstrated in amphibian and avian stomach, but our own work suggests that similar peptides are present only in nerves in the mammalian gut (9).

Endocrine-type cells have been shown to contain CCK, gastrin, GIP, glucagon, enteroglucagon, motilin, neurotensin, PP, somatostatin, secretin, and substance P. The cells that contain these peptides have characteristic patterns of distribution and ultrastructural appearances that have permitted classification of the cell types that contain each peptide. Uncertainties remain about the occurrence of certain other peptides in endocrine cells including gastrin–CCK tetrapeptide, bombesin-like, VIP-like, enkephalin-like, and adrenocorticotropin- (ACTH)-like peptides. Some reasons for differences in results obtained by different workers will be discussed in later papers. False

positive immunocytochemical reactions have been reported for certain gut endocrine cells such as antral gastrin cells that appear under some circumstances to bind immunoglobulins or immunoglobulin-complement complexes in a nonspecific manner. Antisera differ in patterns of specificity and serum from immunized animals may contain several antibodies with differing patterns of specificity. Only one antibody may occur in titers high enough to be revealed by radioimmunoassay, whereas less abundant antibodies may contribute to immunocytochemical reactions. Different methods of fixation may mask some immunoreactive sites and also may dissolve rather than fix certain highly soluble peptides. Radioimmunoassay of tissue extracts combined with chromatographic separation procedures is a high desirable adjunct to immunocytochemical studies. Here again there are possible pitfalls, including differences in efficiency of extraction in various solvent systems and variations in immunological cross-reactivity that may produce misleading estimates of the proportions of large and small molecular forms of a peptide. Demonstration of immunoreactive material in muscle layers of the gut from which the mucosa has been removed is good evidence for neural localization but does not distinguish the neuropeptides that are present predominantly in the submucous plexus and mucosal nerve fibers. Use of well-characterized monoclonal and region-specific antisera may obviate some of the difficulties in interpretation of immunocytochemical results, but such antibodies are not currently available for these peptides. Purification and chemical determination will ultimately be required to prove the nature of each molecular form tentatively identified by immunological methods.

Additional information may be provided by isolation of separated cells and of messenger RNA and cDNA from endocrine cells. Successful extraction of peptide from a distinctive cell fraction will provide evidence that the peptide is not derived entirely from neural elements. Isolation of specific messenger RNA from tissue extracts should permit direct determination of the biosynthetic precursors of the gut hormones either by sequence analysis of the peptides produced by cell-free synthesis with the messenger RNA or by isolation and sequence analysis of cDNA. Agarwal and co-workers recently reported the use of a synthetic deoxynucleotide probe deduced from a known amino acid sequence of gastrin as a primer for synthesis by reverse transcriptase of cDNA for gastrin in the presence of partially purified mRNA (28). Nucleotide sequencing of the cDNA product revealed the correct structure (as recently revised) for the amino terminal portion of G34 plus an amino terminal extension not previously described. The cDNA hybridized to RNA that contained enough base triplets to code for a peptide that could contain more than 200 amino acid residues. Further studies with this technique may lead to identification of a large precursor for the gastrin molecule. Moody and co-workers already have obtained evidence that a peptide containing 100 amino acid residues, glicentin, contains the amino

acid sequences of both pancreatic glucagon and at least one form of enteroglucagon (24). The implications of such studies have not yet been fully realized. It is likely that intracellular processing of peptide precursors leads to production of peptide fragments that have not yet been identified. It is possible that more than one active peptide may be produced from a single precursor, as in the case of pituitary β-lipotropin. Some of the gut peptides may be stored in precursor forms that are not immunoreactive with antisera directed against the fully processed products. Knowledge of the intracellular steps in synthesis and processing for each peptide may be necessary before immunological data can be interpreted fully.

Some tentative conclusions about peptide function can be reached based on information available from chemical, immunological, and physiological data. Those peptides that are present primarily in endocrine cells and released into the circulation during appropriate time periods in the digestive cycle, at concentrations sufficient to produce effects on the digestive system that can be reproduced by exogenous infusions of equivalent amounts of peptide, are likely to function as true hormones (13). There now is good evidence that gastrin, insulin, glucagon, and secretin have specific endocrine functions. There is reasonable evidence that GIP, PP, and motilin also function as circulating hormones. The same conclusion is likely for CCK, but radioimmunoassay data are not yet reliable. Neurotensin, enteroglucagon, and somatostatin are released by food components, but it is not yet known if the amounts present in the circulation are sufficient to cause measurable effects on the gut.

The possible functions for neuropeptides are more difficult to determine. Gut neuropeptides have diverse pharmacologic actions and overlapping distributions. Proof of specific function is not yet available. Evidence in favor of such functions includes storage in secretory granules in nerve endings, release by electrical stimulation of nerves, demonstrable action on neurons, and release from brain synaptosomes by calcium-dependent stimulation with extracellular potassium (36). Specific high-affinity receptors for several neuropeptides have been identified on pancreatic acinar cells and in the brain, but it is not known under which conditions these receptors might be activated *in vivo*. Specific inhibitors of receptor binding are not available for any peptides except the enkephalins. Studies with naloxone administration have shown effects on gastric and pancreatic secretion but are difficult to interpret (32). Specific mechanisms for uptake and degradation of neuropeptides in the region of gut nerve endings generally are insufficiently documented. The best evidence for a specific role for a gut neuropeptide probably is offered by the observation that receptive relaxation of the upper stomach is accompanied by a parallel increase in VIP in the venous drainage of this region and that similar relaxation can be produced by small doses of VIP (11). Until specific antagonists are developed, it appears that a large number of ingenious experiments will be required to build evidence for specific functions of neuropeptides in the gut.

Morphologists have provided a good structural basis for possible paracrine function of at least one peptide, somatostatin. The cellular localization in pancreatic islets and the demonstration by Larsson of long cytoplasmic processes extending from antral somatostatin cells to the vicinity of gastrin cells support the idea that this peptide may act on adjacent cells without a requirement that it enter the circulation (20). There is some evidence from studies with preparations of isolated perfused stomach that peptides that inhibit antral gastrin release produce a parallel increase in somatostatin release (4). However, no data definitively proves that somatostatin or any other gut peptide acts entirely by diffusion through tissue rather than via the circulation.

It is appropriate that this volume is directed at a cellular and morphological approach to investigations of gut peptide function. These studies will provide the clues on which future investigations should be based. Much work is needed before any unifying hypotheses can be set forth for the roles of the various peptides, particularly the neuropeptides, in regulation of gut function.

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The Diffuse Neuroendocrine System: Falsification and Verification of a Concept

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INTRODUCTION

Karl Popper (22), denying the existence of verification procedures for establishing the truth or falsity of scientific theories, declares that such can only be validated by their ability to resist falsification. This harsh doctrine is softened by the views of T. S. Kuhn (7), who declares that "anomalous experiences" do not necessarily equate with falsifications and that the two processes, falsification and verification, cannot be compressed into one. "Verification," he says, "is establishing the agreement of fact with theory."

On that basis, I propose to examine the present status of the so-called APUD (amine precursor uptake and decarboxylation) concept and of the diffuse neuroendocrine system that derives from it.

THE DIFFUSE NEUROENDOCRINE SYSTEM (DNES)

The DNES comprises the cells of the APUD series, presently some 40 in number, which are divided into central and peripheral divisions (17,18). The first of these two divisions contains the cells of the hypothalamo-pituitary axis and pineal gland; the second takes in all the cells situated outside these regions. Most of the cells of the second division are located in the gastrointestinal tract and pancreas, where they comprise the gastroenteropancreatic (GEP) endocrine cells (4). The APUD cells are in fact much more widespread than this, particularly in fetal life, being present in the respiratory and urogenital tracts, in the thyroid and parathyroid glands, in the adrenal medulla and accessory chromaffin tissue, and in the sympathetic

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nervous system itself. Interest has been concentrated, in general, on the GEP endocrine cells, for a number of reasons in addition to the obvious one of numerical superiority.

It should be made clear, at this point, that all the APUD cells are considered to arise in the embryo from neuroendocrine-programmed epiblastic ancestors and to derive their essential characteristics and functions directly from this fact.

In Tables 1 and 2 are listed the cells of the central and peripheral divisions of the DNES, together with their principal peptide and amine components. The coincident possession of both amine and peptide was the basis of the original concept, first proposed in 1966 (16).

The Central Division of the DNES

As can be seen in Table 1, the central division of the DNES contains the peptide-producing cells of the pineal gland, the pituitary gland, and the magno- and parvocellular groups of the hypothalamus.

Since the majority of neurons in the central nervous system possess the amine-handling facility from which the term *APUD* is derived, as shown by Louis (12) in rat brain, it would be possible to embrace a much larger number of cells within the term *APUD*, especially in view of the steady advance in the number of recorded "common peptides." The latter are peptides common to the cells and processes of the central and peripheral divisions of the nervous system, on the one hand, and to the cells and processes of the central and peripheral divisions of the DNES, on the other (19). Such an extension, though logical, has no particular merit in practice. Indeed, if falsifications of the concept are accepted as paramount, the extension itself is falsified. If, on the other hand, its verifications are able to sustain the concept and neuroendocrine function is found, as seems likely, to extend through the entire nervous system, it will be expedient to restrict the appelation APUD to the existing divisions.

If we consider the origins of the individual cells of the central division (Table 1), there is clearly no opposition to the view that the pineal gland arises as a neuroectodermal process from the roof of the diencephalon or that the hypothalamus derives from its alar plate. For many embryologists the adenohypophysis is itself to be regarded as a placode, and this view is consistent with the findings of Takor-Takor and Pearse (25).

The Peripheral Division of the DNES

Before discussing the possible origins of the main component of the peripheral division, the GEP endocrine cells, it may be as well to provide, as in Table 2, a more or less complete list of all its constituent cells.

Considering first the origins of the non-GEP members of the second division, the cells of the adrenomedullary, sympathetic, melanin-producing, carotid body, and thyroid–ultimobranchial groups have all been shown to be

TABLE 1
The Central Division of the DNES[a]

Cell type	Peptide product	Amine product(s)
Pineal	AVT, LRH, TRH, α-MSH	MT, 5-HT
Hypothalamic m.c.	AVT, AVP, SST	
Hypothalamic p.c.	RH's, RIF's, β-LPH, ACTH[a], α-MSH[a], INS, AVP[sc], PP[na], Subs.P[am], NT[po]	DA[pa, a] NA, 5-HT[a]
Pituitary p. dist.	FSH, LH, TSH, STH, PRL, ACTH, α-MSH, β-LPH, β-endorphin, gastrin, NT	DA NA 5-HT
Pituitary p. int.	β-LPH, ACTH, α-MSH, β-endorphin, (calcitonin), Met/Leu-enkephalin	H (T)

[a]Key to abbreviations: RIF's, release-inhibiting factors; RH's, releasing hormones; AVP, arginine vasopressin; AVT, arginine vasotocin; LRH, lutropin-releasing hormone; 5-HT, 5-hydroxytryptophan; DA, dopamine; NA, noradrenalin; H, histamine; m. c., magnocellular; p. c., parvocellular; p. dist., pars diatalis; p. int., pars intermedia; INS, insulin; NT, neurotensin; SST, somatostatin; a, N. arcuatus; pa, N. paraventricularis; sc, N. suprachiasmaticus; am, amygdala; po, N. preopticus; na, N. acumbens; T, tyramine; MT, melatonin; β-LPH, beta lipotropin; ACTH, adrenocorticotropin; α-MSH, alpha melanotropin; TRH, thyrotropin-releasing hormone; PP, pancreatic polypeptide; Subs. P, substance P; FSH, follitropin; LH, lutropin; TSH, thyrotropin; STH, somatotropin; PRL, prolactin.

derived from neuroendocrine-programmed cells arising, at various levels, in the definitive neural crest. Complete proof of such an origin, for most of the cells in this part of the list, derives from the elegant "biological marker" studies of Le Douarin (8) and her colleagues (9,10,11). The endocrine cells of the parathyroid gland, usually considered to arise from the endoderm of the third and fourth pouches (pharyngeal), have been shown in *Rana temporaria* to arise from the ectoderm of the corresponding pharyngeal grooves (20).

There are three morphologically distinct APUD cell types in mammalian fetal lung (5) and one of these types contains bombesin-like immunoreactivity (26). Finally, we must note the APUD cells of the urogenital tract, for which no definitive origin has yet been proved, although, in the chick embryo, cells from the neural crest have been traced to the blastema of the metanephros (3).

Thus the balance of evidence, for the non-GEP cells of division 2 of the DNES, enumerated earlier, is clearly on the side of verification. The situation is quite otherwise for the 18 or 19 GEP endocrine cells, as Table 3 shows. In this table are listed the principal falsifications of the APUD concept, giving in each case the reference and a précis of the authors' conclusions.

The first two papers referred to in the table, and some others published at earlier dates, appear to provide conclusive evidence that the GEP endocrine cells are not derivatives of the neural crest and that they cannot possibly be

TABLE 2
The Peripheral Division of the DNES[a]

Cell type		Peptide product	Amine product(s)
Pancreas	B, A, D, PP (F)	Insulin, glucagon, SST, β-endorphin, PP	5-HT, DA
Stomach	G,	Gastrin, enkephalin, ACTH	—
	AL,	Glucagon	
	EC_1	Substance P	5-HT
	EC		
	D	Somatostatin	H-
Intestine	EC_1	Substance P	5-HT, MT
	EC_2 (M)	Motilin	—
	L, S,	Glicentin, secretin	—
	I, BN	CCK, bombesin,	—
	D, K	Somatostatin, GIP	—
	NT	Neurotensin	—
Lung	K	Bombesin	DA, NA
Parathyroid	Ch.	Parathyrin	—
Adrenomedullary	A, NA	Met-enkephalin	A, NA
Sympathetic	Ga.	VIP, somatostatin	NA, DA
	SIF	—	NA
Carotid body	Type 1	Met-enkephalin	DA, NA
Melanoblast/cyte	—	—	Promelanin
Thyroid/UB	C	Calcitonin, SST	5-HT
UG tract	EC	—	—
	U	—	—
Skin	Merkel	Met-enkephalin	—

[a]Key to symbols: EC, enterochromaffin; ECL, enterochromaffin-like; PP, pancreatic polypeptide; SIF, small intensely fluorescent; Glicentin (formerly enteroglucagon); K, Kultschitzky (Feyrter); UB, ultimobranchial; UG, urogenital; Ga, ganglion. Other symbols as in Table 1.

TABLE 3
Falsifications of the Concept

Author(s)	Conclusions
Fontaine and Le Douarin (1977) (ref. 3)	Gut endocrine cells "do not originate from the neuroectoderm"
Pictet *et al.* (1976) (ref. 15)	"Common endodermal precursor" for exo- and endocrine cells of the pancreas
Cheng and Leblond (1974a) (ref. 1)	Gut endocrine cells "do not come from nervous system or neural crest"
Cheng and Leblond (1974b) (ref. 2)	Crypt-base cell origin for all four epithelial cell types
Melmed *et al.* (1972) (ref. 14) and Melmed (1979) (ref. 13)	Neuroectodermal origin of islet cells "largely refuted"
Rawdon *et al.* (1979) (ref. 23)	Enterochromaffin cells and other gut endocrine cells "not derived from ectoderm"

derived from neuroectoderm. All seven papers provide more or less complete falsifications of the concept except for the proviso added by Pictet and his colleagues to their paper (15), in which they say "our finding does not preclude an ectodermal origin for the endocrine cells of the gut, since these precursors may have colonized the endoderm earlier in development."

Neuroendocrine Markers

A search for specific markers of neuroendocrine determination has occupied the best part of the decade, in my laboratory as well as in several others. Originally based practically exclusively on the acronymous amine-handling characteristics of the APUD cells, the concept of their neural origin has been supported, more recently, by evidence derived from their possession of a neuronal specific enolase (NSE). This property was first demonstrated immunocytochemically by Schmechel, Marangos, and Brightman (24), who reported, in man and monkey, that APUD cells belonging to both central and peripheral divisions of the DNES contained this enzyme. Among the cells that were demonstrated by this means were those of the adrenal medulla, the endocrine pancreas, the pituitary and pineal glands, and the thyroid C cells. The list has been extended to embrace the endocrine cells of the stomach and intestine (21) and also of the lung. At the time of writing, no exceptions have been found with respect to any of the APUD cells listed in Tables 1 and 2 (except the parathyroid), but there are wide variations in the level of NSE contained by individual APUD cells. [In fact, unlike most neurons but in common with many interneurons, they contain both NSE and one of the nonneural enolases, the NNE of Schmechel and his colleagues (24), an isoenzyme of NSE.]

The presence of NSE in a neuron is an expression of its neural determination, whereas in an APUD cell it can be interpreted as evidence for a neuroendocrine determination. NSE is thus a specific marker for the latter quality, exemplifying precisely the property sought by Gardner and Rossant (5) when they observed, with regard to the problem of determination during embryogenesis, that "the development of methods of detecting molecules specific to particular types of differentiated cells at the single cell level in situ would avoid many of the problems inherent in present methods of analysis."

An alternative explanation for the presence of NSE in APUD cells located in endodermal derivatives like gut, pancreas, and lung would necessarily invoke dedifferentiation of an endodermal precursor cell to express the gene coding for the neural isoenzyme. This explanation appears to be less lucid than the other.

Conclusions and Implications

The evidence derived from the use of molecular marker systems, including that derived from earlier work on amine precursor uptake and decarboxylation, provides a positive refutation of the falsifications listed in Table 3, and of many minor ones not so listed. "Verification," says Kuhn (7),

"is like natural selection; it picks out the most viable among actual alternatives." Thus the balance of available evidence supports the establishment of the DNES, specifically its peripheral division and the minor part of the central one, as a *third division of the nervous system.*

The way is now clear for the propagation of a series of investigations to discover the precise degree of integration between the autonomic and endocrine branches of the nervous system and the manner in which they affect, modulate, or control all the functions of the GEP system and of related systems throughout the body. It is already clear enough that control of such functions, formerly considered to be the exclusive province of the adrenergic and cholinergic branches of the autonomic system, in fact depends on the integration of an increasingly complex peptidergic or polyergic nervous system with the peptide and amine-secreting cells of the peripheral division of the DNES. Identical or very similar peptides, used in both systems, thus function equally as neurotransmitters, neurohormones, hormones or local hormones, and their effects must depend to a large extent, therefore, on their mode of delivery (i.e., neurocrine, neuroendocrine, endocrine, or paracrine) and on the nature and number of their target receptors.

Whatever its precise role may eventually be shown to be, the third division of the nervous system must be considered to participate in every aspect of the neural control of organs and tissues. This conclusion need not wait on the provision of a satisfactory embryologic explanation for the neuroendocrine programming of the major component of the peripheral division of the DNES, the so-called endocrine cells of the gastroenteropancreatic regions.

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Phylogeny of the Gastroenteropancreatic Neuroendocrine System: A Review

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Several classical endocrine concepts have recently been overturned by the discovery of the neuronal localization of most gut hormones in the brain of higher vertebrates (27, 28). Even typical islet hormones, like insulin, glucagon, and pancreatic polypeptide (PP), seem to belong to these neurohormonal gastroenteropancreatic (GEP) peptides with dual localization in the brain and the GEP endocrine system (cf. 15). The same applies to several typical gut hormones, for instance, secretin (32) and cholecystokinin (CCK) (35). In a recent review of the evolution of the GEP neuroendocrine system (44), it was stated that the increasing number of GEP neurohormonal peptides found in the nervous system of invertebrates indicates that neuronal production of GEP peptides represents a genuinely primitive mechanism and that the seemingly unexplained presence of such peptides in the mammalian central nervous system (CNS) may be an atavistic feature. The probability that the GEP hormonal peptides have a neuronal ancestry has obtained a strong support from the discovery that their cells of origin in the gastrointestinal (GI) tract contain a neuron-specific enolase, previously thought to be confined to the nervous system (33, 38).

It was against this background that an investigation of the occurrence of some GEP neurohormonal peptides in the brain and the alimentary tract of

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lower vertebrates and invertebrates started as a complement to previous studies of the evolution of the islet hormones (12, 18, 20, 23, 44). This report gives an updated version of a recent review of the evolution of the GEP neuroendocrine system (44) and includes an account of some preliminary observations on the phylogeny of gastrin–CCK–caerulein and neurotensin (36, 37). Lastly, in various parts of the review a brief summary is made of preliminary results of a current research project in insect larvae on the distribution in the alimentary tract and nervous system of cells and nerve fibers showing immunoreactivity with antisera against mammalian GEP neurohormonal peptides (11).

Insulin, Somatostatin, Glucagon, PP

In previous studies on the phylogeny of the four islet hormones (12, 18, 20, 23, 44) a stepwise evolution of the endocrine pancreas was observed that could be clearly illustrated by choosing the following species (see tabulation below):

Protostomian invertebrates	
Mollusca:	*Mytilus edulis* (edible mussel)
	Buccinum undatum (whelk)
Deuterostomian invertebrates	
Tunicata:	*Ciona intestinalis* (sea squirt)
Cephalochordata:	*Branchiostoma lanceolatum* (lancelet or Amphioxus)
Vertebrates	
Cyclostomi:	*Myxine glutinosa* (Atlantic hagfish)
	Lampetra fluviatilis (river lamprey)
Holocephali:	*Chimaera monstrosa* (rabbit fish)
	Hydrolagus colliei (ratfish)
Elasmobranchi:	*Squalus acanthias* (spiny dogfish; spur-dog)
	Raja radiata (starry ray)
Teleostei:	*Cottus scorpius* (daddy sculpin; bull-rout)
	Raniceps raninus (tadpole-fish)

Whenever possible, the occurrence of GEP neurohormonal peptides was investigated as a correlative study, employing both immunohistochemical procedures and radioimmunoassays (RIA). Another objective was to verify and supplement the observations made by means of immunological methods with nonimmunological techniques, such as conventional light and electron microscopy (23, 44), and chemical isolation of the neurohormonal peptide in question, and with experimental investigations of its biological, biochemical, and biophysical properties, as done in the past for hagfish insulin (12, 20).

INVERTEBRATE ALIMENTARY TRACT

In all invertebrates studied, where insulin, somatostatin, and glucagon–gastrin immunoreactive cells were observed in the mucosa of the alimentary tract, the peptide-storing cells were of open type and widely

disseminated (1, 23, 44). Recently, we have got some immunocytochemical evidence that also PP cells occur in the digestive tract of the Amphioxus (M. Reinecke, S. Van Noorden, and F. Falkmer, unpublished) but, so far, not in other strains of invertebrates. None of these GEP neurohormone immunoreactive substances has been isolated and closely studied biochemically. Thus it is not known how they differ from the vertebrate hormones. This was the original situation in the evolution of the endocrine pancreas.

INVERTEBRATE NERVOUS SYSTEM

Insulin, somatostatin, glucagon, and PP immunoreactive cells have been recently observed in the nervous system in protostomian and deuterostomian invertebrates (9, 10, 15, 44). In an attempt to find an evolutionary stage at which it could be determined whether the GEP neurohormonal peptides appear first in the CNS or in the alimentary tract, an immunohistochemical study was started in the fifth instar larvae of a highly developed protostomian invertebrate, namely, an insect, the hoverfly, *Eristalis aeneus* (11). When antisera raised against mammalian GEP neurohormones were used, an astonishingly large number of immunoreactive cells and nerve fibers were found in the brain and the fused ventral ganglia, whereas no immunoreactive structures could be detected in various parts of the digestive tract. Among the neurohormonal peptides supposedly produced and/or stored in these nervous structures were all four islet hormones. This observation gives additional support to the hypothesis outlined in the introduction that the islet hormones all have their phylogenetic origin in the CNS (44).

Insulin immunoreactivity was discovered in a few aldehydefuchsinophil oval-shaped cells in the pars intercerebralis area of the brain but not in nerve fibers (11). They were probably identical to the previously described insulin cells in the brain of the blowfly, *Calliphora vomitoria* (9). The blowfly insulin-like substance is the only islet hormone of the CNS for which some biochemical information is available. It was extracted with acid ethanol from the heads of some 120,000 blowflies (460 g) and further purified by gel filtration and ion-exchange cellulose chromatography. The polypeptide isolated was found to be remarkably similar to mammalian insulin, immunologically, physically, and biologically. When it was tested in membrane-binding assays, ox insulin displacement curves with steep slopes were obtained, similar to those observed in analogous experiments with insulin derivatives. This was interpreted as indicating that the blowfly brain insulin-like material was conformationally somewhat different from ox insulin (10). Its amino acid composition is still unknown.

Somatostatin immunoreactivity was observed both in cells and nerve fibers (11). The somatostatin immunoreactive cells were more widely distributed than the insulin cells, occurring in two areas of the brain and in the anterior part of the fused ventral ganglia. From the two groups of immunoreactive

cells in the brain, bundles of somatostatin immunoreactive nerve fibers extended in various directions. The cells and nerve fibers observed corresponded obviously to those previously described in an adult insect, the grasshopper, *Locusta migratoria* (7).

Glucagon immunoreactivity resembled that of the insulin-like peptide, occurring only in cells and not in fibers (11). Moreover, the glucagon immunoreactive cells were restricted to the brain only. They were rounded or polygonal cells, forming two groups in each protocerebrum hemisphere, situated posterior to the corpora pedunculata. Whether or not these glucagon immunoreactive cells represent the production site for the glucagon-like polypeptides found by RIA in other arthropods (insects, crustaceans) (cf. 44) is still unknown (11).

PP immunoreactivity was also found to be confined to oval or polygonal cells without any connection with PP immunoreactive nerve fibers. The cells, however, were observed in the fused ventral ganglia only, and not in the brain (11). These PP immunoreactive cells are obviously identical to those described in an annelid, a mollusc, and another insect (43, 44; H. Duve and A. Thorpe, personal communication).

EARLIEST VERTEBRATES (THE CYCLOSTOMES)

The first islet organ appears in the earliest vertebrates, the cyclostomes (23, 44); in the hagfish it buds out from the bile duct, forming a whitish swelling, visible to the naked eye (23). It lacks exocrine pancreatic parenchyma and consists of insulin cells (95–99%) and somatostatin cells (1–5%). The glucagon cells still remain in the mucosa of the primitive intestinal tube as cells of open type (44). Recently, we have also observed some PP cells in this location (M. Reinecke, S. Van Noorden, and S. Falkmer, unpublished), both in the hagfish and the lamprey. No insulin cells remain in the gut mucosa, but a few can still be found as cells of closed type in the bile duct epithelium together with most of the somatostatin cells (18, 19, 44). The amount of insulin in the hagfish islet organ is 1 mg/g wet weight and the somatostatin content (by RIA) 8 μg/g (18).

Hagfish insulin is, next to pig insulin, the best known of all insulins with regard to its crystal structure, amino acid sequence and tertiary structure, biosynthesis, *in vivo* and *in vitro* release, receptor binding affinity, and biological activity (12, 20). Specific antibodies against hagfish insulin have been raised and a homologous insulin RIA accomplished (13). It had been previously maintained that the prepeptide of preproinsulin appears to be well preserved throughout vertebrate evolution in terms of both length and hydrophobic character (5). The mRNA for hagfish preproinsulin has been isolated (42) and the complete amino acid sequence of hagfish preproinsulin has also been established (42). An interesting feature is that both the connecting peptide region (37 residues) and the prepeptide (26 residues) are slightly larger than ever seen before. Nevertheless, the amino acid sequence of the prepeptide is similar to that of the mammalian preproinsulins,

consistent with its important role as a leader sequence in the formation of the ribosome-membrane junction and in the vectorial discharge of the peptide (5). In contrast, the connecting peptide shows wide variations during evolution (12, 20), a fact that is difficult to understand against its presumed biological activity in a feed-back regulation of the GIP cells which occur already at the level of the cyclostomes (17).

HOLOCEPHALAN VERTEBRATES

The first exocrine pancreatic gland with islets of Langerhans of higher vertebrate type is seen in the holocephalan cartilaginous fish, considered to represent a kind of "living fossil" (*cf.* 15, 44). It is a flattened, compact organ of triangular or conical shape, attached to the spleen but widely separated from the gut (there is no stomach). Its excretory duct is several centimeters long and runs partly in parallel with the common bile duct; these ducts open close to each other on a papilla-like structure just below the esophageal-gut junction (Figure 1A). The islets are large and closely associated with ducts. They contain large numbers of insulin, somatostatin, and glucagon immunoreactive cells but no PP cells (24, 40) (Figure 2A). PP immunoreactive cells are, however, present in the gut mucosa, and occasionally also in the pancreatic duct epithelium (24, 40). Thus, at this evolutionary stage, the two-hormone islet organ of the cyclostomes has become a three-hormone parenchyma in close association with the exocrine

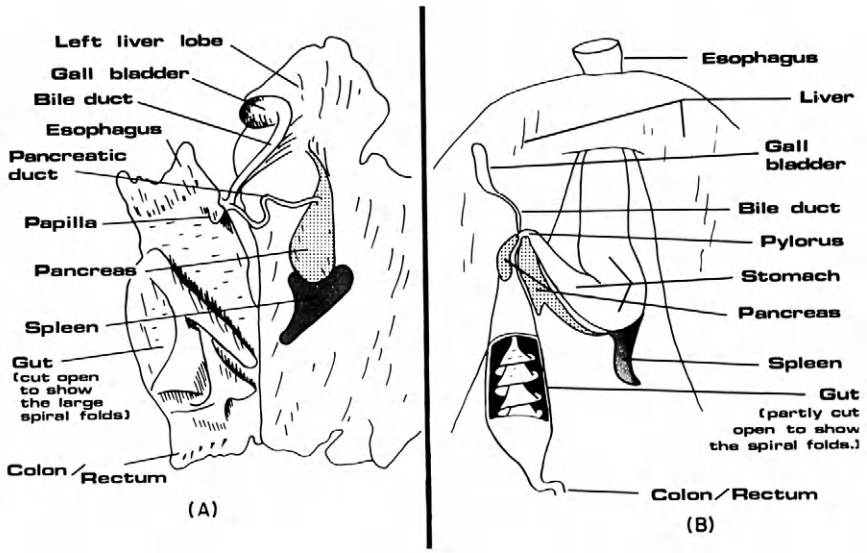

Figure 1. The gross anatomy of the GI tract, the liver with its gallbladder and bile duct, the pancreas, and the spleen, in a holocephalan cartilaginous fish (A) and an elasmobranch (B). The most important difference is the wide distance between the pancreas and the GI tract in the holocephalan fish (where a stomach is lacking) and the close apposition of the caput region of the elasmobranchian pancreas to the duodenum. Note the long slender pancreatic duct in Figure 1A.

pancreas but separated from the gut. In the gut mucosa, somatostatin and glucagon cells are also found, but no insulin cells are seen (24, 40).

ELASMOBRANCHIAN VERTEBRATES

Already at this evolutionary level the final type of vertebrate islet parenchyma appears. Now the large, branched, compact pancreas is intimately associated with the duodenum, almost encircling it (Figure 1B). Still, its caudal region is attached to the spleen. The corpus parts are closely apposed to the greater curvature of the stomach. The duct-associated islets of Langerhans have become a four-hormone parenchyma, with islet cells showing bright immunoreactivity with antisera against all the four mammalian islet hormones (24, 39, 40) (Figure 2B). In contrast to the endocrine pancreas of higher vertebrates, there are no regional differences with regard to the cellular composition of the islet parenchyma. In the gut mucosa, somatostatin, glucagon, and PP immunoreactive cells occur, but no insulin cells are seen.

TELEOST BONY FISH

Wide variations seem to exist in the structural organization of the endocrine pancreatic parenchyma in the large group of teleost bony fish. In the most highly developed species, like *Cottus scorpius*, the islet parenchyma is concentrated in one or two so-called Brockmann bodies or "principal islets." They are whitish spherical structures with a diameter of 1–2 mm. In *Cottus* one is situated close to the spleen, the other in the wall of duodenum at the pylorus (14, 16). In addition, islets of common microscopic size occur within the exocrine pancreatic parenchyma (14). Marked regional differences exist between the two "principal islets" of *Cottus scorpius* (14, 39, 41). Thus the PP cells are located exclusively in the "principal islet" close to the pylorus, whereas the other three islet hormone cells are evenly distributed in the two Brockmann bodies (Figure 2C). In both, the insulin cells occupy the central region of the parenchyma, whereas the PP and glucagon cells occur mainly in the peripheral region. The PP cells in particular have a distinctly peripheral localization (41), a fact that, since cell renewal of the principal islets seems to occur from the periphery (22), might be interpreted as an illustration that they are the last to inhabit the islet parenchyma. The somatostatin cells are found both in the central and peripheral regions. The immunohistochemical observations have been confirmed by the results of RIA of extracts of pooled "principal islets" from each of the two regions (Table 1). In the GI mucosa, somatostatin, glucagon, and PP cells occur, but no insulin cells are seen. (*cf.* 44).

A regional difference in the distribution of PP cells has also been observed in the endocrine pancreas of some mammals (e.g., rat, dog, and man) (31, 34). The general pattern is similar to that described earlier in the *Cottus* islet parenchyma, namely, that PP cells occur preferentially in those parts of the

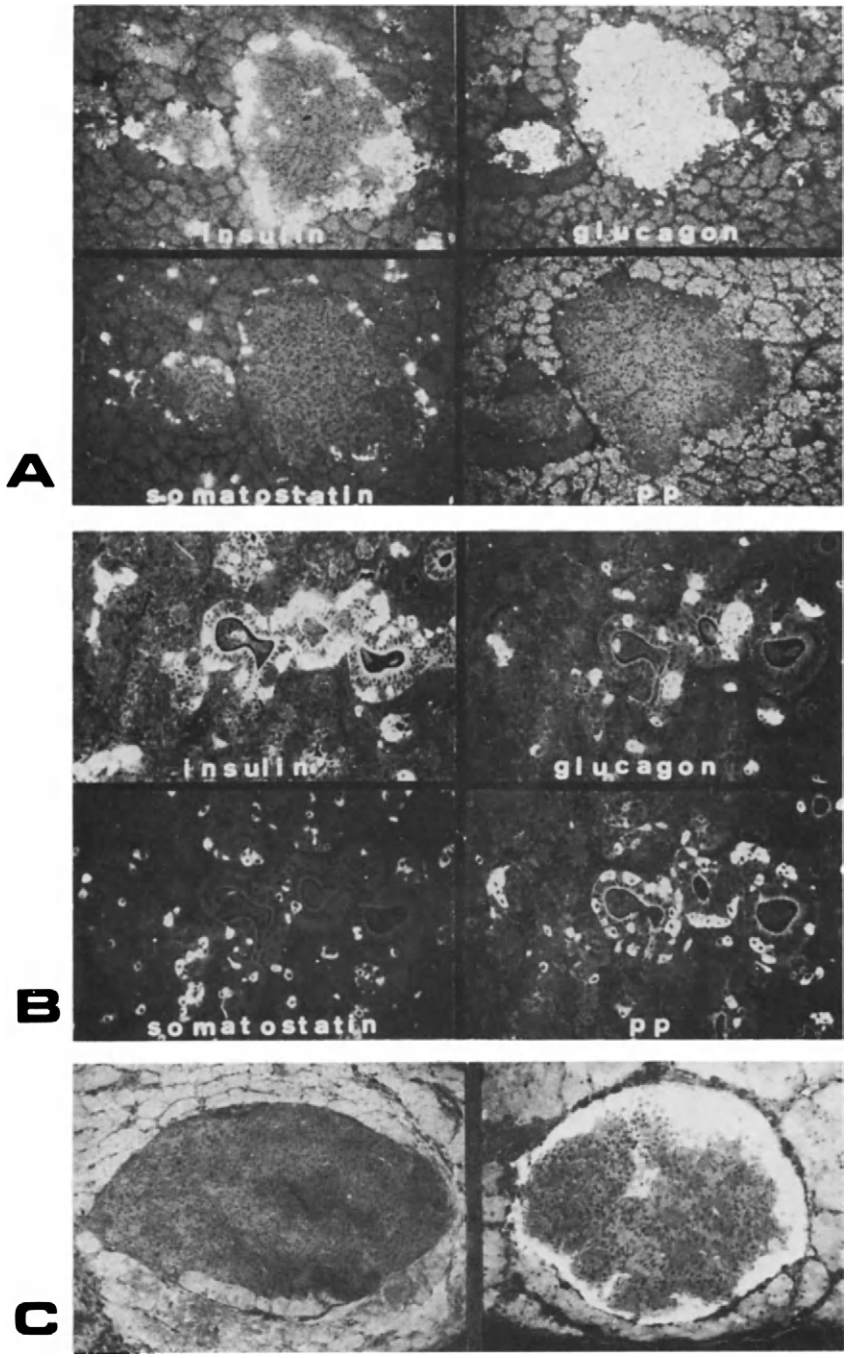

Figure 2. Immunofluorescence medium-power photomicrographs of consecutive serial sections of pancreatic islets in a holocephalan cartilaginous fish (A) and an elasmobranchian (B). The sections have been incubated with antisera against the four islet hormones of mammalian origin. The lower-most panel (C) shows immunofluorescence photomicrographs of two small satellite islets around the giant Brockmann bodies or "principal islets" in the teleost fish, *Cottus scorpius* (the daddy sculpin), one from the vicinity of the spleen (left), the other from the pyloric region. Both have been incubated with antisera against oxPP. Panels A and B demonstrate that the islet parenchyma evolves from a three-hormone organ in the holocephalan fish to a four-hormone organ in the elasmobranch. Panel C shows that PP immunoreactive cells only occur in the teleostean islets close to the GI tract and not in those close to the spleen. (From work done together with Yolande Stefan.)

endocrine pancreas in close contact with the duodenum, possibly derived from the ventral anlage, and supplied by a separate main branch of the visceral arteries (39, 41). This observation may shed some light on the physiological function of PP, which is still essentially unknown (24), and also on the ontogeny of the mammalian endocrine pancreas (31, 34).

Cottus scorpius is a fish readily available in large quantities in the circumpolar half of the Northern Hemisphere (14). Its two Brockmann bodies can be rapidly excised and they do not contain any admixture of exocrine parenchyma inside their connective tissue capsule (14, 16). Their contents of insulin and somatostatin are the highest known for any anatomical structure, so far (18). Judging from immunocytochemical observations (41), their glucagon and PP contents are also high. Consequently, they represent an easily available source of the four islet neurohormones, allowing several kinds of investigations, for instance, biochemical studies like those made with the hagfish islet organ (12, 16, 20) or other kinds of experimental diabetes research (2, 14). The concentration of several peptides in hagfish and sculpin islet parenchyma are given in Table 1.

SUMMARY AND CONCLUSIONS

The four islet hormones are obviously phylogenetically old. They all seem to appear first in the CNS. Later on, but still fairly early, they somehow come to inhabit the digestive tract, first as cells of open type, widely disseminated in the mucosa, and later on as cells of closed type in the bile duct or pancreatic duct epithelium, budding out to form the vertebrate islet organ. Their order of appearance in the vertebrate islet parenchyma is (1) insulin, (2) somatostatin, (3) glucagon, (4) PP. In the most highly differentiated endocrine pancreas the islet PP cells are located close to the gastrointestinal (GI) tract.

SECRETIN, VIP, GIP

The members of the secretin family seem to differ considerably in their phylogenetic age. Thus whereas secretin and vasoactive intestinal polypeptide (VIP) immunoreactivities have been described in nervous tissues of invertebrates (*cf.* 44), gastric inhibitory polypeptide (GIP) seems to be a hormone restricted to the upper GI tract of vertebrates (17).

A confirmation of this suggested difference in phylogenetic age has recently been obtained from preliminary observations of secretin immunoreactive cells and nerve fibers in both the brain and the fused ventral ganglia of the flowerfly larva (11). In the brain, a group of seven to nine cells in each protocerebrum hemisphere was observed, located posteriorly to the corpora pedunculata, where the secretin immunoreactive nerve fibers occurred. The cells in the ventral ganglia were detected in the anterior part outside the central neuropile (Figure 3). No secretin immunoreactive cells or nerve fibers were found in the digestive tract (11).

At this evolutionary stage no VIP or GIP immunoreactive cells or nerve fibers could be detected, either in the CNS or in the digestive tract. The

TABLE 1
Islet Hormone Contents in the Islet Organs of the Atlantic Hagfish and the Daddy Sculpin

Phylum (subclass)	Species	Type of islet organ	Insulin[a]	SOM[b]	GLI[c] "11–15"[c]	GLI[c] "24–29"[c]	PP[d]
Cyclostomi (Myxinoidei)	*Myxine glutinosa* (Atlantic hagfish)	(Bile duct origin)	1000	8	—	—	—
Osteichthyes (Teleostei)	*Cottus scorpius* (Daddy sculpin)	"Splenic"	1040	212	119	22	0
		"Pyloric"			126	8	0.2

[a]Yield (μg/g wet weight) of monocomponent hormone (18).

[b]SOM, somatostatin. RIA (synthetic SOM) (μg/g wet weight) (18).

[c]GLI, glucagon-like immunoactivity. GLI 11–15; RIA (avian glucagon + gut GLI); GLI 24–29: RIA (mammalian glucagon). Both are given in μg eqv/g wet wt.). From S. Falkmer, A. J. Moody, and F. Sundby, unpublished.

[d]PP, pancreatic polypeptide. RIA (ox PP) (μg/g wet weight). From J. R. Kimmel, H. G. Pollock, and S. Falkmer, unpublished.

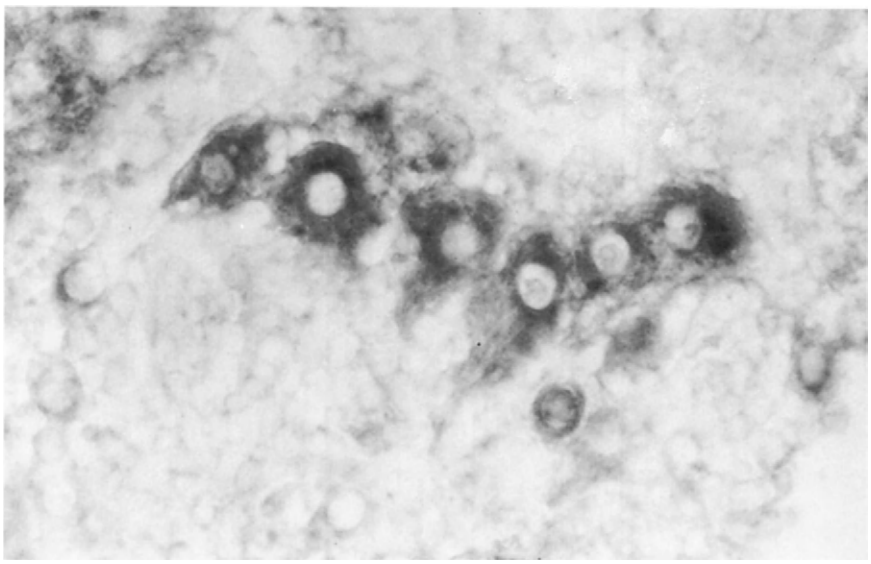

Figure 3. Medium-power photomicrograph of a PAP-stained section of the brain of the fifth instar larva of the hoverfly, *Eristalis aeneus*, after incubation with antisera against mammalian secretin (11). Distinctly immunoreactive nerve cells (as shown in the photomicrograph) and nerve fibres (not shown) were found, both in the brain and in the ventral ganglia (not shown).

presence of VIP or GIP in the CNS and the digestive tract of protostomian and deuterostomian invertebrates is not well established. Whereas the presence of secretin immunoreactive cells and nerve fibers, as well as secretin-like bioactivity, have been reported earlier in various nervous and digestive tissues in both protostomian and deuterostomian invertebrates (44), and now also in pig brain (32), apparently only one report exists on the occurrence of VIP cells in protostomian invertebrate nerve cells and fibers (43). However, in the gut mucosa of one of the lower vertebrates, namely, the ray, *Raja radiata*, VIP immunoreactive cells have been observed in large numbers (21).

Thus it can be concluded that of the three members of the secretin family, secretin and VIP may show the same distribution pattern during evolution as the islet hormones, whereas GIP seems to be a phylogenetically younger GEP hormone, namely, a typical representative of the true GI hormones, confined to the vertebrates and not appearing in the CNS at any evolutionary stage (17). In a pilot study no VIP immunoreactive material was discovered in either of the two principal islets of *Cottus scorpius* (J. Fahrenkrug and S. Falkmer, unpublished).

Gastrin-CCK-Caerulein

The phylogeny of the gastrin family of the GEP neurohormonal peptides is also incompletely known, and, because of the close similarities in the molecules of the various forms of gastrin, CCK, and caerulein, some controversies exist. In an earlier review (44) it was stated that it seemed as if

some kind of gastrin–CCK–caerulein-like polypeptides are produced, or at least stored, not only in the GI mucosa of all vertebrates and several invertebrates but also in the CNS of some species of both groups. Since that review was finished, two kinds of comparative studies have been started, one essentially based on RIA of tissue extracts, the other immunohistochemical.

RIA STUDY

Using both boiling water extracts and acetic acid extracts, and four different antisera, the occurrence of neurohormonal peptides of the gastrin family was studied in various parts of the digestive tract and in the CNS of lower vertebrates and some invertebrates (Table 2). One antiserum ("2604") was considered specific for gastrin, being raised against the region 2-17 of the synthetic human gastrin-17 molecule and showing no reactivity with CCK caerulein (30, 35). With this antiserum no immunoreactive substances were discovered in any of the extracts. A second antibody was directed against the central parts of the CCK-33 molecule; a third and a fourth were directed against the N- and the C-terminal CCK octapeptides, respectively (30, 35). With the latter three antisera, the results obtained were essentially similar to each other, indicating that the immunoreactive peptide found in various extracts was most closely related to CCK-8 (or caerulein). Particularly high values were obtained with the antibody directed toward the N-terminal CCK octapeptide, which was an unexpected observation. The tissue contents have been summarized in Table 2, expressed as pmol equivalents of CCK-8 per g wet weight.

TABLE 2
CCK–Caerulein-Like Immunoreactivity[a]

Species	Digestive tract mucosa				
	Stomach	Duodenum	Gut	Pancreas	Brain
Invertebrates					
Mollusca		0		n.s.p[b]	n.s.p.
Buccinum undatum					
Mytilus edulis					
Protochordate (Tunicata)		94		n.s.p.	n.s.p.
Ciona intestinalis					
Vertebrates					
Jawless fish		0		0	0
Myxine glutinosa					
Cartilaginous fish					
Squalus acanthias	0	348	342	3	336
Teleost bony fish					
Cottus scorpius	261	385	109	n.t.[b]	80

[a]Data in pmol eqv. CCK-8/g wet weight.
[b]n.s.p., No such parenchyma present in these species; n.t., not tested.

The observations given in Table 2 need to be supplemented in several respects, for instance, by RIA of extracts of the molluscan and protochordatan invertebrate homologues of the CNS of vertebrates. Some alternative extraction procedures should also be tried in order to study the possible occurrence of other molecular forms (35). So far, however, it appears that CCK-8-like peptide is present in roughly equal concentrations in both the brain and GI tract mucosa of bony and cartilaginous fish, but not at all in jawless fish and in the two protostomian invertebrates studied. A low but significant content was found in the digestive tract of the deuterostomian invertebrate *Ciona intestinalis*, where so many other neurohormonal polypeptides previously have been found, namely, insulin, somatostatin, secretin, substance P, neurotensin, calcitonin, and adrenocorticotropin (ACTH) in the digestive tract and nerve ganglia (1, 25, 44). The minimal amounts observed in shark pancreas are probably not significant. They are in keeping with the borderline contents obtained by a biological gastrin assay used in an earlier study (2). In that study no gastrin was observed in the *Cottus* islet parenchyma (2).

IMMUNOHISTOCHEMICAL STUDY

The corresponding immunohistochemical analysis of the tissues examined in the preceding RIA study remains to be finished. Meanwhile, another immunohistochemical gastrin-CCK-caerulein investigation has been started (11) using specimens from insect larva CNS parenchyma and the alimentary tract. The background was the report by Kramer and colleagues (29) that in boiling water extracts of insect brain, but not in those of insect gut, a peptide occurred that was gastrin-like in its antigenicity, size, and susceptibility to degradative enzymes.

The antiserum raised against the 9-20 central part of the CCK-33 molecule was also used in our PAP procedure. Immunoreactive cells and nerve fibers were found in the brain by means of the "4562" antiserum specific for the C-terminus common for gastrin and CCK but not with the CCK-9-20 antiserum (11). No gastrin-CCK-caerulein immunoreactive cells or nerve fibers were observed in any part of the digestive tract. The immunoreactive cells in the CNS were rather widely distributed, forming three groups of cells in the brain and two in the fused ventral ganglia, each group consisting of two to four cells (Figure 4) (11). In contrast to the RIA observations by Kramer and colleagues (29), no gastrin-CCK-caerulein immunoreactive structures were found in the *corpus cardiacum-corpus allatum* (CC/AA) complex. An explanation for this discrepancy could be that the immunoreactive material found by RIA in the CC/CA complex (29) might have been transported there from the production sites in the brain or fused ventral ganglia (11).

It is difficult to draw conclusions from these still rather preliminary and incomplete observations, but it can be hypothesized that the members of the gastrin family of GEP neurohormonal peptides may also have originated in

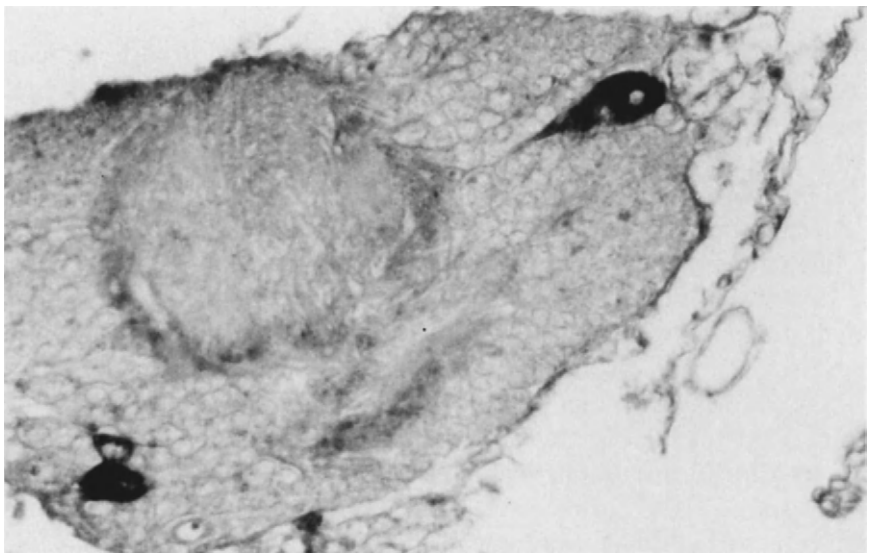

Figure 4. Gastrin-CCK-caerulein immunoreactive cell in the *protocerebrum* hemisphere of the hoverfly larva, revealed by a technique analogous to that described in Figure 3. Such cells and nerve fibres were also discovered in other parts of the brain and in the fused ventral ganglia (11).

the brain and not begun to inhabit the digestive tract mucosa until the level of the protochordates and earliest vertebrate (30). They remain as a CNS neurohormone throughout the whole of evolution (26). Further aspects on the roles of various molecular forms of gastrin and CCK as gut hormones and as neurotransmitters or neuromodulators are given elsewhere in this volume (6).

Substance P, Neurotensin, Enkephalin, β-Endorphin

SUBSTANCE P

As stated in a preceding review (44), substance P was among the first of those GEP neurohormones that were shown to occur in both the CNS and GI tract of bony and cartilaginous fish (in 1956), as well as in hagfish and sea squirt (in 1959). Later, substance P, or a closely related peptide, was also isolated from nervous tissue and digestive tract glands of some protostomian invertebrates (*cf.* 44) and even in coelenterates (26). So far, no insects or other arthropods have been studied.

When antisera raised against synthetic substance P of human type were used in the PAP immunohistochemical procedure applied on the hoverfly larva (11), immunoreactivity occurred only in the brain and not in the digestive tract or in the fused ventral ganglia. The immunoreactive nerve cells appeared in pairs in each half of the protocerebrum hemisphere, posterior to the corpora pedunculata. No substance P immunoreactive nerve fibers were found (11).

NEUROTENSIN

During the course of purification of substance P from ox hypothalamic extracts, a vasoactive substance was detected that clearly separated from the sialogogic activity of substance P. The peptide, named neurotensin (NT), was isolated, sequenced, and synthesized and a RIA was developed. It occurs in identical molecular forms both in neurons of the CNS and in epithelial cells of the gut (cf. 3,4).

The phylogeny of gut NT has recently been traced all the way down to the teleost bony fish (36). Now these studies of the phylogeny of NT have been extended to comprise the digestive tract of lower vertebrates and invertebrates (37) and to include also the islet parenchyma, pancreas, and CNS. Several observations are still preliminary; some NT contents determined by means of RIA of acetic acid tissue extracts remain to be corroborated by an immunohistochemical analysis of the same tissues.

Two antisera, "HC-8" (directed against the 7-8 C-terminal residues of the NT molecule) and "PGL-4" (directed against the 4 C-terminal residues), were used for RIA of 2 *M* acetic acid tissue extracts (3, 4). One of these two antisera (HC-8) together with one ("Neu-6") directed toward the C-terminal region of the NT molecule and one ("Xen-6") raised against xenopsin (an amphibian skin peptide, structurally closely related to NT) were employed for immunohistochemical investigations, by the PAP procedure (36, 37). The RIA contents of NT in the spectrum of tissues studied are given in Table 3, and typical examples of the immunoreactive NT cells in the GI mucosa of bony and cartilaginous fish are shown in Figures 5A and B.

The widely divergent results obtained with the two antisera used for RIA (Table 3) indicate that these crude tissue extracts from lower vertebrates and invertebrates contain several NT-like substances, each with its own PGL-4/HC-8 ratio. Methodological errors also contributed to the divergent results, as is obvious from the duplicate values given for the splenic and pyloric "principal islets" of *Cottus scorpius*. Further information on the specificity of the RIA values in Table 3 is obtained when they are compared with the observations made by quantitative immunohistochemistry (36). As partly reported elsewhere, the number of NT immunoreactive cells found in the digestive tract was almost identical with the HC-8 and Neu-6 antisera and only about 15% higher with the Xen-6 (37).

By the PAP procedure it could be confirmed that the highest density of NT immunoreactive cells (24 and 18 cells/mm^2 mucosa surface in *Cottus* and *Raniceps*, respectively) was present in the pyloric–duodenal region of the two bony fish; that in the cartilaginous fish only a few NT cells occurred in the GI mucosa, mainly in the spiral valve gut (less than 1 cell/mm^2); and that only a few NT immunoreactive cells could be detected in the whole digestive tract of hagfish, amphioxus, and sea squirt (37). In the mucosa of the alimentary tract of echinoderms or molluscs no immunoreactive NT cells at all could be observed. The immunohistochemical analysis of the hagfish and sculpin islet parenchyma, the molluscan hepato-pancreas, and the CNS is still not

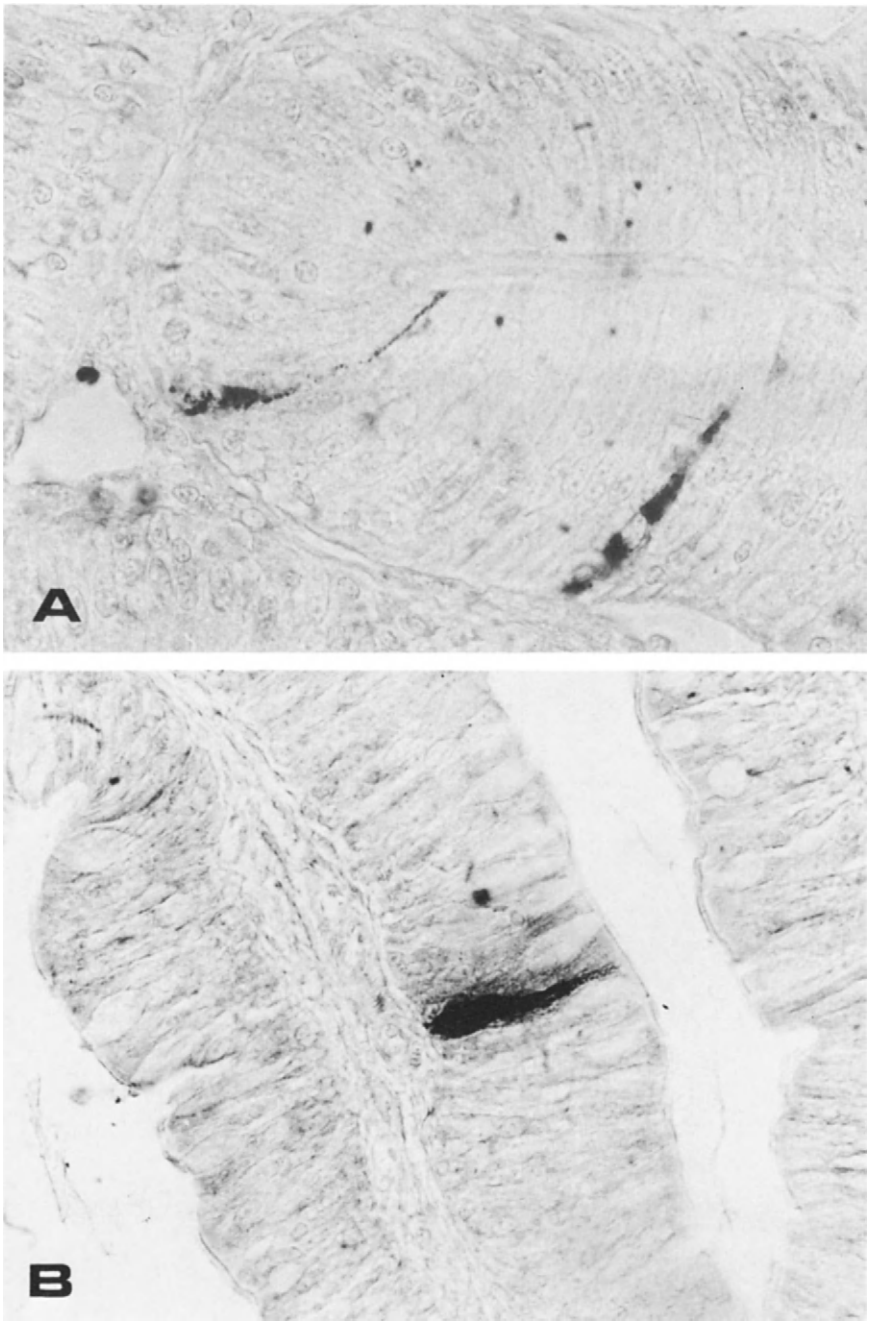

Figure 5. Neurotensin-immunoreactive cells of open type, demonstrated by the PAP technique with antisera directed to the C-terminal region of the neurotensin molecule. The cells in Figure 5A occur in the duodenal mucosa of the elasmobranchian cartilaginous fish, *Squalus acanthias* (the spiny dogfish) (antiserum HC-8), and those in Figure 5B are from the small intestine of the bony fish, *Cottus scorpius* (antiserum Neu-6).

TABLE 3
Neurotensin Immunoreactivity in Acetic Acid Extracts[a,b]

Species	Alimentary tract								Islet parenchyma			
		Stomach										
	Total	Antrum	Pyloric ceca	Duodenum	Spiral valve gut	Colon, rectum	Invertebrate hepato-pancreas	Vert. pancreas	B (+ D) cells	A + B + D cells;	A + B + D + PP cells	Brain
Invertebrates												
Molluscs												
Buccinum undatum	<0.1						1.4					
	68	—	—	—	—	—	2550	—	—	—	—	—
	—						1820					
Mytilus edulis	2.1						2.3					
	816	—	—	—	—	—	1300	—	—	—	—	—
	390						460					
Protochordate (tunicate)												
Ciona intestinalis	1.5											
	223	—	—	—	—	—	—	—	—	—	—	—
	150											

Vertebrates														
Jawless fish														
Myxine glutinosa	1.4								0.3					1.7
	1240	—	—	—	—	—	—	—	63	—		—		260
	890								210					150
Cartilaginous fish														
Squalus acanthias				<0.1	0.6	0.3		0.3						1.5[c]
	—	—	—	115	240	60	—	220	—	—		—		131
				—	400	180		730						90
					6.0			3.0						12
Raja radiata	—	—	—	—	466	—	—	39.2	—	—		—		44.5
					78			13						3.7
Teleost bony fish														
Cottus scorpius	—	4.4	3.1	8.8						1.0[d]	0.66[d]	0.8[e]	0.3[e]	0.28
		931	253	320	—	—	—	—	—	387	142	255	30	47.8
		212	82	36						390	215	340	100	171
Raniceps raninus	—	12.7	—	2.2		1.3	—							4.0
		7000		430	—	270		—	—		—		—	480
		550		200		210								120

[a] Data in pmol/g wet wt.

[b] First figure gives the RIA value with antiserum "HC-8" (7–8 C-terminal residues). Second figure gives the RIA value with antiserum "PGL-4" (4 C-terminal residues). Third figure gives the PGL-4/HC-8 ratio. A ratio of 1.0 is characteristic of mammalian neurotensin (3,4).

[c] For rhinencephalon, the HC-8 value was less than 0.1.

[d] Results of two separate experiments.

[e] Results of two separate experiments.

finished. Therefore it remains to be settled whether or not the RIA values of Table 3 reflect the actual presence of NT cells in these tissues. In immunohistochemical studies of hoverfly larva, using the same antisera, no NT immunoreactive cells or nerve fibers were observed, either in the alimentary tract or in the CNS (11).

From these observations, and from other reports (36, 44), it might be hypothesized that NT-like peptides have a dual occurrence in the CNS and the GI tract in all vertebrates. In the nervous system, NT-like peptides seem to occur already in coelenterates (C. J. P. Grimmelikhuijzen, personal communication). Moreover, they also seem to be present in the digestive tract of higher, but apparently not in that of lower, deuterostomian invertebrates, and probably not in protostomian invertebrates. NT thus seems to be of an intermediate phylogenetic age. In the GI tract, where the NT cells are of open type, three stages of evolution can be discerned in the distribution pattern (37), namely, (1) a few, widely scattered cells, evenly distributed over most of the digestive tract (in protochordates and lowest vertebrates); (2) a concentration of NT cells in the pyloric–duodenal region (in the highest developed bony fish and other submammalian vertebrates); (3) a predominant localization to the ileum (in mammals). Whether this hypothetical evolution pattern in the GI tract and/or the putative occurrence of NT in the islet parenchyma of lower vertebrates (Table 3) can give some new insights into the still poorly known physiological function of NT, remains to be seen. If the occurrence of NT in islets is true, it supports the hypothesis that NT participates in carbohydrate metabolism by controlling the release of islet hormones (8).

ENKEPHALIN, β-ENDORPHIN

The opiate peptides, enkephalin and β-endorphin, are known to occur both in the CNS and in cells and nerves of the gut of mammals, amphibia, and bony fish (44). A strong immunohistochemical reaction has also been reported with antisera against enkephalin in nerves of the posterior part of the intestine and in visceral and cerebral ganglia of a mollusc (44).

That a CNS localizations of these opiate peptides is a phylogenetically old feature is supported also by the preliminary results of immunohistochemical studies in hoverfly larva (11). Using an antiserum raised against a mixture of synthetic human Met- and Leu-enkephalins and one against synthetic human β-endorphin, groups of immunoreactive cells occurred in the pars intercerebralis region. Whether or not one and the same cell stored both enkephalin and β-endorphin—as described for other GEP neurohormones (27)—is still not known. There was, however, another group of cells located posterior to corpora pedunculata which was immunoreactive only with the enkephalin antiserum. Enkephalin–β-endorphin immunoreactive nerve fibers also were found in the CNS of the hoverfly larva, particularly in the calyx cell area (11). In the digestive tract, however, no enkephalin–β-endorphin immunoreactive structures were observed.

Thus enkephalin and β-endorphin may be additional examples of how, during evolution, GEP neurohormone-containing cells first appear in the CNS and later begin to inhabit the digestive tract mucosa as cells of epithelial type, at the phylogenetic stage of the first vertebrates.

SUMMARY

Studies of the counterparts of mammalian GEP neurohormonal peptides in lower vertebrates and in deuterostomian and protostomian invertebrates by means of immunohistochemical and radioimmunochemical procedures, supplemented by nonimmunological techniques, show that they differ from each other regarding their distribution pattern during evolution. Preliminary observations in an insect larva indicate that insulin, somatostatin, glucagon, PP, secretin, gastrin-CCK-caerulein, substance P, and enkephalin-β-endorphin all appear in nerve cells (and partly also in nerve fibers) of the central nervous system before they begin to inhabit the digestive tract. For the islet hormones the order of appearance in the digestive tract mucosa and in the islet parenchyma is (1) insulin, (2) somatostatin, (3) glucagon, and (4) PP. Gastrin-CCK-caerulein peptides remain throughout the whole of evolution as CNS peptides. They appear first in the digestive tract mucosa of the highest developed protochordates and lowest vertebrates. A similar pattern is seen as regards the phylogeny of neurotensin-like peptides. In contrast, GIP is a phylogenetically more recent GEP hormone, occurring only in the upper gastrointestinal mucosa in vertebrates. The phylogeny of the remaining GEP neurohormones is even more incompletely known. As a general rule, however, it seems that most of the vertebrate GEP neurohormones have originated in neural tissue before the development of the vertebrate lines of evolution.

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Membrane Recycling in Secretory Cells: Existence of Multiple Pathways Involving Golgi Cisternae, Secretory Granules, and Lysosomes

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INTRODUCTION

At the time of exocytosis a considerable amount of membrane derived from secretory granules is inserted into the cell membrane or plasmalemma (Figure 1). The question is, what happens to that membrane? Is it recovered and reutilized? Is it completely degraded? Or is it partially dismantled and subsequently reassembled and used again? All three ideas have been proposed. The concept of membrane reutilization or recycling was introduced over 20 years ago (24), but the experimental evidence that bears on this problem has been contradictory and confusing until recently. This chapter presents a brief summary of the work done on this topic by our group in the last few years. The presentation will be brief and can best be considered an extended abstract included in this volume to introduce readers to this problem area. Those who wish to delve deeper into the topic can consult a recent review (8) or the original references (6, 7, 14, 23) for more detailed information.

BACKGROUND INFORMATION

The question of the fate of the granule membrane after exocytosis has been investigated in two ways: (1) the turnover of the components of the granule membrane has been determined and compared to that of the granule contents and (2) various electron-dense tracers (usually ferritin and

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IN THE DIGESTIVE SYSTEM

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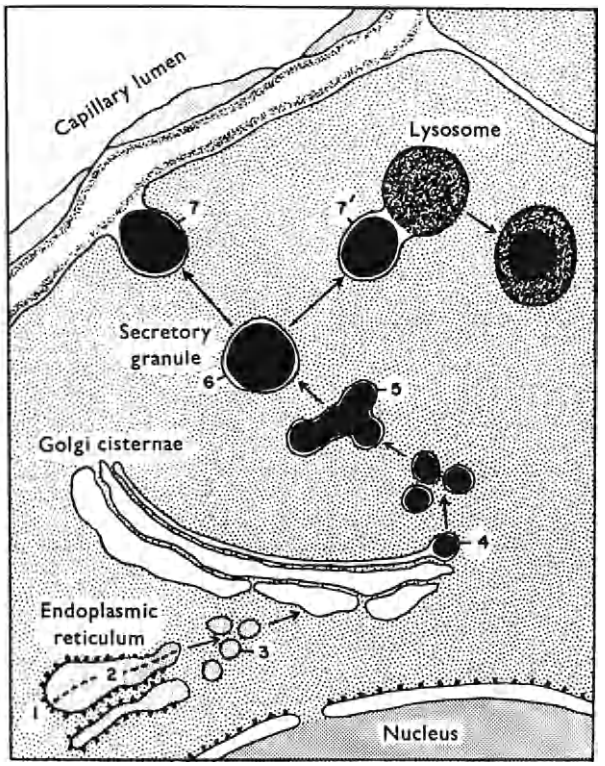


Figure 1. Diagram showing the steps (1–7) in the synthesis and secretion of prolactin by mammotrophs of the anterior pituitary gland. The basic pathway (rough ER → Golgi → secretion granules) is the same as that in other secretory cells (25). During active secretion the secretory granules fuse with the cell membrane and their content is discharged into the perivascular spaces by exocytosis (step 7 in diagram). The net result is the relocation of a considerable amount of Golgi-derived granule membrane to the plasmalemma. Evidence has been obtained (see text and refs. 6–8) that indicates that following exocytosis in mammotrophs (as well as other cells), surface membrane is recovered and reutilized. The diagram also shows that when secretory activity is suppressed (e.g., by removal of suckling young) and the cell must dispose of excess stored hormone, some granules fuse with lysosomes (crinophagy) and are degraded (step 7′ in diagram). Since the size of the lysosomes (like the overall cell size) remains fairly constant, membrane must also be continuously recovered from lysosomes to compensate for that added during crinophagy. Diagram taken from Smith and Farquhar (30). The numbering of the stages and the legend have been modified in accordance with more recent work (5, 6, 9).

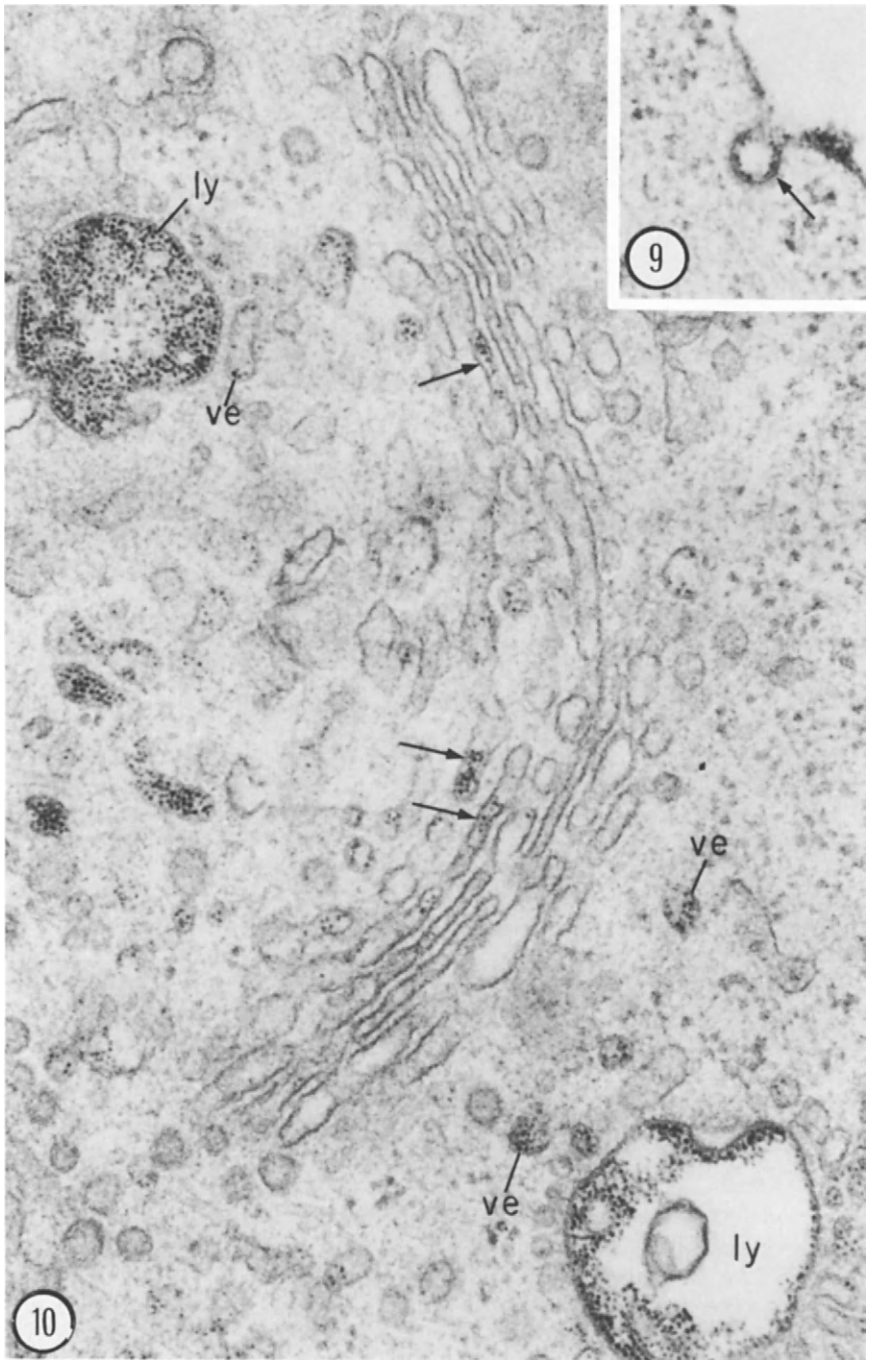
horseradish peroxidase) have been used to trace directly the fate of the membrane. The early turnover data (based on analysis of membrane preparations heavily contaminated with content proteins) indicated that the turnover of the granule membrane and its contents were similar and led to the erroneous conclusion that surface membrane was degraded rather than reutilized. The early tracer experiments clearly indicated that following exocytosis the surface membrane is recovered intact by endocytosis but led to the conclusion (also erroneous) that the recovered membrane was degraded within lysosomes (because these tracers subsequently appeared largely or

exclusively in lysosomes). Thus the early turnover data and the early tracer data appeared to be in agreement; both supported the notion that the granule membrane is recovered intact and degraded in lysosomes. As a consequence, this idea has prevailed and gained wide acceptance until quite recently [see reviews by Holtzman (18) and Meldolesi (20) and their colleagues].

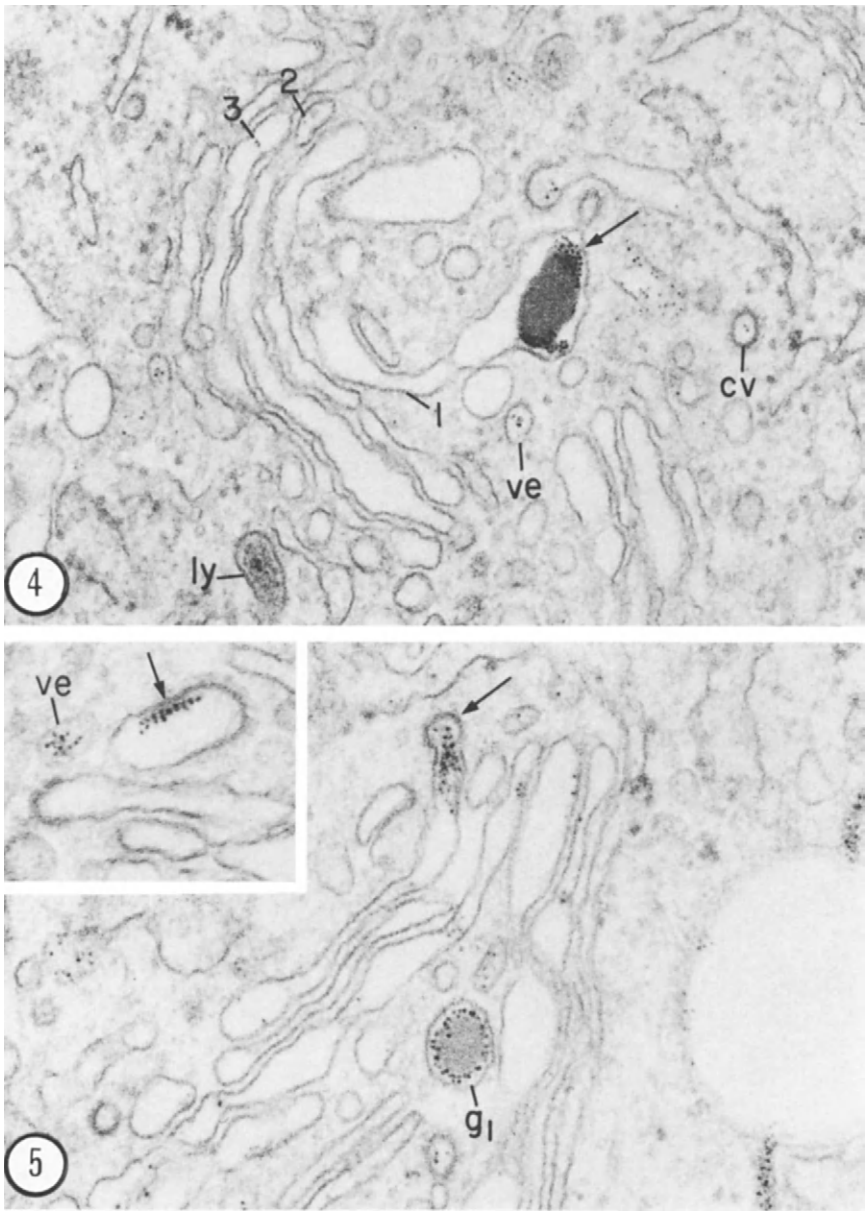
Work from Our Laboratory Demonstrating Involvement of the Golgi Complex in Membrane Recycling

Some time ago we (10 [as well as Pelletier (26)] obtained some preliminary results on anterior pituitary cells suggesting that in addition to lysosomes, other cell compartments, primarily the Golgi complex, might be involved in the recovery of surface membrane (assumed to be derived, at least in part, from secretion granules). As a result of these initial intriguing findings we began to study systematically membrane recovery in secretory cells using several newer electron-dense tracers (dextrans, cationized ferritin) that had not been used to investigate this problem previously.

With these tracers we have obtained evidence on several different glandular cell types that points to the existence of recycling of granule membranes. The evidence obtained is based on findings in nine different cell types of varied nature that include exocrine cells from parotid or lacrimal glands (14), endocrine cells of the anterior pituitary (6) (mammotrophs, somatotrophs, gonadotrophs, thyrotrophs, and corticotrophs), and immunoglobulin-secreting cells (23) (both plasma cells from normal lymph nodes and mouse myeloma cells). In all these cases we showed that following exocytosis surface membrane is recovered by endocytosis and fuses directly or indirectly with multiple-stacked Golgi cisternae as well as with lysosomes. Fusion appears to take place preferentially with the dilated rims of the Golgi cisternae where concentration of secretory products usually occurs. The tracers used to follow the fate of the surface membrane were dextrans for parotid and lacrimal gland cells and cationized ferritin (CF) for anterior pituitary and immunoglobulin-secreting cells. In all these cases the tracer was found at early intervals (2–15 min) in endocytic vesicles near the discharging surface (Figure 2), and at later intervals (1–2 h) in multiple-stacked Golgi cisternae (Figures 4–8 and 10), in forming secretory granules or vacuoles (Figures 3 and 6) and in lysosomes (Figures 4 and 10), indicating that the incoming vesicles had fused with all these compartments. The presence of tracers within forming secretion granules strongly suggests that this traffic is connected with the recycling of the membrane containers for secretory products, i.e., secretion granule or secretion vacuole membranes. It is of considerable interest that recent data obtained on improved granule membrane preparations (in which great care was taken to remove contaminating content proteins) indicate that the turnover of the granule membrane proteins is much slower than that of the content proteins, a finding that is compatible with the membrane or its constituents being reutilized. Thus at present the tracer data and turnover



Figures 2 and 3. Mammotrophs from estrogen-treated female rats incubated with cationized ferritin (CF) (0.1 mg/ml) to trace the surface membrane. Figure 2 shows that initially (after 15 min of incubation at 37°C), CF binds to the cell membrane (cm) and is taken up by endocytosis. Numerous endocytic vesicles (ve) containing CF are seen in the cytoplasm near the plasmalemma. CF is aggregated on the free cell surface (arrows) but forms a regular layer one or two molecules deep in the intercellular spaces (Is). Inside the vesicles (ve′) the CF is also aggregated; as a consequence some CF molecules are closely associated with the vesicle membrane, whereas others are not. Figure 3 shows that after longer periods of incubation (1 h at 37°C), CF is seen within immature secretory granules (g_1 and g_2) as well as in endocytic vesicles (ve). The incoming CF-labeled vesicles apparently fuse preferentially with the dilated rims of the transmost Golgi cisternae (see Figures 1 and 4) and the CF becomes trapped within the forming granules, where it is typically located at the periphery of the dense content. G = Golgi cisternae. From Farquhar (6). Figure 2, ×70,000; Figure 3, ×76,000.



Figures 4 and 5. Fields from pituitary mammotrophs incubated with CF for 60 min. Figure 4 shows CF within three Golgi cisternae (1–3). The tracer is particularly concentrated around a secretory granule forming within the transmost Golgi cisterna (arrow), suggesting that the incoming vesicles carrying CF fuse preferentially with these Golgi elements. Note that the CF is concentrated at the periphery of the forming granule adhering to its dense contents. CF is also seen within several vesicles (ve), one of which is coated (cv) in the Golgi region and within a lysosome (ly). Figure 5 shows CF within multiple (4–5) stacked Golgi cisternae and within a forming granule (g_1). One of these cisternae loaded with CF has a coated region at its tip (arrow), suggesting that a CF-loaded vesicle has just fused with the Golgi cisternae. The inset depicts another Golgi cisterna with a row of CF molecules attached to its membrane, which appears coated on part of its surface (arrow). From Farquhar (6). Figure 4, ×70,000; Figure 5, ×87,000; inset, ×100,000.

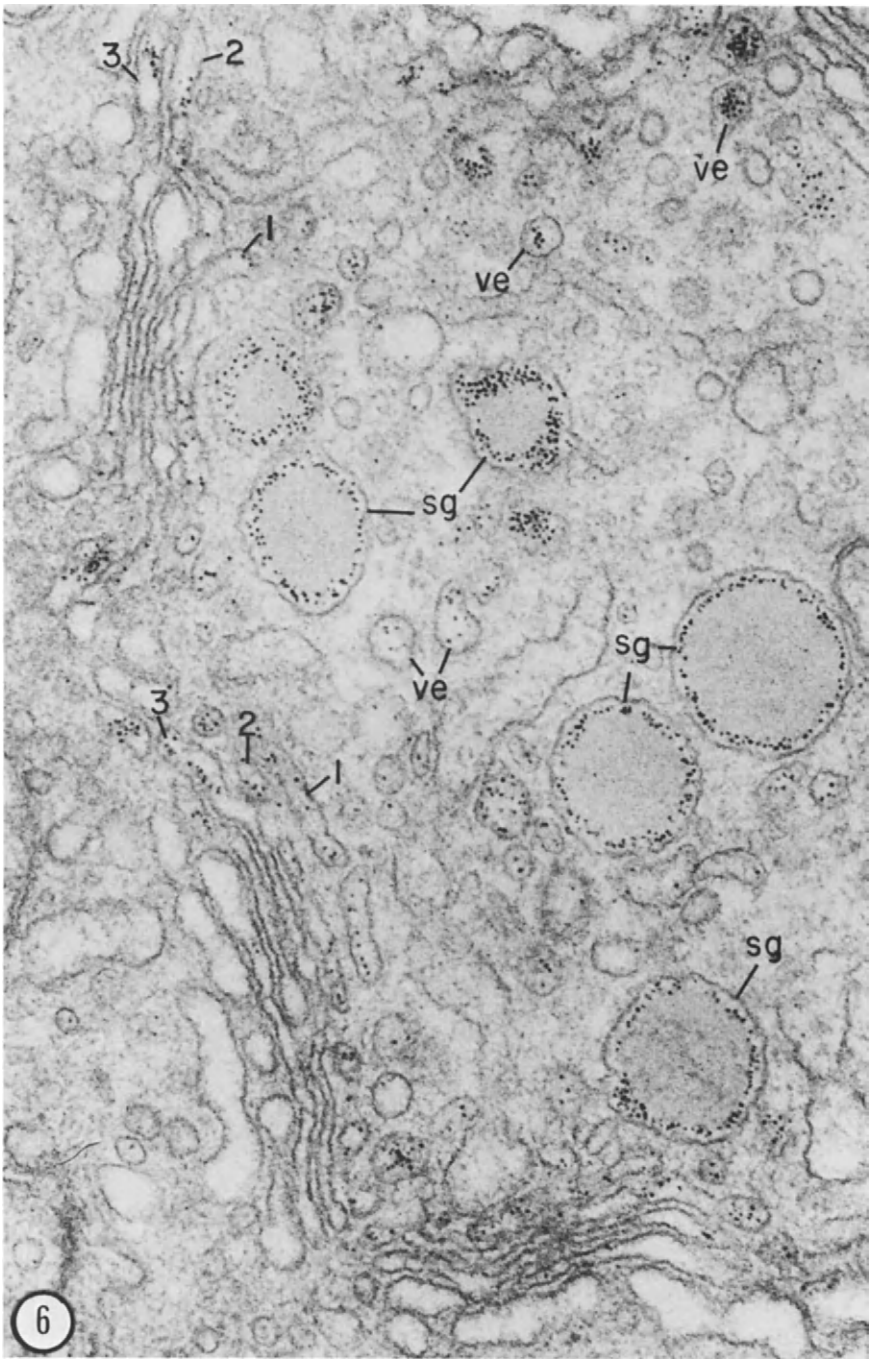
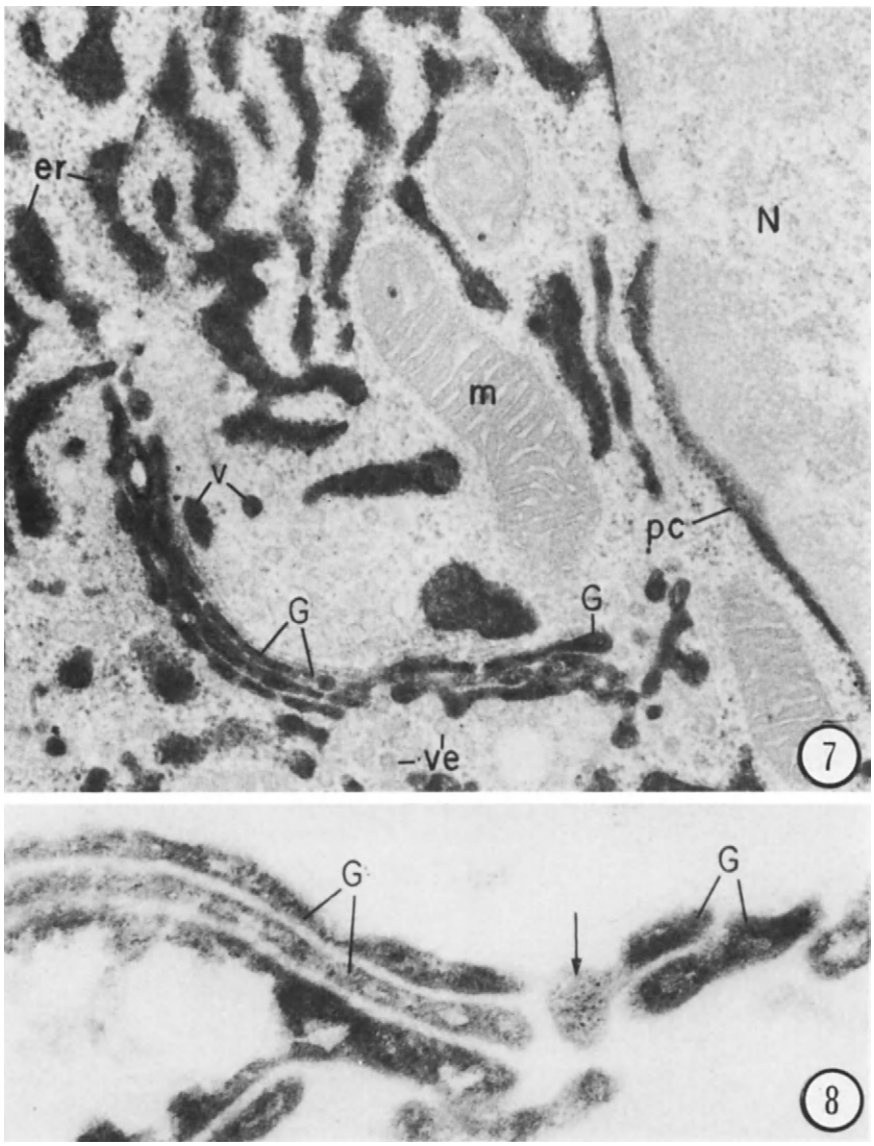
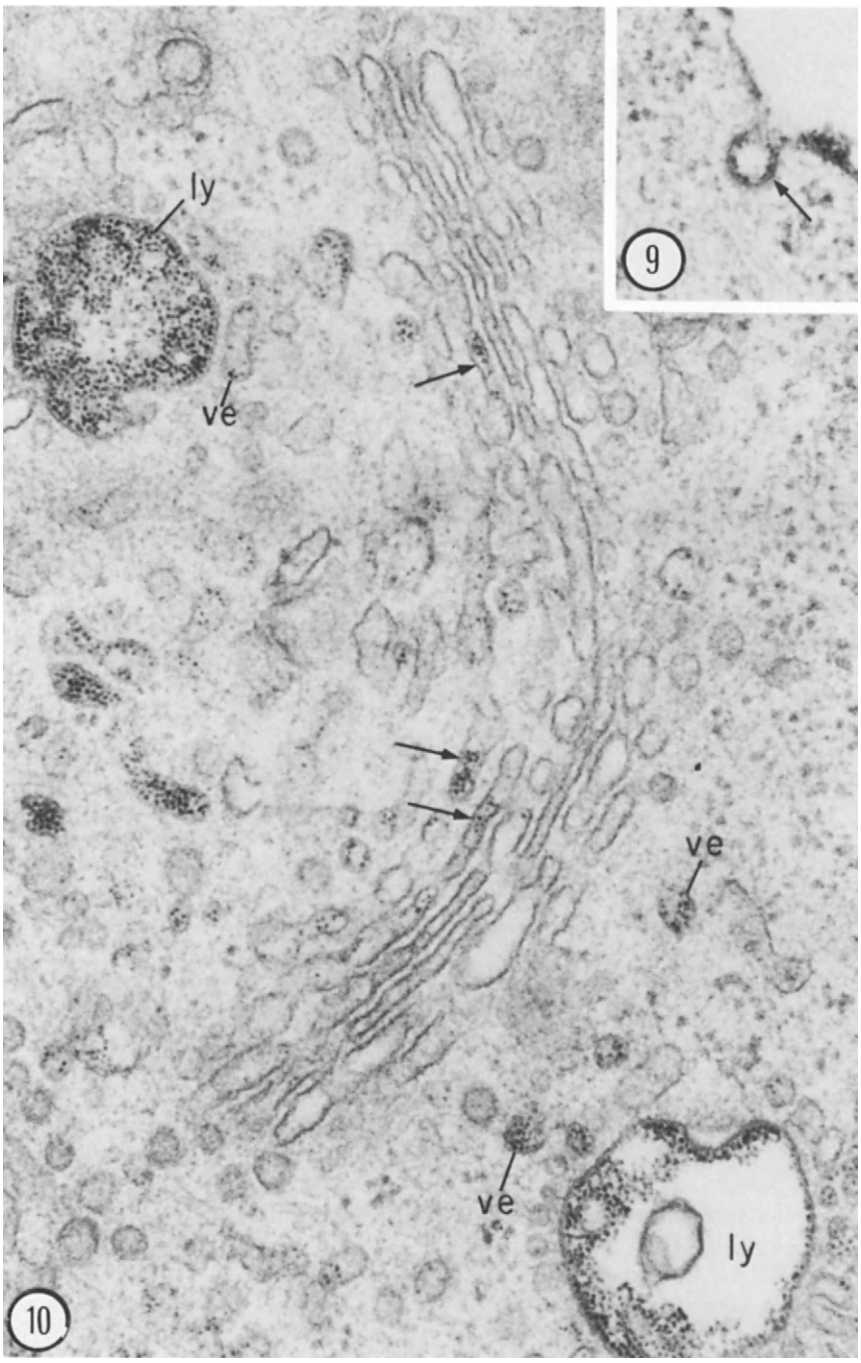


Figure 6. Somatotroph or growth hormone-secreting cell from a male rat incubated 60 min in CF (0.05 mg/ml), showing uptake of CF into multiple Golgi cisternae and secretion granules (sg). CF molecules are present within several stacked Golgi cisternae (1–3), within multiple smooth vesicles (ve) in the Golgi region, and within numerous secretion granules (sg) of varying size. Note that the CF molecules are located exclusively at the periphery of the dense granule contents. From Farquhar (6). ×85,000.



Figures 7 and 8. Portions of plasma cells from the lymph nodes of rats immunized with horseradish peroxidase (HRP). The distribution of anti-HRP immunoglobulins, (IgG), the cell's secretory product, is demonstrated by immunostaining. Figure 7 illustrates the presence of IgG throughout the rough ER (er) including the perinuclear cisternae (pc), in the stacked Golgi cisternae (G), and in small vesicles or vacuoles (v) in the Golgi region. Other small vesicles (ve) are not stained. Figure 8 is an enlargement of the Golgi region from a plasma cell from the same experiment as that in Figure 7 except that the cell had been incubated with CF for 60 min prior to fixation and immunostaining. IgG are present throughout the stacked Golgi cisternae (G). Note the presence of CF molecules in one cisterna that also contains IgG (arrow). Specimen fixed in glutaraldehyde, sectioned on a cryostat, and incubated with HRP, followed by incubation in diaminobenzidine (DAB). From Ottosen *et al.* (23). Figure 7, ×22,000; Figure 8, ×75,000.



Figures 9 and 10. Portion of mouse myeloma cells (RPC 5.4 cell line), fixed after incubation in 0.05 mg/ml CF for 10 min (Figure 9) or 60 min (Figure 10). Figure 9 shows that CF binds to the cell surface and is taken up by endocytosis, often in coated invaginations, as seen here (arrow). Figure 10 shows CF in several stacked Golgi cisternae (arrows) and in small vesicles (ve) located near the Golgi cisternae or near lysosomes (ly). The two large lysosomes in the field also contain considerable CF. From Ottosen *et al.* (23). Figure 9, ×76,000; Figure 10, ×57,000.

data are in accord, and although the evidence is still indirect, there is little reason to doubt the existence of recycling of granule membranes in secretory cells.

Widespread Nature of Membrane Recycling and Multiplicity of Recovery Routes

We have just summarized our results on nine different types of secretory cells that indicate that after exocytosis surface membrane is recovered and returned to multiple stacked Golgi cisternae, presumably to be reutilized as containers for newly synthesized secretory products. Similar data have also been obtained by others on three additional cell types: β-cells of the endocrine pancreas (22), thyroid epithelial cells (15), and exocrine pancreatic cells (16).

As far as the routing of the traffic is concerned in secretory cells, the precise routes taken by surface membrane to reach the Golgi complex remain to be established. However, the available evidence renders it likely that both a direct route (plasmalemma → Golgi) and an indirect route involving lysosomes (plasmalemma → lysosomes → Golgi) are used in different cell types.

In addition to recycling of granule membranes, there is also evidence for recycling of synaptic vesicle membranes in neurons (17, 18, 20) and of the membranes of pinocytic vesicles in macrophages and fibroblasts (28, 29). In these cases, the pathway taken, as far as is known, does not involve the stacked Golgi cisternae. In neurons the recovered membrane either is

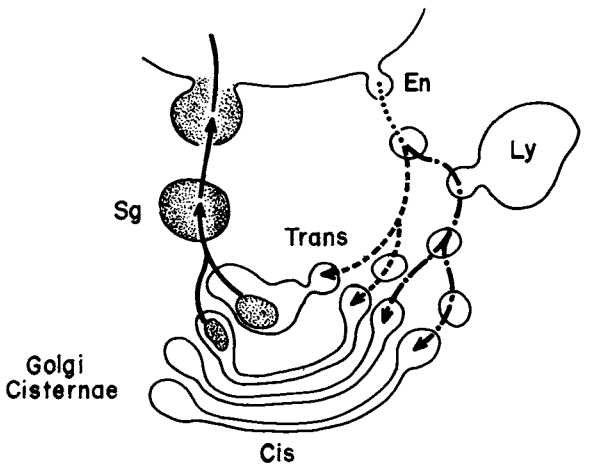


Figure 11. Diagram showing two possible routes that can be taken by surface membrane to reach the stacked Golgi cisternae in secretory cells. Following exocytosis of secretory granules (———), patches of surface membrane are recovered by endocytosis and fuse with the dilated rims of multiple stacked Golgi cisternae. Membrane recovered by endocytosis either can fuse directly with the cisternae (- - - -) or can fuse first with lysosomes and then with the Golgi (——.——). The available evidence suggests that both routes are used in different cell types. From Farquhar (8).

directly reutilized or makes a stopover in an intermediate compartment [special endoplasmic reticulum (ER) cisterna], whereas in macrophages and fibroblasts it clearly involves a lysosomal stopover (plasmalemma → lysosomes → plasmalemma). In addition to the pathways already outlined involving Golgi and lysosomes, it has been reported that in basophilic granulocytes (4) and β-cells of the pancreatic islets (Orci, personal communication) surface membrane fuses with mature secretion granules. Hence at present it can be assumed that (1) membrane reutilization is a widespread phenomenon (in all likelihood even more widespread than can be documented at present); (2) multiple recovery routes are used; and (3) individual cell types direct the traffic according to their activities or functional state. The precise pathways and the types and amounts of membrane involved remain to be worked out in individual cell types.

Implications of Membrane Recycling to Golgi Complex

Currently there is a great deal of interest and excitement, stimulated by the work of Goldstein and Brown (11), on the role of traffic directed to lysosomes (plasmalemma → lysosomes) in regulation of intracellular metabolic events (through receptor-mediated endocytosis and lysosomal digestion of LDL).[1] The validation of the existence of a pathway from the cell surface to Golgi cisternae is equally exciting and has even broader implications because it provides a route whereby molecules from the cell surface can reach a biosynthetic compartment.[2] Thus a mechanism is provided whereby various informational molecules from the extracellular environment (hormones, catecholamines, and other agents) can reach the Golgi and influence intracellular events. Indeed, several hormones—prolactin (19), insulin (27), and melanotropin (21)—have already been demonstrated to reach the Golgi complex, although to date the biological consequences of their uptake into Golgi elements is not clear.

The existence of recycling membrane traffic to the Golgi also provides a mechanism whereby surface membrane components (receptors, enzymes, and other membrane proteins) could potentially be modified (e.g., reglycosylated, sulfated, phosphorylated) in passage. To date no examples of these phenomena have been validated, but there is no reason on the face of it why they could not take place if the molecules are brought into contact with the right Golgi compartments or subcompartments.

[1]Besides low-density lipoprotein (LDL), several hormones [epidermal growth factor (EGF) (12, 13), insulin (2), and human chorionic gonadotropin (hCG) (1, 3)] are also known to be taken up by endocytosis and to be subsequently concentrated within lysosomes. The biological consequences of LDL uptake are well worked out (digestion of incorporated LDL in lysosomes releases free cholesterol, which depresses the synthesis of cholesterol and LDL receptors). However, the biological consequences of hormone internalization and segregation in lysosomes remain to be established.

[2]Among the known functions of the Golgi complex (see refs. 7 and 25) are the terminal glycosylation of secretory and membrane proteins, sulfation of secretory products (glycopeptides and glycosaminoglycans), synthesis of polysaccharides in plant cells, and partial proteolysis of prohormones such as proinsulin.

Summary and Conclusions

At present there is considerable evidence, albeit indirect, suggesting that the membranes of secretory granules or vacuoles used as containers for secretory products are reutilized in the packaging of successive generations of secretory products, i.e., they are recycled. The implications of the existence of such recycling membrane traffic from the cell surface to elements of the Golgi complex are potentially far-reaching. However, the precise pathways taken, the amounts and types of membrane involved in recycling, and the physiological consequences of this phenomenon remain to be worked out in individual cell types.

Acknowledgment

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Use of EM Autoradiography and Organ Culture to Identify Pathways of Amine Synthesis in Human and Murine Gastric Endocrine Cells

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Several morphological types of endocrine cells can be identified by electron microscopy (EM) in the mammalian stomach—at least four in man (8) and five in the mouse (13) and rat (3). Some of these cells synthesize and store serotonin (5-hydroxytryptamine, 5-HT) (2), and, at least in the rat and mouse, some of them synthesize and store histamine (5,17) (Figure 1). To determine which of the gastric endocrine cells synthesize 5-HT, some years ago we injected mice with DL-5-hydroxy[^{3}H]tryptophan ([^{3}H]-5-HTP), the immediate precursor in the biosynthesis of 5-HT (Figure 1), sacrificed the animals 2 h later, and processed their stomachs for light (LM) and EM autoradiography (13). When the stomachs were washed, fixed, and embedded in these studies, essentially all the [^{3}H]-5-HT was shown to be retained in the tissue while essentially all other ^{3}H-labeled substances, namely, [^{3}H]-5-HTP and [^{3}H]-5-HT-*o*-glucuronide, were washed out, so that the silver grains observed by the autoradiography reflected the presence of only [^{3}H]-5-HT (13). In this way we found that all morphological types of mouse gastric endocrine cells were comparably labeled and thereby demonstrated that all types could take up exogenous 5-HTP, could convert it to 5-HT by the enzyme DOPA decarboxylase, and could retain the 5-HT (13), that is, all types possessed amine precursor uptake and decarboxylation (APUD) ability (7).

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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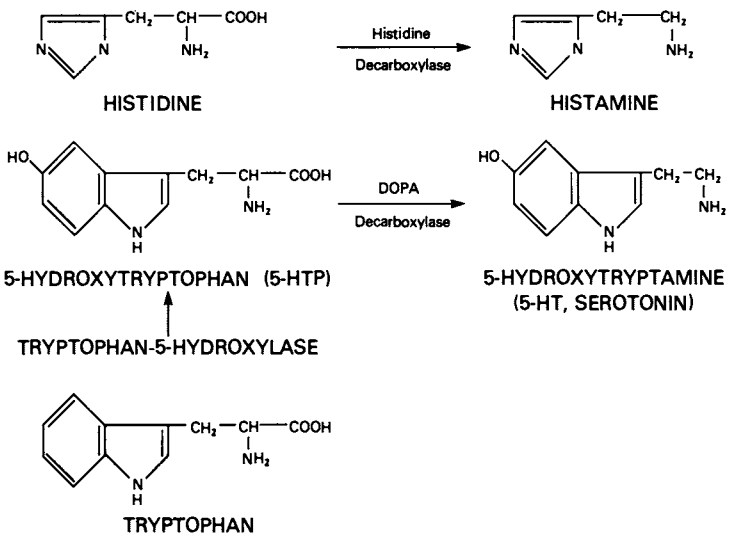

Figure 1. Biochemical pathways for the synthesis of histamine and serotonin.

In order to study pathways of amine synthesis in the endocrine cells of the human gut, the organ culture system of Browning and Trier (1) was adapted (Figure 2) so that specimens could be incubated with ^{3}H-labeled substrates *in vitro*. This *in vitro* system not only permits the study of human tissue, but also markedly reduces the amounts of expensive ^{3}H-labeled substrates needed by exposing the endocrine cells directly to relatively large quantities of precursors of amine synthesis, such as [^{3}H]tryptophan and [^{3}H]histidine, which if injected systemically into the living animal would be markedly diluted and rapidly metabolized systemically into protein and other pathways.

Small pieces of gastric mucosa are obtained from mice and rats after sacrifice and from human volunteers at surgery or by oral biopsy (14). The specimens are gently washed with culture medium, composed of 9 parts Trowell T-8 and 1 part fetal calf serum, and are placed on the organ culture grids (Figure 2). Approximately 1 ml of culture medium is added to the central wells of the culture dishes so that a thin layer of medium is drawn over the surface of the specimens by capillary action. The culture medium also contains the appropriate ^{3}H-labeled substrates and inhibitors described below and some of the cultures also contain pyridoxal phosphate (10^{-5}–10^{-4} *M*) to stimulate the enzymes DOPA decarboxylase and histidine decarboxylase. Filter paper, saturated with water, is placed in the outer well of the culture dishes, and the dishes are covered and placed in a Torbal jar (Torsion Balance Co., Clifton, N.J.). The jar is gassed with 95% O_2—5% CO_2 for 5 min, sealed, and placed in an incubator at 37°C for 1–3 h. The specimens are sometimes preincubated with inhibitors for a short period (usually 0.5–2 h), and then incubated also with the ^{3}H-labeled substrates for an additional 1–3 h. After incubation the pH of the culture medium is tested (it usually remains

between 7.2 and 7.4), and the specimens are removed and washed for 10 min with several changes of 0.2 M, pH 7.4 phosphate buffer. The specimens are then fixed for 3 h at 0–4°C with 2.5% glutaraldehyde in 0.1 *M*, pH 7.4 phosphate buffer and postfixed for 3 h at 0°–4°C with 1% osmium tetroxide in the same buffer. They are dehydrated in graded alcohols, cleared in propylene oxide, and embedded in Epon 812. Serial thick (about 1 μm) and thin (silver to pale yellow) sections are cut so that they extend from the surface to the submucosa of the gastric specimens (14), and the sections are processed for LM and EM autoradiography by previously published methods (10,13,15). Both the thick and thin sections are coated with Ilford L4 emulsion and are kept in dark slide boxes containing desiccant at room temperature or at 4°C. The thick sections are exposed for 7–21 days, developed in D19 for 4 min, stained with aqueous toluidine blue, and studied by light microscopy. The thin sections are exposed for 1–36 weeks, developed in Microdol-X for 4 min, stained with uranyl and lead, and examined by EM. Grain densities (numbers of grains per unit cross-sectional area) and density ratios for the different types of epithelial cells are determined for individual grids and are compared.

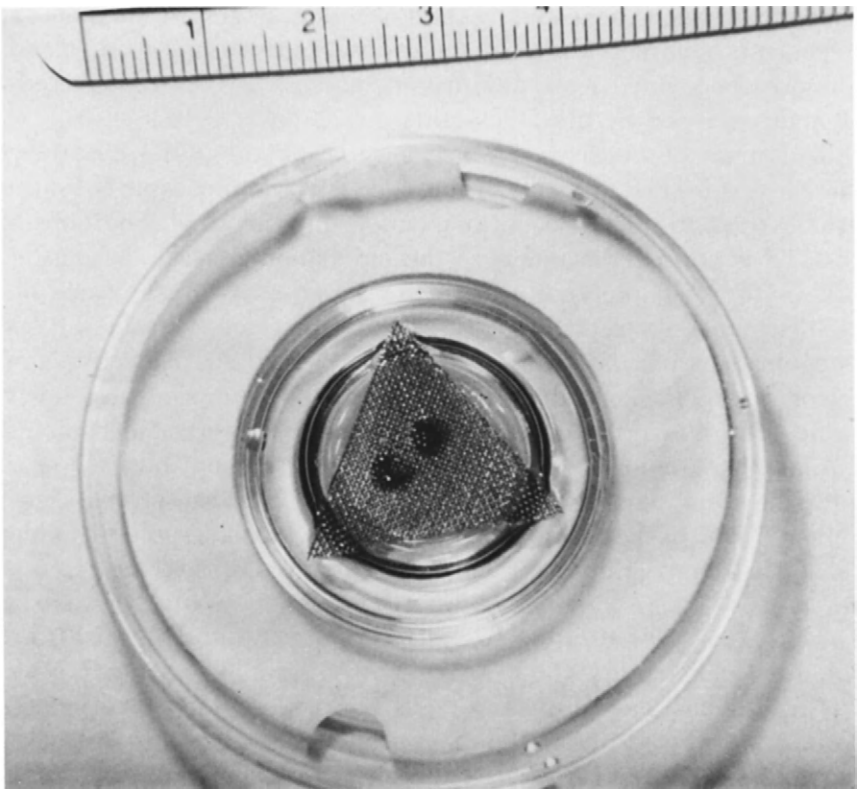

Figure 2. The organ culture. The small pieces of tissue are placed on the metal grid and the culture medium with ^{3}H -labeled substrates and inhibitors is placed in the central well below.

To identify the endocrine cells that can convert 5-HTP to 5-HT (the ones with APUD ability) specimens are incubated with DL-[^{3}H]-5-HTP (50 μCi/ml). Additional cultures also contain carbidopa (10^{-5}–10^{-2} *M*), an inhibitor of the enzyme DOPA decarboxylase, used to block the conversion of the [^{3}H]-5-HTP to [^{3}H]-5-HT. A final group of specimens is incubated with [^{3}H]-5-HT (50 μCi/ml) to determine where exogenous [^{3}H]-5-HT localizes in this tissue.

Using this *in vitro* system, all morphological types of mouse (adult male CFl) gastric endocrine cells were again shown to possess APUD ability (10); they were all comparably labeled by the [^{3}H]-5-HTP (Figure 3). This labeling was prevented when the conversion of [^{3}H]-5-HTP to [^{3}H]-5-HT was blocked with carbidopa, and exogenous [^{3}H]-5-HT failed to label the endocrine cells significantly (10). When rat (adult male Sprague-Dawley) oxyntic mucosa was studied, however, the [^{3}H]-5-HTP heavily labeled the EC and ECL cells, but the A-like cells appeared unlabeled (10, Figure 4). This finding was unexpected since the gastric endocrine cells of the rat and mouse had been thought to be similar in their structure and properties and because previous studies had suggested that the rat A-like cells also had APUD ability (6,10). However, the presence of non-APUD endocrine cells within the rat oxyntic mucosa had also been suggested by the LM autoradiography (10) and also by the presence of nonfluorescing pale cells observed within oxyntic glands by phase-fluorescence microscopy after oxyntic mucosa had been incubated with 5-HTP and processed by the Falck-Hillarp technique (9,10).

Although many of the gastric endocrine cells contain DOPA decarboxylase and can convert 5-HTP to 5-HT, all these cells do not necessarily synthesize appreciable quantities of 5-HT normally under physiological conditions, since little 5-HTP is normally available in the circulation. In fact, Håkanson and co-workers (5) had pointed out several years ago, by means of the Falck-Hillarp technique, that many of the endocrine cells in rat oxyntic glands could decarboxylate 5-HTP or L-DOPA to their respective amines, but few of these cells normally contained demonstrable quantities of 5-HT or dopamine. The endocrine cells that do synthesize appreciable amounts of 5-HT under physiological conditions must also be able to take up L-tryptophan and convert it to 5-HTP by means of the enzyme tryptophan-5-hydroxylase (Figure 1); that is, they must be able to synthesize 5-HT directly from tryptophan (Figure 1). To identify these cells, pieces of rat gastric mucosa were incubated in the organ culture system with L-[^{3}H]tryptophan (50 μCi/ml, 8.3×10^{-6} *M*) (12). Additional cultures also contained cycloheximide (0.05 to 0.5 mg/ml) to inhibit the incorporation of the [^{3}H]tryptophan into protein; or either *p*-chlorophenylalanine (PCPA) (10^{-4}–10^{-2} *M*), an inhibitor of the enzyme tryptophan-5-hydroxylase, or carbidopa, the DOPA-decarboxylase inhibitor, to block the conversion of the [^{3}H]tryptophan into [^{3}H]-5-HT (12). The [^{3}H]tryptophan labeled all EC cells heavily, but the other types of endocrine cells only lightly (12). When protein

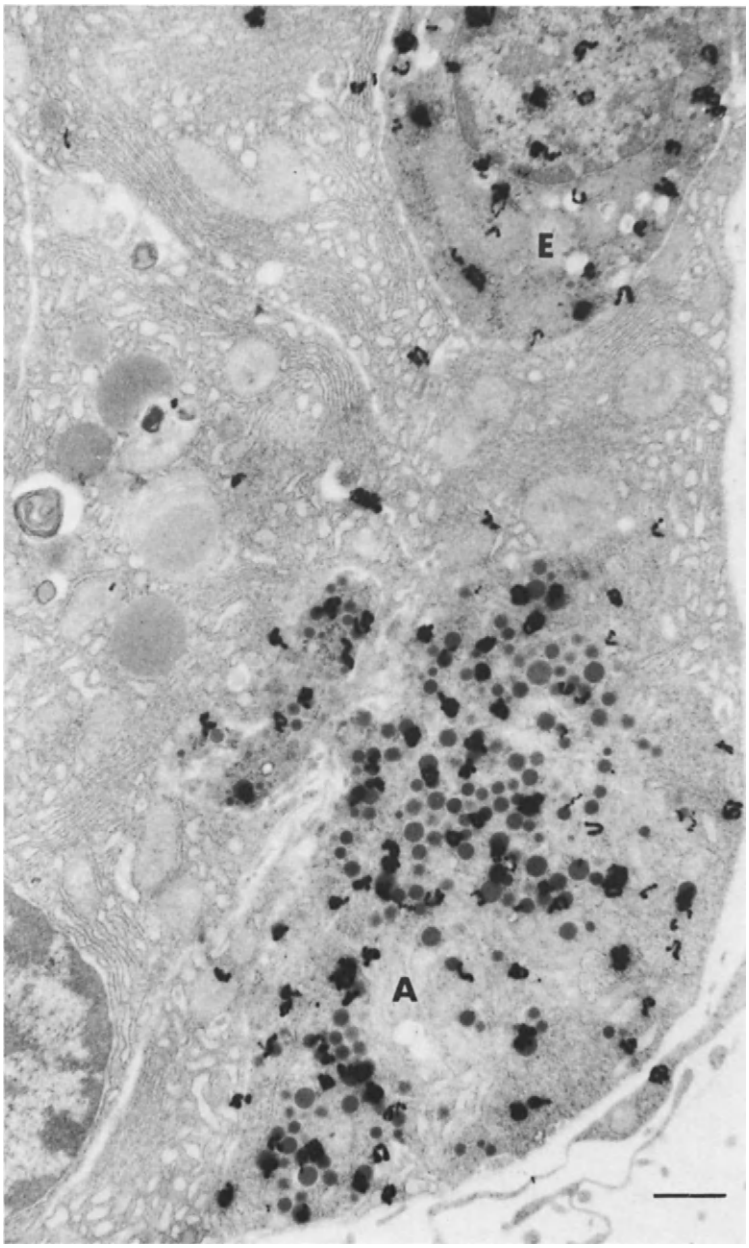

Figure 3. Mouse oxyntic mucosa incubated with [^{3}H]-5-HTP and processed for EM autoradiography reveals comparable labeling of the ECL (E) and A-like (A) cells, thereby indicating that both types of mouse endocrine cells can readily decarboxylate 5-HTP to 5-HT. From Rubin and Schwartz (10), by permission from Elsevier North Holland.

synthesis was inhibited with cycloheximide, heavy labeling of the EC cells persisted, but the labeling of the other endocrine cells, with the exception of the ECL cells, was essentially eliminated (12). When 5-HT synthesis was

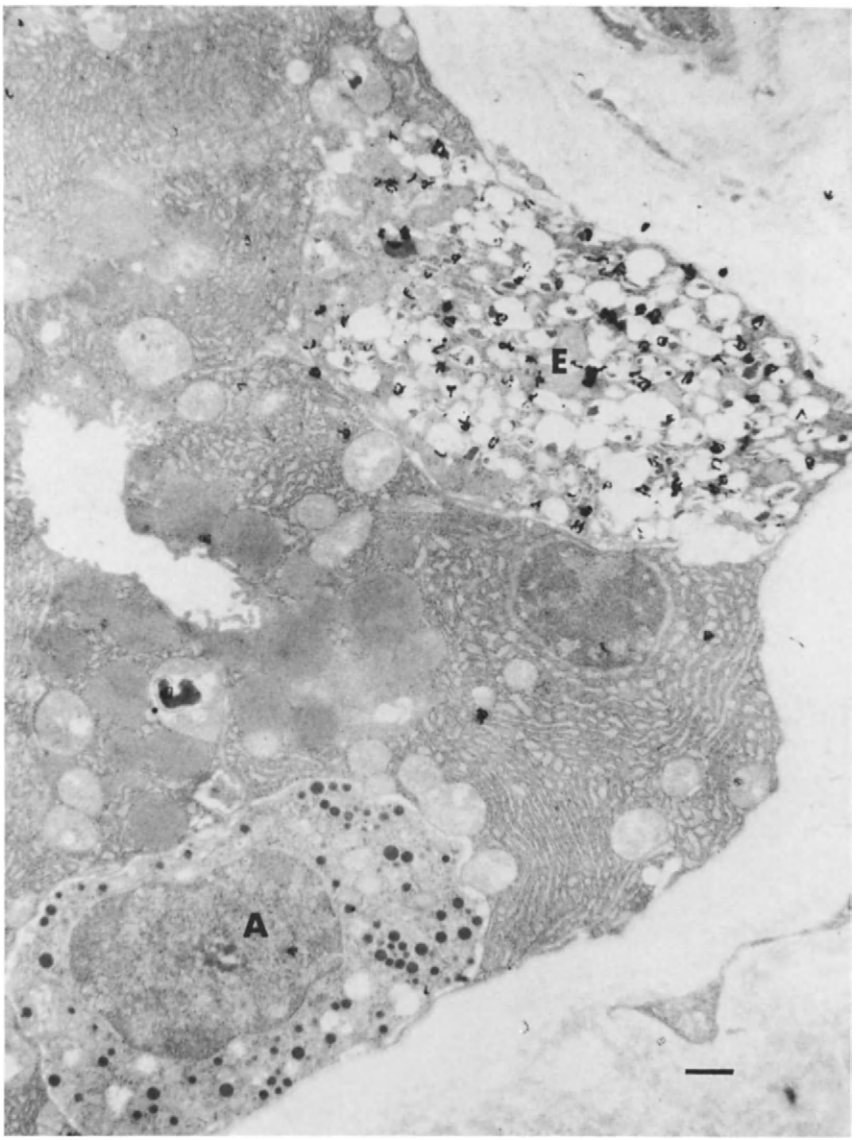

Figure 4. Rat oxyntic mucosa incubated with [^{3}H]-5-HTP and processed for EM autoradiography reveals good labeling of the ECL cell (E), but the A-like cell (A) appears unlabeled, thereby suggesting that the rat A-like cell is unable to decarboxylate 5-HTP to 5-HT. From Rubin and Schwartz (10), by permission from Elsevier North Holland.

inhibited with either PCPA or carbidopa, the labeling of EC cells was markedly reduced to approximately the level of the other endocrine cells (12). Thus this study (12) demonstrated that all rat gastric EC cells can readily synthesize appreciable amounts of 5-HT from tryptophan and that, as suggested by previous investigators (3,16), the EC cells represent the classical argentaffin or enterochromaffin cells. The labeling of ECL cells by

[^{3}H]tryptophan was significantly greater than the labeling of the other non-EC endocrine cells, was greater than the labeling of ECL cells produced by [^{3}H]leucine, and remained significant even after cycloheximide (12). These observations suggested that the ECL cells can also synthesize and store some 5-HT, but not enough normally to be detected by conventional argentaffin or amine-fluorescent techniques.

Håkanson and co-workers (5,17) have described numerous endocrine cells, located predominantly in the oxyntic glands of the rat stomach, which synthesize and store histamine and which he termed "enterochromaffin-like" cells (4). To identify by EM autoradiography the endocrine cells that synthesize histamine in the rat stomach, pieces of rat stomach were incubated in the organ culture system with [^{3}H]histidine (50 μCi/ml, 1.8×10^{-5} *M*) (11). Additional cultures also contained cycloheximide to inhibit the incorporation of the [^{3}H]histidine into protein; or carbidopa (10^{-5}–10^{-2} *M*) the inhibitor of DOPA decarboxylase; or NSD-1055 (4-bromo-3-hydroxybenzyloxyamine) (10^{-4}–10^{-2} *M*), an inhibitor of histidine decarboxylase as well as DOPA decarboxylase (11). A final group of cultures contained [^{3}H]histamine (50 μCi/ml, 0.9×10^{-5} *M*) to determine where exogenous histamine accumulates in this system (11). The [^{3}H]histidine heavily labeled the ECL cells, as much or more so than the chief cells, which synthesize much protein and which after 3 h of incubation tend to concentrate the label in the supranuclear area of granule formation (Figure 5). In contrast, the other types of endocrine cells were only weakly labeled, usually not much more than the neighboring parietal cells (Figure 6). The heavy labeling of ECL cells persisted when protein synthesis was inhibited with cycloheximide, even though the labeling of the other endocrine and other epithelial cells was essentially eliminated (11). The labeling of ECL cells by [^{3}H]histidine was not appreciably affected by carbidopa but was markedly reduced by the NSD-1055, which inhibits histidine decarboxylase as well as DOPA decarboxylase (11). Exogenous [^{3}H]histamine failed to label endocrine cells significantly (11). Thus this study (11) showed that the ECL cells are the histamine-synthesizing endocrine cells of the rat stomach.

As illustrated earlier, the organ culture and autoradiographic techniques have been very useful in the study of pathways of amine metabolism in the gastric endocrine cells. They have permitted us to identify in the rat stomach the cells that can synthesize 5-HT from tryptophan (12) and the cells that produce histamine (11), and in both the mouse and rat stomachs, the cells that can decarboxylate 5-HTP to 5-HT (10). The human gastric endocrine cells, for whose study the organ culture system was originally adopted, are now being successfully studied in like manner. The validity of the *in vitro* system has been demonstrated in two instances—in studying the labeling of mouse and rat gastric endocrine cells by [^{3}H]-5-HTP, the *in vitro* system has been shown to produce the same results as when the animals were injected *in vivo*. The success of the autoradiographic techniques described is dependent

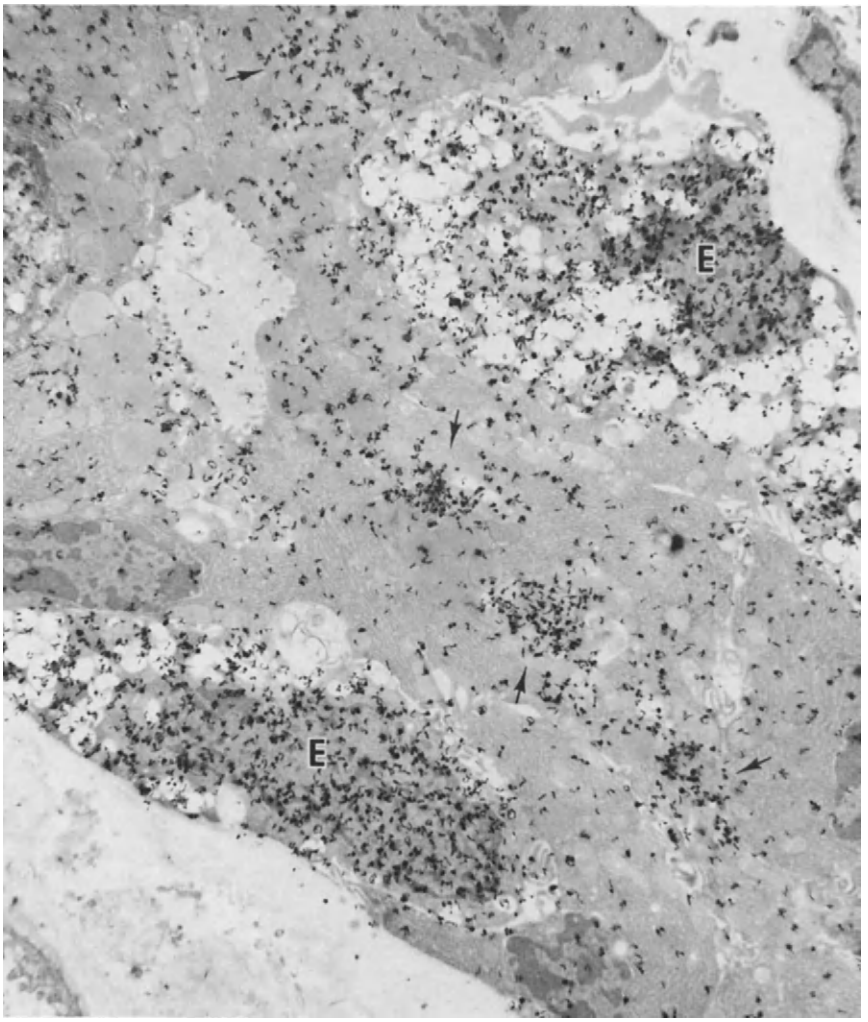

Figure 5. Rat oxyntic mucosa incubated for 3 h with [^{3}H]histidine and processed for EM autoradiography reveals heavy labeling of the two ECL cells (E). This labeling persists even when protein synthesis is inhibited with cycloheximide but is markedly reduced by NSD-1055, an inhibitor of the enzyme histidine decarboxylase. Thus the heavy labeling of ECL cells is largely due to the synthesis of histamine. After 3 h of incubation, the labeling of the chief cells, which is inhibited by cycloheximide and therefore represents protein synthesis, is heavily concentrated in the supranuclear areas of granule formation (arrows). From Rubin and Schwartz (11), by permission from Elsevier North Holland.

on the intracellular binding of the amines, small water-soluble molecules, so that they are not extracted during tissue washing, fixation, and dehydration, and also on the elimination from the tissue of other [^{3}H]-labeled substances that could obscure the results. The inhibitors described have been used successfully *in vitro*, a feature usually necessary for the study of human tissue, but for animal studies these or other inhibitors, especially if they pose solubility or pH problems, can be administered *in vivo* prior to the sacrifice of the animals.

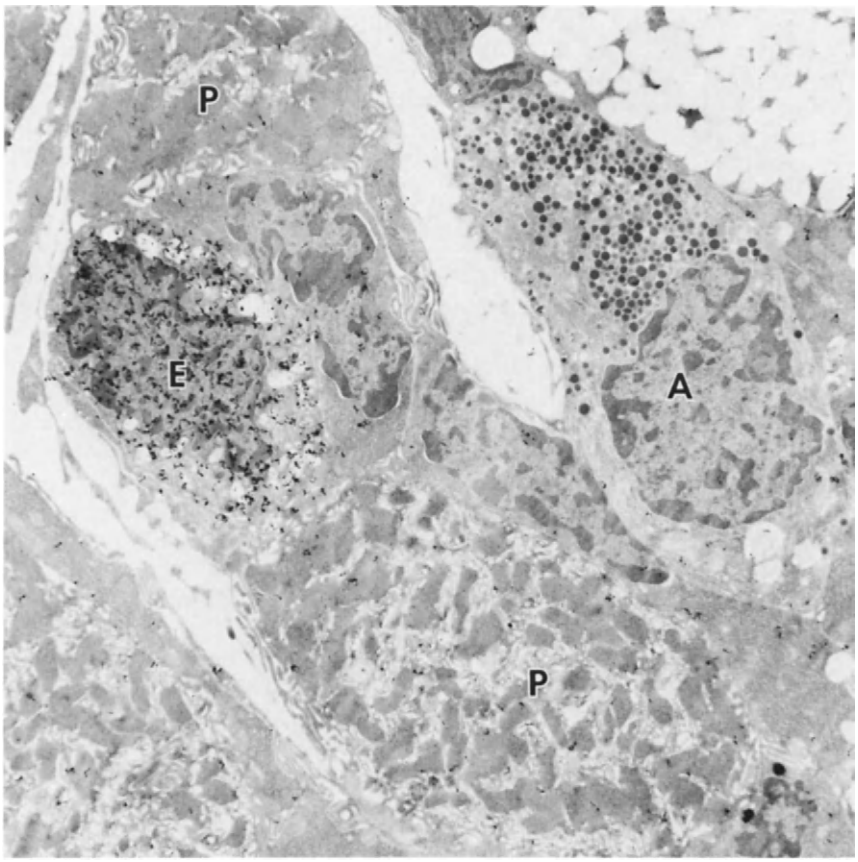

Figure 6. Rat oxyntic mucosa incubated for 3 h with [^{3}H]histidine and processed for EM autoradiography reveals heavy labeling of the ECL cell (E), but the A-like cell (A) is only weakly labeled, no more so than the neighboring parietal cells (P). The light labeling of A-like cells can be abolished with cycloheximide, and therefore probably represents protein synthesis. Thus the ECL but not the A-like cells seem to synthesize histamine in the rat stomach. From Rubin and Schwartz (11), by permission from Elsevier North Holland.

The organ-culture system described has worked very successfully for the maintenance of pyloric and small and large intestinal mucosae, usually preserving these tissues well for at least 24 h. The preservation of oxyntic mucosa, however, has been less successful. The tissue usually does not look good after even 12 h of culture and rarely survives satisfactorily for 24 h. For the relatively short incubations employed in the described studies, 1–3 h, the mucosa is, however, usually well maintained.

ACKNOWLEDGMENTS

This work was supported by Research Grant AM25495 from the National Institute of Arthritis, Metabolic, and Digestive Diseases, Department of Health, Education, and Welfare, and by Grant No. IN-125 from the American Cancer Society.

We should like to thank Miss Nanette Brason and Mrs. Ruth Palmer for their able technical assistance.

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Immunocytochemistry of Secretory Peptides: Introduction of a New Immunocytochemical Technique

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INTRODUCTION

Immunocytochemistry has contributed greatly to the identification of cells and neurons producing biologically active peptides. Thus three main types of cells—neurons, endocrine cells, and paracrine cells—have been found to produce a variety of secretory peptides. Some peptides may be simultaneously produced by all three types of cells, and, conversely, some cells may produce more than one type of peptide.

These data challenge our traditional concepts of neuronal and hormonal communication. Continued immunocytochemical studies will aid in identifying new cellular peptides and in elucidating the destinations of neuronal and paracrine cell processes. In this way cytochemistry may provide us with valuable ideas about the physiology of the many new peptidergic systems.

It is therefore important to analyze critically the limitations and potentials of immunocytochemistry. In the following pages, we will concentrate on problems with defining specificity in immunocytochemistry and will discuss briefly a novel technique, which may increase the specificity and reliability of immunocytochemical data.

ANTIBODY SPECIFICITY

Antibodies used for peptide immunocytochemistry are almost invariably of the IgG class. IgG-molecules possess two equivalent antigen-combining sites. Since the size of these sites is limited, they will react only with a limited

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region of most peptide antigens. Such a region may be composed either of a continuous sequence of amino acids (continuous antigenic site) or of amino acids that come close together for steric reasons (discontinuous antigenic site) (1). The size of such regions usually averages three to eight amino acids. The specificity of an antibody for a given peptide will, thus, depend on the relative uniqueness of this small region. Since many secretory peptides and proteins show great similarities in their amino acid sequences, perhaps reflecting their evolution through gene duplication, and since, conceivably, many peptides and proteins have still to be isolated and characterized, the uniqueness of a particular antigenic region cannot be assessed. Similar problems relate to all immunological techniques, including radioimmunoassays. The latter techniques, however, lend themselves to analysis of the observed immunoreactivity by various chromatographic systems. Characterization of immunoreactants by their size and/or charge is, of course, not possible at the tissue section level, and therefore radioimmunoanalytical techniques have often been used for supplementing immunocytochemical data.

The use of one single type of antibody allows only limited conclusions about the nature of the immunoreactive molecules. It has, however, been proved possible to raise antibodies to several distinct regions of many peptides. Combined use of several types of region-specific antibodies allows a "micro-dissection" of immunoreactive molecules and hence contributes significantly to immunocytochemical credibility (3,4). It is, of course, mandatory that such antibodies be characterized by extensive absorption against synthetic or natural peptide fragments. Peptides to be tested should be coupled preferably to a solid phase, like sepharose, since, otherwise, differential degradation by serum or interactions of soluble antigen–antibody complexes with tissue may interfere with the results. Sepharose-bound peptides not only constitute attractive immunoabsorbents but can also be used for model staining experiments, aimed at verifying the antibody specificity (6,9). Many peptides seem to have one or two preferential antigenic sites, and immunizations with different conjugates or with synthetic fragments may often be necessary.

Pure Antibodies and Impure Antisera

It is evident that use of region-specific immunocytochemistry requires pure antibodies. Unfortunately, most antisera contain an admixture of different natural and induced antibodies. The desired "monospecific" antisera may sometimes be produced by absorptions against known contaminants. Most often, however, the nature of the contaminating antibodies is not known, and in this case, purification by affinity chromatography against the desired antigen may be attempted. Antibodies of high avidity are, however, difficult or impossible to elute from affinity columns under nondenaturing conditions (8). Hence many affinity-purified antisera will be relatively enriched in low-avidity antibodies. Although useful in many situations, such

antibodies are potentially harmful in immunocytochemistry, since they may dislodge during staining and produce falsely negative or spurious results.

A possible alternative to affinity purification is offered by the use of monoclonal antibodies. Sets of region-specific monoclonal antibodies of sufficient avidity should constitute attractive immunocytochemical detection reagents. Production of such antibodies is, however, time-consuming and requires specialized equipment.

A more simple and more thoroughly tested alternative is given by the labeled antigen detection techniques (2,5). These techniques take advantage of the fact that IgG molecules possess two identical antigen-combining sites. If a surplus of IgG molecules is allowed to react with a limited amount of antigen, they will bind antigen with only one of their two sites. In its most recent modification, the gold-labeled antigen detection (GLAD) technique

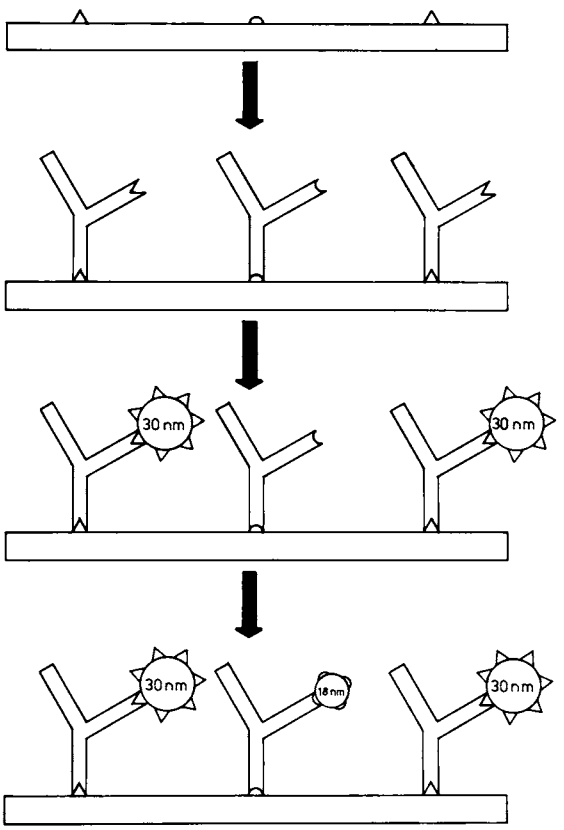

Figure 1. The principle of the GLAD method. Antibodies react with tissue-bound antigen with one of their combining sites, leaving the remaining site free to react with specific antigen absorbed onto colloidal gold particles. The gold particles, hence, mark the site of tissue-bound antigen. In this example antibodies of different specificities have reacted with their corresponding tissue-bound antigens. Use of colloidal gold particles of different diameters, each coated with the respective antigen, allows us to distinguish between the two types of tissue-bound antigens. In conventional immunocytochemistry the two antigens would be confused.

(2), antibodies are first allowed to react with tissue-bound antigen with one of their antigen-combining sites, leaving the remaining site free to react with antigen-coated colloidal gold granules (Figure 1). The colloidal gold granules are electron-impermeable and are easily visualized under the electron microscope even when optimally fixed and contrasted specimens are used (Figure 2). Nonspecific antibodies will not be detected, since they will be unable to bind specific antigen on the gold granules. In this way prepurification of antisera is not necessary, since the labeled antigen selects the desired antibody population. In contrast, all conventional immunocytochemical techniques detect all antibodies, specific and unspecific, that react with tissue sections. Initially, when developing the GLAD technique, we expected that the antiserum dilutions should be low in order to assure that the antibodies reacted with tissue-bound antigens with only one of their combining sites. This was not borne out, however, and optimal dilutions equaled or exceeded those of indirect immunoenzymatic techniques and were about ten-fold lower than those used in the peroxidase–antiperoxidase (PAP) method. This observation suggests that even in conventional immunocytochemistry, many antibodies do not react with tissue-bound antigen with both of their combining sites.

Gold granules may be manufactured in many narrowly determined sizes, which makes it possible to absorb different antigens to gold granules of differing diameters. Hence multiple antigens may be simultaneously demonstrated at the ultrastructural level with this technique (2). It is possible to quantitate specific staining versus background by simply counting gold granules. Since optimally contrasted specimens are routinely used, it is also

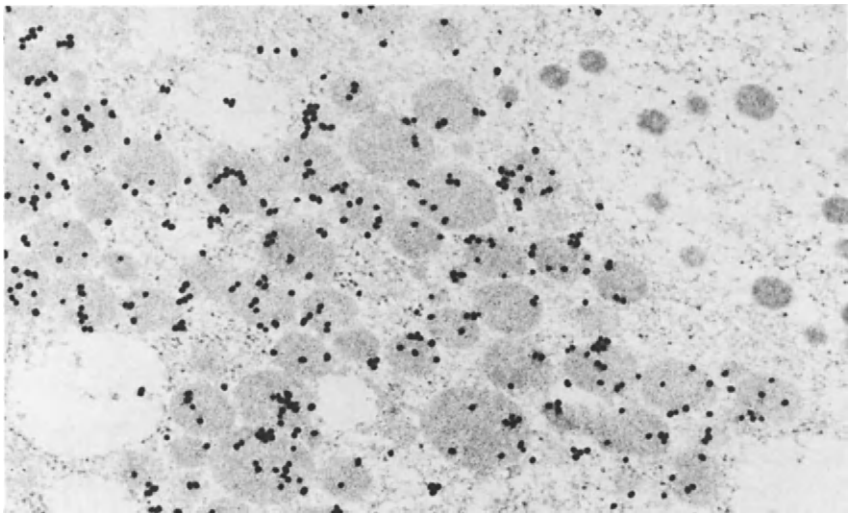

Figure 2. Pituitary somatotroph stained with an antiserum to human growth hormone. The site of antigen–antibody reaction is revealed by growth hormone-coated colloidal gold particles (18 nm) that are predominantly accumulated over the cytoplasmic granules.

possible to quantitate gold granules over identified subcellular organelles. At present, model experiments are under way to determine whether a linear relationship exists between numbers of gold granules and antigen concentrations.

Although it could be said that the GLAD method already contains a built-in control, several additional controls are necessary. These, as detailed elsewhere (2), serve to confirm the specificity of the immunological reaction between the antibodies and the tissue-bound antigen as well as between the antibodies and the labeled antigen. Controls for excluding nonimmunological binding (e.g., receptor-mediated, cf. 7) of antigen to tissue can be carried out by omitting the antiserum from the staining sequence.

Since the GLAD method detects only specific, desired antibodies, it is possible to pretreat the sections with high concentrations of serum from the same species as the one donating the immune serum. Such pretreatment is, of course, only possible with labeled antigen detection techniques and has empirically been found to inhibit nonimmunological binding of specific antibodies to the sections.

The preceding features make the GLAD method an attractive technique for ultrastructural immunocytochemistry. The method is simple to carry out, requires no specialized equipment, and has an inherent specificity. Its ability to demonstrate multiple peptides simultaneously at the ultrastructural level should prove valuable for studies of the transport and secretion of biologically active peptides.

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Electron Immunohistochemistry of Gut Peptides in Tissues Processed for Routine Electron Microscopy

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INTRODUCTION

Numerous methods have been devised for the immunohistochemical localization of peptides by electron microscopy (3, 6, 7). One major flaw of most such methods has been the unsatisfactory preservation of the histological structures owing to the need for special, usually less than ideal, fixation techniques. This situation has improved recently with the appearance of a few reports in which the peroxidase-antiperoxidase (PAP) immunoperoxidase method popularized by Sternberger (11) has been successfully applied to osmium (OsO_4) postfixed tissues from rat (1), ovine (4), and human fetal (10) pituitary.

Research in our laboratory during the last few years has been directed toward the localization of hormonal peptides in the digestive system by ultrastructural immunohistochemical methods. This chapter deals with the localization of bombesin, somatostatin, and gastrin, or closely related cross-reacting peptides, in digestive tissues routinely fixed and embedded for electron microscopy. These three particular peptides were chosen as models because reliable information as to the cell types involved was already available from previous work. We utilized a "sandwich" method that enabled us to study one given cell by light microscopy immunoperoxidase, electron microscopy immunoperoxidase, and conventional electron microscopy. Our results indicate that treatment of tissues with OsO_4 does not irreversibly

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impair the immunoreactivity of endocrine cells containing bombesin, somatostatin, or gastrin.

Materials and Methods

TISSUES

Human duodenal bulb mucosa, removed at the time of partial gastric resection for peptic ulcer, was used to localize gastrin and somatostatin-containing cells. Antral gastric mucosa from *Rana catesbeiana* (bullfrog) was used for the localization of the bombesin-containing cells. All tissues had been fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer for 1 h, postfixed in 1% OsO_4 for 1 h, dehydrated through an ascending series of ethanols, and embedded in Epon 812. The tissue blocks had been in our files for four years or longer.

ANTISERA

The antisera against bombesin, somatostatin, and gastrin were all raised in rabbits and were kindly contributed by Dr. John H. Walsh. The antibombesin antiserum, lot No. 1078, was directed against the COOH-terminal portion of the synthetic bombesin nonapeptide. The specificity of this antibody was corroborated by both radioimmunoassay and immunofluorescence in an earlier publication (9). The antisomatostatin antiserum, lot No. 7812, was raised against synthetic whole somatostatin molecule conjugated with Keyhole limpet hemocyanin. The specificity of this antibody has been characterized elsewhere (13). The antigastrin antiserum, lot No. 1802, was raised against synthetic human gastrin conjugated with bovine serum albumin. The specificity of this antibody was tested elsewhere by radioimmunoassay (5) and by immunofluorescence (12). All these antibodies were diluted to working concentration using 10% decomplemented nonimmune goat serum.

PROCEDURE

A semithin (1 μm thick) section was obtained, mounted on a glass slide, and stained with toluidine blue to ensure that the appropriate histological structures would be observed. Then 7–10 serial sections, 40–60 nm in thickness, were obtained with an LKB Ultrotome using glass knives. The first four to six thin sections were picked up on a 200-mesh nickel grid for electron microscopy immunoperoxidase. The next two to four thin sections were placed on a copper single-hole grid coated with a Formvar film for conventional electron microscopy. Finally, a serial semithin section was cut and mounted on a glass slide for light microscopy immunoperoxidase (Figure 1).

The first step was to perform the light microscopy immunoperoxidase treatment of the last semithin section. After a circle was drawn around the section with a diamond pencil the tissue was handled according to the

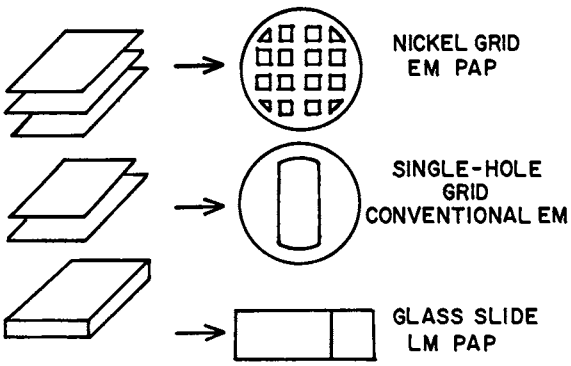

Figure 1. Diagrammatic representation of the "sandwich" method used to identify peptide-producing cells in digestive tissue sections. The first serial thin sections are mounted on a nickel grid for ultrastructural immunoperoxidase; the following ones are mounted on a single-hole copper grid for conventional electron microscopy; and a serial semithin section, taken at the end, is mounted on a glass slide for light microscopy immunoperoxidase.

following schedule: (a) saturated NaOH in absolute ethyl alcohol (NaOH-ETOH), 2 min (this solution should be aged for a week before use); (b) 100%, 95%, 70%, and 50% ETOH, 2 min each; (c) distilled H_2O, 2 min; (d) 10% H_2O_2, 5 min; (e) phosphate-buffered saline (PBS) pH 7.2, two changes of 2 min each. The slide was then placed in a Petri dish containing a circle of filter paper soaked in PBS and the specific first antibody was applied to the section. For light microscopy the antibombesin antiserum was diluted 1:80, the antisomatostatin antiserum 1:40, and the antigastrin antiserum 1:40. After an overnight incubation at room temperature, the sections were treated for immunoperoxidase staining by the PAP method (11) following this schedule: (a) PBS wash for 45 min under constant stirring; (b) goat antirabbit IgG diluted 1:10 with PBS, incubate for 1.5 h; (c) PBS wash for 45 min under constant stirring; (d) rabbit antiperoxidase complexed with peroxidase (PAP—Cappel Laboratories) diluted 1:10 with PBS, incubate for 1.5 h; (e) PBS wash for 45 min; (f) 3′3′-diaminobenzidine (DAB) solution was prepared by dissolving 4 mg of DAB (Sigma) in 10 ml Tris buffer, pH 7.6, and adding a few drops of 0.15% H_2O_2; (g) freshly made DAB solution, incubated for 7 min or until color reaction was properly "developed"; (h) distilled H_2O, wash for 1 min; (i) mount with permount and cover slip. After observation under the light microscope a map of the section was drawn, carefully delineating the position of each peroxidase-positive cell.

The thin sections mounted on single-hole grids were double-stained with uranyl acetate and lead citrate. Then conventional electron microscopic observation was carried out with a Siemens Elmiskop IA electron microscope and photographs were taken at magnifications ranging from ×1500 to 8000. Using the map drawn from the light microscopic observation of the serial semithin section, the PAP-positive cells were identified in the corresponding electron micrographs.

The following immunohistochemistry procedure was carried out on the

sections mounted on 200-mesh nickel grids (in steps c, e, f, h, j, and l the grids were floated on drops of the solution; in the others they were immersed): (a) NaOH-ETOH diluted 1:10 with 100% ETOH, dip for 1.5 min; (b) 100%, 95%, 70%, 50% ETOH, and distilled H_2O, dip for 1 min in each; (c) 10% H_2O_2 float for 5 min; (d) distilled H_2O and PBS, two 1-min dips in each; (e) decomplemented nonimmune goat serum diluted 1:30 with PBS, float for 5 min; (f) specific antiserum, float overnight at room temperature. For electron microscopy, the antibombesin antiserum was diluted 1:80, the antisomatostatin antiserum 1:40, and the antigastrin antibody 1:100. All sera (immune and nonimmune) were filtered through an HA 0.45 μm millipore filter before use. The following morning the immunoperoxidase reaction was carried out as follows: (g) PBS, three 1-min dips; (h) goat antirabbit IgG diluted 1:10 with PBS float for 30 min; (i) PBS, three 1-min dips; (j) rabbit PAP diluted 1:50 with PBS, float for 8 min; (k) PBS, three 1-minute dips; (l) float grid on Tris buffer drops; (m) DAB solution, prepared fresh by dissolving 6.3 mg of DAB in 50 ml of Tris buffer and adding 0.042 ml of 3% H_2O_2, dip for 3 min under constant stirring; (n) distilled H_2O, three 1-minute rinses, and then allow the grids to air-dry. Without further staining, the grids were observed and photographed under the electron microscope: all peroxidase-positive cells were recorded and later matched with the serial thin sections observed under conventional electron microscopy.

Immunohistochemical controls included (a) substituting normal rabbit serum for the first antibody at the same dilutions; (b) substituting normal goat serum, diluted 1:30 with PBS, for the goat antirabbit IgG; and (c) for gastrin, preincubating the specific antiserum with an excess of synthetic 2-17 human gastrin prior to exposure to the tissues. Other technical controls consisted of omitting either the NaOH-ETOH or the H_2O_2 exposure steps for the light microscopy, and the NaOH-ETOH etching step for electron microscopy.

Results

Because all thin as well as the adjacent semithin sections were serially cut, no two sections were more than 2 μm apart in most cases. This determined that the majority of our "sandwich" section sets were well within one given cell, thus enabling us to establish clear correlations among the three approaches in practically every cell studied. Occasional broken membranes were exceptions to the rule.

The bombesin-containing cells of the bullfrog gastric mucosa were identical to those described previously (8). They had a round or ovoid profile and a large, characteristically indented nucleus. The cytoplasm, often protruding into the surrounding stroma, contained abundant microfilaments and variable numbers of basally located secretory granules. These were characterized by a round or slightly ovoid profile, high electron density, a closely apposed limiting membrane, and a diameter ranging between 140 and 180 nm (Figure 2).

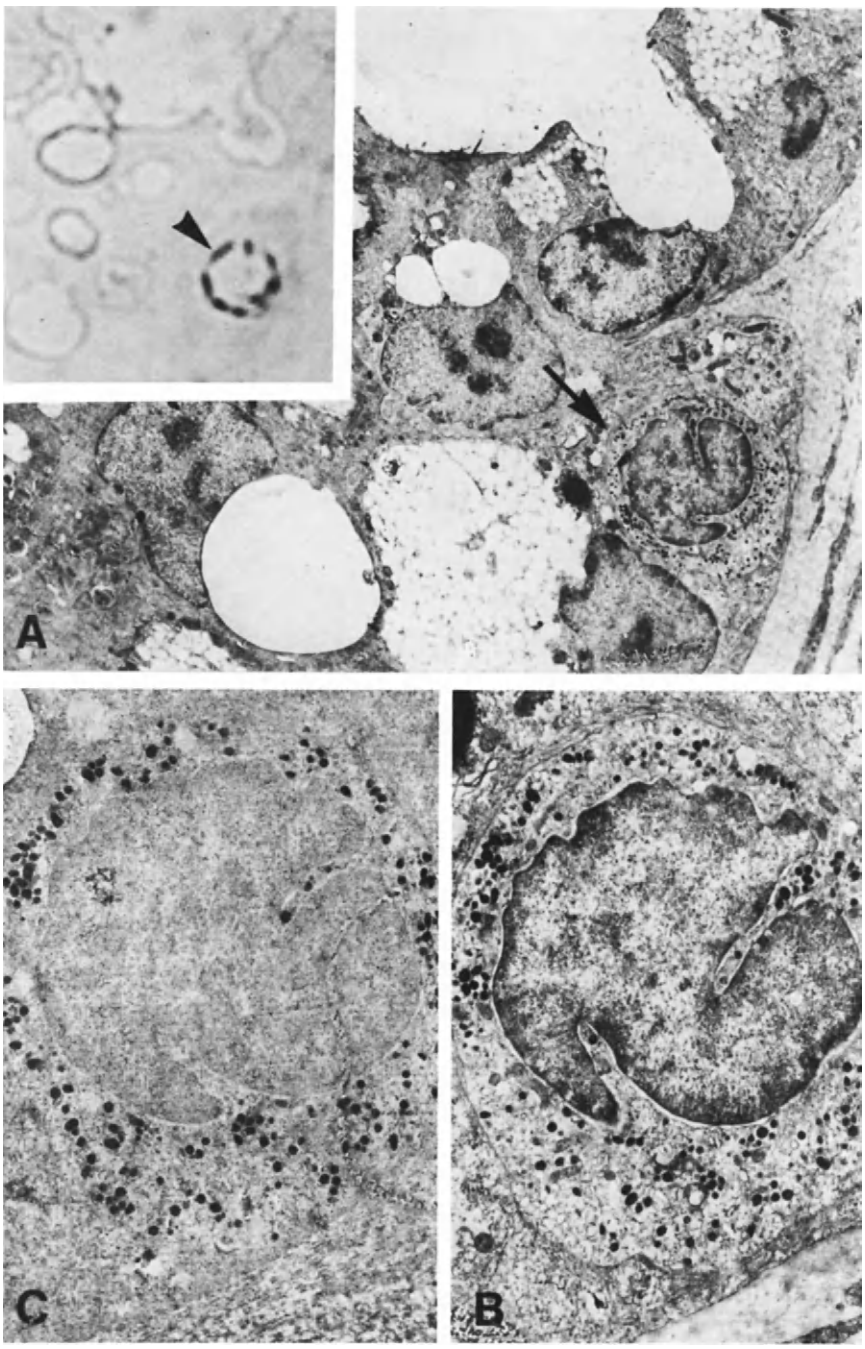

Figure 2. (A) A bombesin-containing cell in bullfrog antral mucosa shown by light microscopy immunoperoxidase in the inset (arrowhead) is identified with an arrow in the serial thin section viewed by conventional electron microscopy. ×2100. (B) Higher-power magnification of the bombesin-containing cell shown above displaying a characteristically infolded nucleus and numerous electron dense cytoplasmic granules. ×5300. (C) Serial thin section of the same cell treated with PAP immunoperoxidase shows intense immunoprecipitate over the bombesin-storing secretory granules. ×5300.

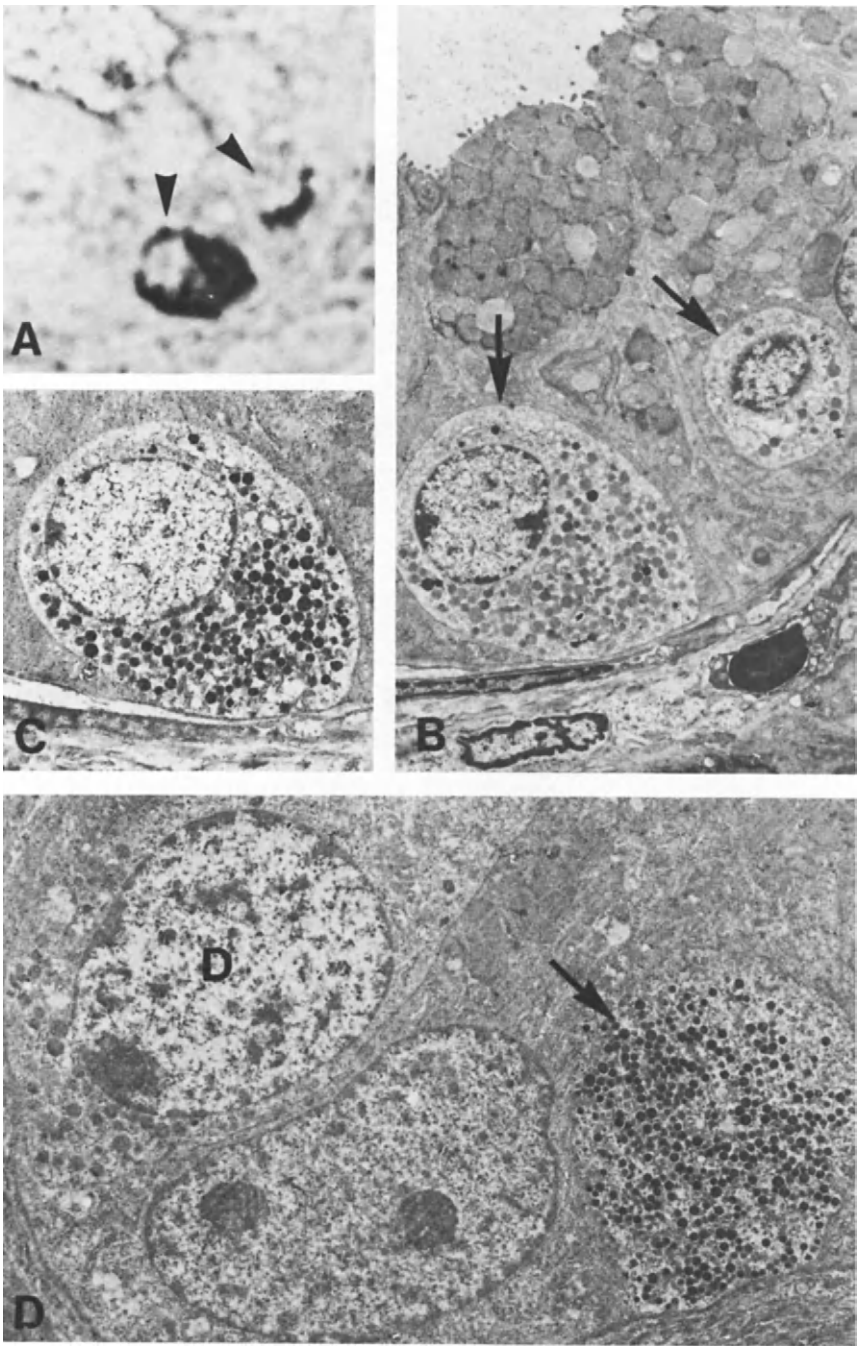

Figure 3. (A) Two somatostatin-containing cells (arrowheads) are identified by light microscopy immunoperoxidase in a human duodenal gland. (B) The same two cells (arrows) seen in a serial section processed for conventional electron microscopy, show the large pale cytoplasmic granules that characterize the D cell. ×2900. (C) Serial thin section demonstrating the D cell to the left after treatment with PAP immunoperoxidase. An intense immunoprecipitate can be appreciated over the secretory granules. ×3200. (D) Thin section of human duodenum treated with PAP immunoperoxidase for gastrin. Although the granules of the intestinal gastrin cell to the right (arrow) are intensely immunostained, those of the D cell to the left (D) show only faint nonspecific staining, thus attesting to the specificity of the immunoperoxidase deposits in the D-cell granules shown above. ×4000.

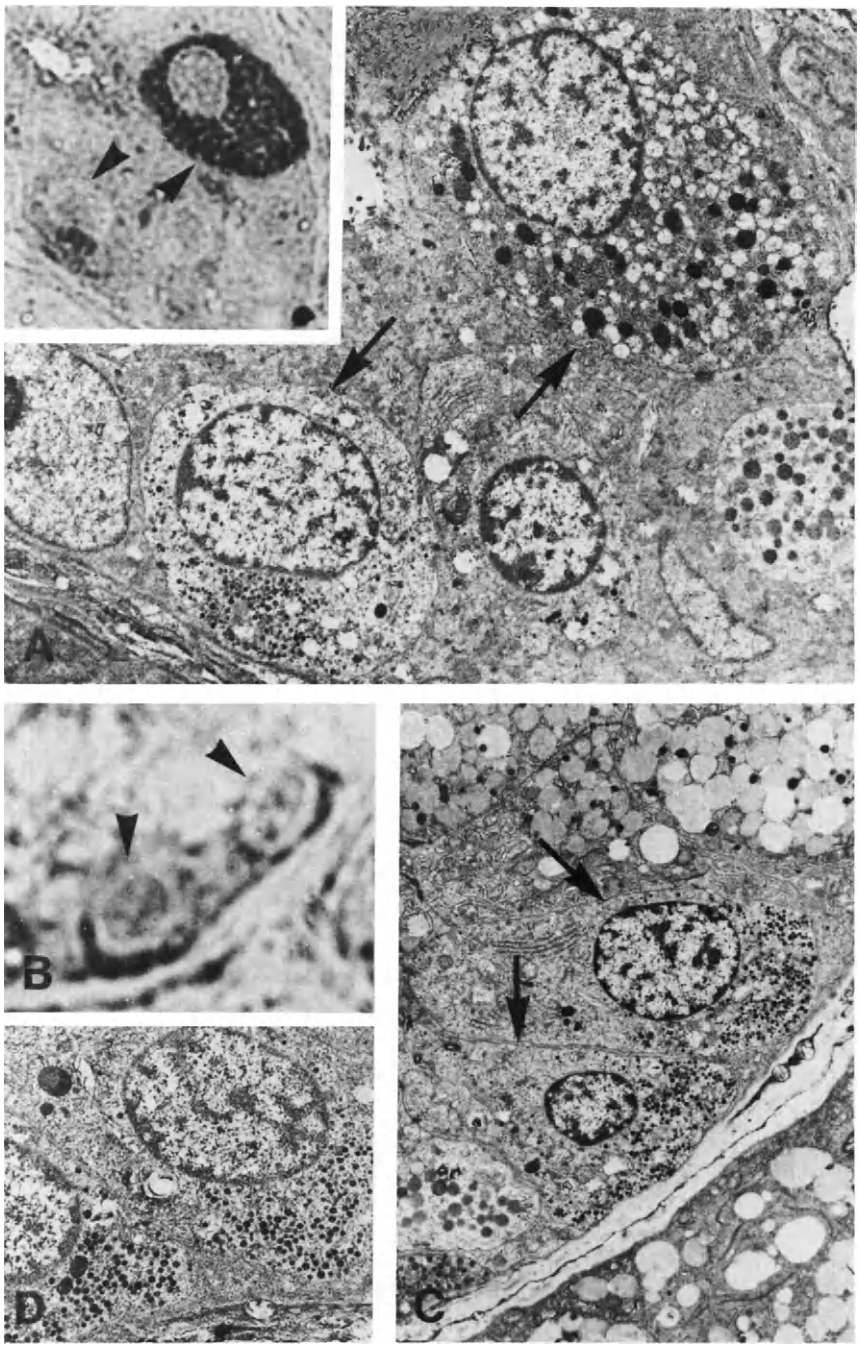

Figure 4. (A) Two gastrin-containing cells in the human duodenal bulb mucosa identified by light microscopy immunoperoxidase in the inset (arrowheads), are correlated with the corresponding cells shown in a semiserial thin section treated with PAP immunoperoxidase (arrows). The cell in the top right corner contains large granules of variable density, characteristic of G cells. The cell in the lower left corner possesses smaller but densely stained granules that typify the intestinal gastrin cell. ×3200. (B) Two gastrin-containing cells in another section of human duodenal mucosa are shown by light microscopy immunoperoxidase (arrowheads). (C) The same two cells (arrows) are identified in a semiserial thin section processed for conventional electron microscopy. ×2800. (D) Serial thin section showing the same two intestinal gastrin cells after treatment with PAP immunoperoxidase. Note the intense immunoprecipitate over the secretory granules, comparable to that shown in Figure 3D. ×3600.

The somatostatin-containing cells, as expected, were found to be the D cells of the human duodenal mucosa. These were characterized by the presence of secretory granules of moderate electron density, with a closely apposed limiting membrane, and measuring between 250 and 400 nm in diameter (Figure 3).

Two populations of immunoreactive cells were identified in the mucosa of the human duodenal bulb with the antigastrin antiserum. Some cells displaying intensely stained coarse cytoplasmic granules by light microscopy immunoperoxidase were further characterized as G cells by ultrastructural observation. These cells were typified by the presence of numerous cytoplasmic granules of highly variable electron density, measuring between 160 and 300 nm in diameter (Figure 4). The other, more frequent, cell type showed very fine immunoperoxidase reactivity in the cytoplasm and was identified under the electron microscope as one of the so-called D_1 cells, later characterized as the intestinal gastrin cell (2). This cell possesses round granules of high electron density that measure between 150 and 200 nm in diameter (Figure 4).

When either the first or the second antiserum was replaced by nonimmune serum from the same species, no specific immunoperoxidase reaction was noted in the granules of the corresponding cells. A similar absence of specific reaction was noted in the case of the gastrin-containing cells when the specific antiserum was preincubated with excess antigen. It should be pointed out that the immunoperoxidase reaction often behaved as a nonspecific stain at the ultrastructural level, revealing with considerable detail all histological structures. Nevertheless, comparison between nonspecifically and immunostained tissues in control situations showed unequivocal differences in the texture and intensity of the granule staining (Figure 3).

In other technique controls, when etching with NaOH-ETOH was omitted, very little background clearing and no immunostaining took place at the light microscopic level. When, after etching wtih NaOH-ETOH, exposure to H_2O_2 was omitted, the nonspecific background staining remained and no immunostaining of the cells took place. At the ultrastructural level, if the preliminary etching with NaOH-ETOH was not carried out prior to H_2O_2 exposure, some immunoperoxidase staining still took place, but it was less intense and its appearance was more haphazard than after the complete treatment.

Discussion

The results obtained here show that the immunoreactivity of bombesin, somatostatin, and gastrin is not abolished when tissues are fixed with glutaraldehyde, postfixed in OsO_4, and embedded in Epon during routine electron microscopy processing. This is in agreement with recent results obtained by others using rat prolactin, (PRI) and growth hormone (GH) (1), ovine luteinizing hormone, (LH) (4), and human fetal GH (10) pituitary cells

as models. In light of this cumulative evidence, it is quite possible that a number of other peptide substances may also be amenable to this same methodology and, therefore, be demonstrable in tissues previously processed for conventional electron microscopy.

In past work dealing with the ultrastructural identification of the frog bombesin cells, we found that etching with NaOH-ETOH is sufficient to remove part of the Epon from the section and to expose the specific antigenic sites in tissues that had been fixed with glutaraldehyde only (8). However, when these frog bombesin cells as well as somatostatin and gastrin cells were examined in the present study following postfixation with OsO_4 after NaOH-ETOH treatment only, we found that there was heavy background staining but no specific immunoperoxidase reaction. When these etched sections were subsequently exposed to H_2O_2, the nonspecific background cleared and, instead, a strong immunoperoxidase reaction appeared, confined to the specific immunocytochemical sites. We assume that the oxidizing properties of H_2O_2 resulted in removal of the OsO_4, thus not only eliminating considerable background staining but also making possible the immunological reaction that up to that point could not take place because of the "blocking" effect of OsO_4 on the specific antigenic sites.

The "sandwich" method used in this work is reliable and relatively simple. An advantage of this method is that all immunohistochemically identified cells can be referred to an adjacent serial section fixed and stained for conventional electron microscopy showing optimal morphological preservation of the histological structures. This, in turn, allows reliable comparisons with results derived from other work using conventional morphological approaches. In addition, this procedure will enable researchers to study retrospectively some peptide-producing tumors and other lesions, spontaneous or experimentally induced, which had been processed for routine electron microscopy and are already available in their files.

ACKNOWLEDGMENT

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Immunocytochemical Investigations of Gastroenteropancreatic Endocrine Cells Using Semithin and Thin Serial Sections

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INTRODUCTION

Light or electron microscopic and immunocytochemical investigations performed on serial sections have been used mainly for the correlative identification of gastroenteropancreatic (GEP) endocrine cells at the light microscopic and ultrastructural level and for the proof of the existence of biogenic monoamines or polypeptide hormones in certain endocrine cell types (3, 6, 7, 8, 18, 29, 30, 31; recent reviews: 20, 47). In addition, appropriate histologic techniques may contribute to the examination of other questions in the field of gastrointestinal endocrinology. Hence the interest of histologists has recently been focused in particular on two problems:

THE MODE OF FUNCTION OF GEP ENDOCRINE CELLS

Apart from an endocrine pathway on more or less remote target organs, the peptides released from these cells are believed to act also via local "paracrine" (11) regulatory mechanisms on cells located in their immediate proximity. Histologic findings on somatostatin cells of the stomach have revealed the morphological correlate of these putative paracrine relationships between endocrine cells and neighboring "target" cells (1, 28, 34).

MULTIPLE HORMONES IN GEP ENDOCRINE CELLS

As demonstrated by immunocytochemical investigations, the same GEP endocrine cell type may contain various peptide hormones (21, 22, 25, 32,

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IN THE DIGESTIVE SYSTEM

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42). Analogous to the findings on pro-ACTH–endorphin in the pituitary, various peptides may be synthesized as a common precursor protein that subsequently is cleaved enzymatically into several different hormones (9, 38, 49). Since GEP endocrine cells also synthesize hormone precursor proteins (37, 39, 41), it seems reasonable to assume that multiple hormones found in the same endocrine cell type may be segments of a common precursor protein.

Immunocytochemical investigations performed on semithin and thin serial sections are especially suitable for contributing to the solution of the previously mentioned questions. The present study is designed to demonstrate the usefulness of this method and to give a review of findings thus far obtained in GEP endocrine cells.

Material and Methods

For light microscopic immunocytochemical studies samples from the pyloric mucous membrane and the pancreas from rat, dog, and man (surgical or endoscopic biopsy specimen) were snap-frozen in melting Freon 22. After freeze drying for 72 h the tissues were fixed with vapor phase *p*-formaldehyde and embedded in Araldite. Serial semithin sections (0.5 μm) were cut and mounted on microscope slides by heat (+90°C for 30 min). After removal of the resin (36) the sections were immunostained using the unlabeled antibody immunoenzymatic technique involving the soluble peroxidase–antiperoxidase complex (48) with omission of the postosmication step.

Antisera raised against the following peptides were used: gastrin, somatostatin, glucagon, glucagon-like immunoreactants (GLI), bovine pancreatic polypeptide (BPP), insulin, ACTH, α-endorphin, and Met-enkephalin.[1]

To exclude any cross-reactivity of the antisera and nonspecific staining, a variety of specificity controls was performed, including the use of increasing dilutions of the antisera; preadsorption of the antibodies with various peptides; replacement of the first antibody of the immunocytochemical staining sequence by α-fetoprotein antiserum (Dakopatts). Other controls included the use of various nonimmune sera [nonimmune rabbit sera; IgG fractions of sera from nonimmunized rabbits; $F(ab')_2$ and Fc fragments of the IgG fraction of sera from nonimmunized rabbits, kindly provided by Behringwerke, FRG]; and use of phosphate-buffered saline (PBS) with various salt content as diluent for the sera or as rinsing solution (for details see 19, 23).

The sections were mounted without counterstaining in Eukitt and viewed with bright-field illumination, phase-contrast optics, or interference-contrast

[1]The antisera against glucagon, GLI, and insulin were obtained commercially (Novo, Denmark). For the gift of the other antisera we are indebted to A. Arimura, New Orleans, USA; R. E. Chance, Indianapolis, USA; B. A. Eipper and R. E. Mains, Denver, USA; W. Schlegel, Münster, FRG; K. H. Voigt, Ulm, FRG; E. Weber, Stanford, USA.

optics (according to Nomarski) in a Leitz Orthoplan microscope. Phase contrast and interference contrast optics greatly enhance the density of DAB polymers, thus leading to an increased efficiency of immunoperoxidase methods. In addition, immunoreactivities not discernible by bright-field illumination are also visualized.

Somatostatin and pancreatic polypeptide (PP) immunoreactive cells of the canine endocrine pancreas were reconstructed graphically at a total magnification of 4000 × from a series of about 50–100 semithin sections stained with either antiserum. The same immunocytochemical staining methods as in light microscopy were applied at the electron microscopic level except that the tissue (rat pancreas) was not lyophilized but fixed by immersion in 2% phosphate-buffered (0.1 *M*, pH 7.4) glutardialdehyde. Thin (about 50 nm) sections were cut serially and collected on nickel grids. Adjacent sections were stained for glucagon or endorphin using a staining schedule as described (48) and viewed in an electron microscope Zeiss EM 9S-2.

Results and Discussion

Shape of GEP Endocrine Cells and Their Relationship to Neighboring Structures

The endocrine cells of the gastrointestinal mucosa—at least the "open type" ones (15)—are considered to be bipolar in structure and function ("receptosecretory cells"; 14, 15). Following stimuli coming from the gastrointestinal lumen, the "chemical messengers" (17) of enteroendocrine cells are secreted by exocytosis at the lateral or basal portions of the cells (13–15, 20). Because of their structural and functional similarities with neurons or sensory cells, enteroendocrine cells have also been named "paraneurons" (13, 14). Correspondingly long basal processes seen in various enteroendocrine cell types (1, 18, 34) were interpreted as residues of simple neurons (13).

In contrast to enteroendocrine cells, the shape of pancreatic endocrine cells has received comparatively little attention, although occasionally a bipolar configuration (10) or long cytoplasmic processes (4) have been described as properties of these cells. We found that particularly somatostatin and PP cells of the islets of Langerhans possess long processes extending in most cases to blood capillaries (Figures 1 and 2). Immunostained semithin sections observed with phase contrast optics or interference contrast optics reveal another peculiarity of these cell types (to be seen also, although less pronounced, in insulin and glucagon cells): The immunoreactive material is distributed unevenly within the cytoplasm; cell portions or cytoplasmic processes facing blood capillaries show strong immunoreactivities, whereas other cell portions or processes (in most cases short) show only moderate or

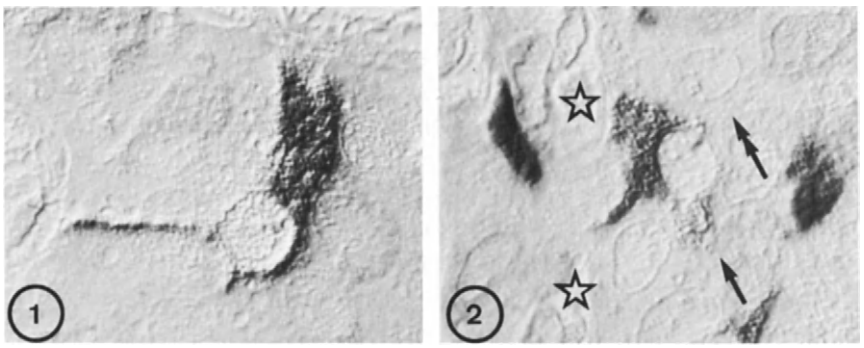

Figure 1. PP immunoreactive cell in the human endocrine pancreas. Freeze-dried, vapor phase *p*-formaldehyde-fixed, epoxy-resin-embedded tissue; semithin (0.5 μm) section stained by the PAP method and viewed with interference contrast optics. Note the slender process of this cell extending to a capillary on the left (×1400).

Figure 2. Somatostatin immunoreactive cells in the canine endocrine pancreas (methods as in Figure 1). The cell in the middle of the figure shows uneven distribution of immunoreactive material in the cytoplasm. Cell portions facing capillaries (asterisks) exhibit strong immunoreactivity; other cell portions show moderate staining (arrow) or no staining at all (lanceolate cytoplasmic process, double-headed arrow) (×1200).

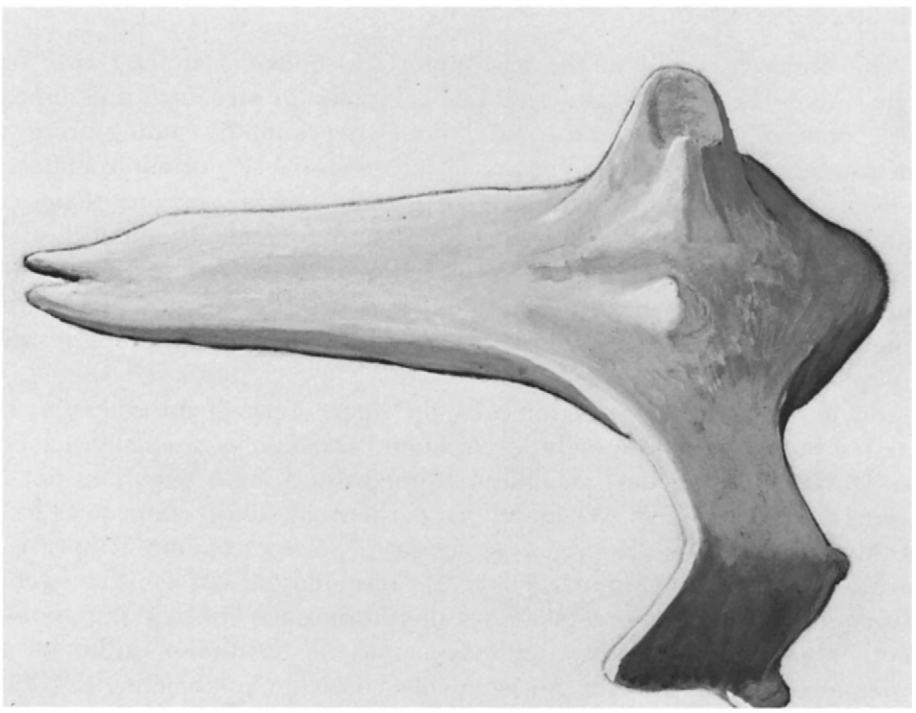

Figure 3. Three-dimensional structure of a PP cell from a canine pancreatic islet. The cell was reconstructed graphically from 38 serial semithin (0.5 μm) sections of epoxy-resin-embedded tissue. The long, sheetlike process on the left showed strong immunoreactivity and was running next to a capillary; other processes were situated between islet cells (×5000).

no staining (Figure 2). These "silent" (with respect to immunostaining) processes were only discernible at high magnifications in semithin sections when phase-contrast optics or interference-contrast optics were used. Since nonimmunoreactive cell portions will escape detection in immunostained paraffin sections or in semithin sections stained by immunofluorescence methods, former immunocytochemical investigations have accordingly shown an incomplete image concerning the shape of pancreatic endocrine cells (similar "silent" processes may also occur in enteroendocrine cells).

When concerned with the shape and number of processes of pancreatic endocrine cells and their relationship to neighboring structures (including other endocrine cells, blood vessels, and nerve fibers) it is not sufficient, however, to observe single immunostained sections. More complete information can be obtained from semithin serial sections or from three-dimensional reconstructions from the semithin serial sections. As yet we have performed such reconstructions of canine pancreatic somatostatin and PP cells. As shown in Figure 3, the cytoplasmic processes of these cells are often not round but rather flat and sheetlike.

The three-dimensional reconstruction of GEP endocrine cells from semithin sections is indeed laborious and time-consuming. Our preliminary experience, however, was encouraging, and results obtained with this technique will certainly lead to a better understanding of the organization of islet cells.

In conclusion, it should be emphasized again that pancreatic endocrine cells, in analogy with enteroendocrine cells, are bipolar in structure and therefore most likely also in function. When the proposed function of pancreatic islets is considered as a neurosecretory system (16), those parts of islet cells showing strong immunoreactivities may represent the effector pole and "silent" processes, the receptor pole of these "neurosecretory" elements.

MULTIPLE HORMONES IN GEP ENDOCRINE CELLS

Results of immunocytochemical studies reported by several laboratories suggest that certain GEP endocrine cell types may contain various peptides in addition to their "established" hormones. These cell types include gastrin (G) cells showing immunoreactivities for "pituitary" hormones (12, 21, 26, 32, 42); pancreatic glucagon cells that can be immunostained with antisera against cholecystokinin (CCK), gastric inhibitory polypeptide (GIP), and α-endorphin (2, 24, 25, 44), and intestinal GLI cells exhibiting immunoreactivity for PP (46).

We have studied the immunoreactivities of G, glucagon, and GLI cells using semithin serial sections alternatively immunostained with various antisera. During these investigations special attention has been paid to specificity controls since all these cell types show nonspecific binding of immunoglobulins (5, 19, 22, 23, 25). In addition, investigations concerning the immunoelectron microscopic localization of peptides in rat pancreatic glucagon cells (22, 25) were continued.

Gastrin Cells

We confirmed that G cells show immunoreactivities against various antisera raised against corticotropin-lipotropin related peptides, i.e., CLRP (Figure 4a, b). However, these cells can also be stained with antisera against several enteric or pancreatic hormones. Moreover, G cells were "immunoreactive" toward various control sera, including α-fetoprotein antiserum, nonimmune sera, and IgG fractions from nonimmune sera or $F(ab')_2$ fragments of immunoglobulins (Figure 4c). Thus immunocytochemical findings in G cells should be interpreted with certain reservations, because the possibility that binding of specific immune sera to G cells by the same mechanism as nonspecific binding of IgG to G cells cannot be excluded. In this connection it is of particular importance that "adsorption controls" (use of antisera preadsorbed with their corresponding peptides), a specificity test commonly used in immunocytochemistry, may give misleading results. This test is indeed suitable to prove the specificity of the antisera in question. However, adsorption controls will be no definite proof for the specificity of the mechanisms by which the antibodies are bound to G cells (5, 19).

Our findings indicate that CLRP antibodies are bound to G cells rather by nonspecific than by specific mechanisms. These conclusions are based mainly on two findings: (1) The immunoreactivities of G cells toward CLRP antisera depend on their dilution, in contrast to results obtained in pituitary ACTH/endorphin cells, which were investigated concomitantly; and (2) the number and staining intensities of CLRP immunoreactive G cells are strongly reduced when the NaCl content of the PBS used as diluent for the antisera or as rinsing solution is increased (Figures 4b, 5b). In particular, this displacement of CLRP antibodies from their binding sites in G cells by salts is an indication that nonspecific binding mechanisms (most probably ionic interactions) are involved in the binding of CLRP antibodies to G cells. Similarly, the "immunostaining" of G cells by control sera was greatly reduced by rinsing of the sections with PBS of relatively high salt content after serum incubation (Figure 5c). In contrast, staining of G cells by antigastrin (or of pituitary ACTH/endorphin cells by CLRP antisera) was not influenced by this modification of the immunocytochemical staining sequence (Figures 4a, 5a).

Should G cells contain any ACTH-related peptides (33) these, according to our immunocytochemical results, are different from the molecular forms hitherto found in the pituitary.

Intestinal GLI Cells

According to various findings from biochemical as well as immunocytochemical studies, intestinal GLI immunoreactive endocrine cells are related to pancreatic glucagon cells (27, 37, 43). GLI and glucagon are believed to be synthesized within a precursor protein common to both cell types (41).

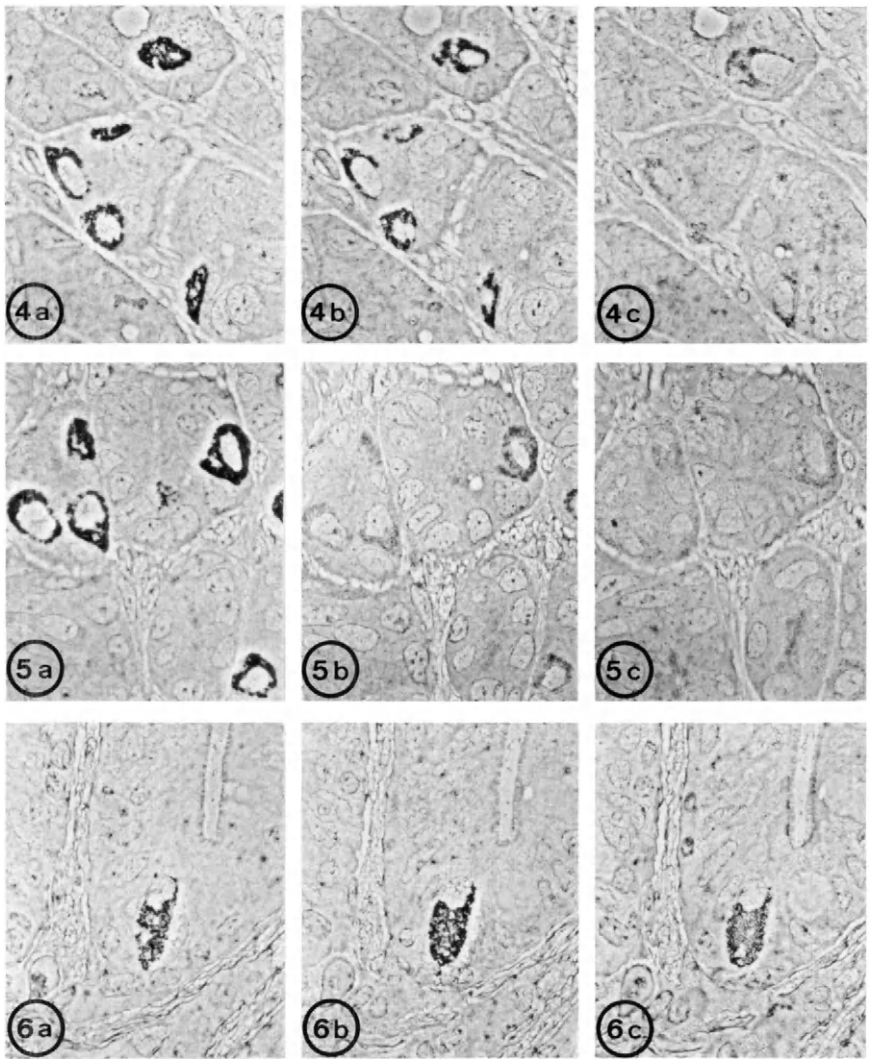

Figure 4. Immunoreactivities of rat pyloric gastrin cells. Freeze-dried, vapor phase *p*-formaldehyde-fixed, epoxy-resin-embedded tissue. Three adjacent semithin sections were stained (PAP method, phase-contrast microscopy) (a) with gastrin antiserum diluted 1:1000 in PBS, (b) with ACTH antiserum diluted 1:1000, and (c) with $F(ab')_2$ fragments of the IgG fraction of nonimmune rabbit sera, diluted 1:500. All G cells stained by gastrin or ACTH antiserum were also stained by $F(ab')_2$ fragments (×640).

Figure 5. Immunoreactivities of rat pyloric G cells under modified staining conditions. The sections were stained as those in Fig. 4 except that PBS with 0.5 *M* NaCl (instead of 0.15 *M* NaCl) was used as rinsing solution subsequent to serum incubation. Gastrin immunoreactivity (a) is unaltered, whereas ACTH immunoreactivity (b) and binding of $F(ab')_2$ fragments (c) is decreased (×640).

Figure 6. Immunoreactivities of canine intestinal GLI cells (methods as in Figure 4). Three adjacent sections were stained with (a) GLI antiserum diluted 1:2000, (b) BPP antiserum diluted 1:2000, and (c) $F(ab')_2$ fragments diluted 1:500. Apart from specific immunoreactivities, GLI cells show nonspecific binding of immunoglobulins (×640).

We have found, independently from Solcia and colleagues (46), immunoreactivities of intestinal GLI cells (dog, man) against BPP antibodies (Figure 6a, b). In contrast to the immunoreactivities of G cells toward CLRP antisera, the PP immunoreactivity of GLI cells was independent of the dilution of the antibodies within a wide range (antisera diluted 1:8000 gave positive staining). Moreover, the PP immunoreactivity was barely influenced by variations of the salt content of the PBS used as diluent. Thus, even though intestinal GLI cells can be "immunostained" by various control sera [e.g., *a*-fetoprotein antiserum, IgG fractions from nonimmune sera or $F(ab')_2$ fragments; see Figure 6c], it seems probable that the PP immunoreactivity of these cells is specific. Provided that this specificity can be confirmed by further investigations, PP cells—hitherto considered as a distinct endocrine cell type—will have to be regarded as related to glucagon or GLI cells. Apart from similarities concerning the intrainsular distribution pattern of glucagon and PP cells (40) this speculation is supported by our previous findings, which had shown that a minority of pancreatic glucagon cells (rat, dog) show also PP immunoreactivity (22). Consequently, if PP is contained in the same endocrine cell type as GLI or glucagon and possibly also in the same precursor protein, PP most likely is present "unmasked" in intestinal GLI cells and "masked" in the majority of pancreatic glucagon cells. These differences may be caused by the set of cleaving enzymes localized in the two cell types.

Pancreatic Glucagon Cells

Following the detection of α-endorphin immunoreactivity in rat pancreatic glucagon cells (25), we also examined pancreatic endocrine cells of other species (dog, man) for endorphin immunoreactivity. Moreover, additional studies were necessary on the specificity for endorphin immunoreactivity of the rat glucagon cell, since this endocrine cell type also exhibits nonspecific binding of immunoglobulins (5, 22, 25). Finally, immunoelectron microscopic studies performed in rat glucagon cells should yield information concerning the subcellular distribution of glucagon and α-endorphin immunoreactivities within these cells.

The specificity of endorphin-like immunoreactivity in rat glucagon cells was examined, in addition to adsorption controls (25), by running ascending dilutions of the antisera and using PBS with various NaCl content as diluent or as rinsing solution. The results of all these controls indicated that the binding of α-endorphin antibodies to rat glucagon cells is caused by specific (immunological) mechanisms. However, neither canine nor human pancreatic endocrine cells showed immunoreactivities toward endorphin antibodies. Thus, provided that an endorphin-like peptide is contained in the glucagon precursor protein, either the amino acid sequence of this protein or its secondary and tertiary structure show interspecies variations.

As revealed by immunoelectron microscopy, the endorphin immuno-

reactivity, together with glucagon immunoreactivity, is confined to the secretory granules of rat glucagon cells (25). However, in all thin sections immunostained with either antiserum, some secretory granules with less dense or no staining are present (Figure 7). These differences between individual secretory granules may have one of the following causes: either the faintly stained granules were sectioned tangentially and the hormone-containing core was not "opened" by the section or the secretory granules of glucagon cells were not uniform with respect to their hormone content. To answer this question thin serial sections were immunostained with glucagon antiserum or purified α-endorphin antibodies. Comparing now adjacent sections, it was found that on an average, two or three thin sections passed through

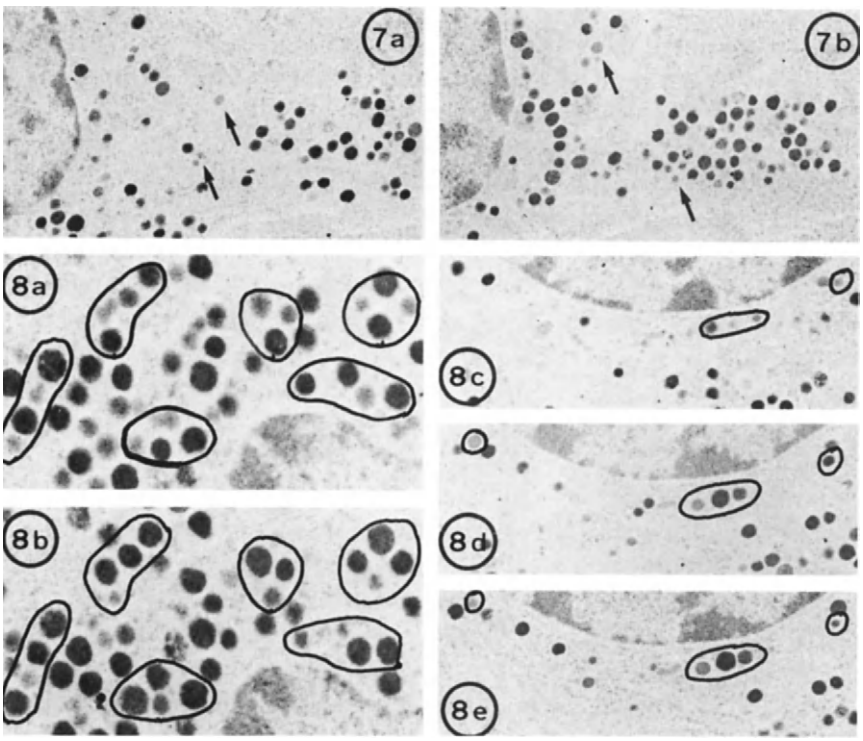

Figure 7. Immunoelectron microscopy of rat pancreatic glucagon cells. Semiadjacent thin sections through the same rat pancreatic glucagon cell were immunocytochemically stained with the PAP method for (a) glucagon and (b) α-endorphin. Staining for both hormones is clearly confined to the secretory granules. Note some granules with faint staining (arrows) (×9000).

Figure 8. Immunoelectron microscopy of rat pancreatic glucagon cell secretory granules in adjacent thin sections. The same secretory granules stained for glucagon can be identified in two or three adjacent sections. (a, b) A set of glucagon immunoreactive granules in two adjacent sections. Numerous granules (encircled) are seen in both sections. Granules faintly stained in one section are strongly immunoreactive in the other (×19,000). (c–e) Three adjacent thin sections stained for glucagon. Some secretory granules can be identified in two or all three sections. Some of the encircled granules show faint staining in consecutive sections, indicating deficiency of immunoreactive material within these granules. (×9000).

the same secretory granules (Figure 8). In most instances, secretory granules which were stained less densely in the one section showed strong immunoreactivity in the adjacent section (Figure 8a, b). Hence in most cases the moderate or faint immunostaining of secretory granules is obviously caused by the fact that the granules were sectioned tangentially. However, secretory granules were sporadically detected exhibiting the same faint staining on two or three adjacent sections (Figure 8c–e). From these findings it may be concluded that a minority of glucagon cell secretory granules either does not contain any hormone or that the amino acid sequences of glucagon and endorphin are present in a "masked" form within these granules. If so, this can be taken as an indication, although indirect, that active cleaving enzymes for the degradation of precursor proteins might not be distributed uniformly in the glucagon cell secretory granules.

Finally, concerning the synthesis of multiple hormones by GEP endocrine cells, it should be emphasized that all cell types hitherto regarded as "candidates" for the synthesis of multiple hormones show nonspecific binding of immunoglobulins. This nonspecific binding of IgG most likely occurs via ionic interactions between cationic (35) IgG or Fab fragments and anionic constituents within the secretory granules of these cells (5, 19, 23, 45). On account of this peculiarity, immunocytochemical investigations of GEP endocrine cells need many other specificity controls in addition to the commonly performed ones. In any case, definite information concerning the types of peptides present in these cells will result only from the analysis of the amino acid sequence within their hormone precursor proteins.

Summary

When semithin sections are cut from epoxy-resin-embedded tissues, about 20–50 sections will pass through the same GEP endocrine cell. In thin serial sections even the same secretory granules can be identified in two or three adjacent sections. In the present study immunostained semithin and thin serial sections were used to examine various questions about the morphology and function of GEP endocrine cells.

It could be shown that pancreatic endocrine cells are bipolar units exhibiting the morphologic correlates of putative receptor and effector poles. In particular, the three-dimensional reconstruction of single endocrine cells from semithin serial sections has proved to be a method suitable for the investigation of relationships between endocrine cells and surrounding structures.

Concerning the alleged synthesis of multiple hormones by certain GEP endocrine cell types, semithin and thin serial sections were immunostained alternatively with antisera against pituitary, enteric, and pancreatic hormones as well as with a variety of control sera. These studies revealed that all GEP endocrine cell types, hitherto regarded to be the source of multiple hormones, bind immunoglobulins by nonspecific mechanisms (most probably

by ionic interactions). This peculiarity limits the validity of immunocytochemical methods as a proof of the presence of peptides within a given cell type. Nevertheless, there is some evidence that canine and human intestinal GLI cells contain PP immunoreactivity in addition to GLI. A minority of rat and dog pancreatic glucagon cells also shows immunoreactivity against PP antisera. Pancreatic glucagon cells of the rat (but not of dog or man) exhibit immunoreactivity for α-endorphin. Both glucagon and endorphin immunoreactivities are confined to the same secretory granules. On the other hand, the binding of antibodies against CLRP to gastrin cells of the stomach, which has been investigated most extensively, seems to be mediated by nonspecific mechanisms.

ACKNOWLEDGMENTS

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Function and Morphology in Gastric Endocrine Cells

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With few exceptions the mechanisms of activation and deactivation in the various endocrine/paracrine cells of the digestive tract are unknown. For many of these cells the hormone remains unidentified. Other cells produce known hormones, but the signals that cause activation or deactivation have not yet been identified. It is likely that the many diverse peptide hormone-producing cells of the gut respond in a similar manner, cytochemically and ultrastructurally, to activating and deactivating stimuli. Once the typical cytochemical and ultrastructural features of activation and deactivation have been identified, predictions can be made as to what physiological functions the many poorly known endocrine cell systems of the gut may have.

Peptide hormone-producing cells, like protein-secreting cells in general, display characteristic structural and ultrastructural features, depending on their functional state. Such features and their relation to cellular function have been studied in some detail in different cell types of the adenohypophysis, in thyroid C cells, and in pancreatic endocrine cells, the insulin cells in particular.

Endocrine cells are very numerous in the gastric mucosa. They are of at least five or six different types (Table 1). Except for the gastrin cells very little is known of their functional significance. Also the relationship between functional state and morphology of the various gastric endocrine cells is poorly understood, and the data available pertain to only a few cell types. The word *morphology* is used here in a broad sense to cover all features of the cells that can be documented with histological, histochemical, or electron

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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TABLE 1
The Predominant Endocrine Cell Populations in the Stomach

Gland	Population
Pyloric	Gastrin cells
	Somatostatin cells
	Enterochromaffin cells[a], D_1 cells[a]
Oxyntic	ECL cells[a]
	A-like cells[a]
	Somatostatin cells
	(Glucagon cells)[b]

[a]The peptide hormone produced remains unidentified. The cells are identified as peptide hormone-producing on ultrastructural and cytochemical grounds.

[b]Glucagon cells are within parenthesis because in many species, e.g., the rat, they are absent or very few.

microscopic techniques. The cells to be discussed in the following are the gastrin cells, the ECL and A-like cells, and the somatostatin cells. Attention was focused on the following problems:

1. The topographic distribution of gastrin cells in the pyloric glands is known to differ in a characteristic manner from one species to another. Why?
2. The gastrin cells respond readily to changes in the conditions in the antral lumen. Which factors are important?
3. The ECL cells and the A-like cells comprise the major endocrine cell populations in the rat oxyntic mucosa. Their physiological roles are unknown. Under various experimental conditions they undergo marked cytochemical and ultrastructural changes. What mechanisms are involved?
4. Several endocrine cell populations in the gut, notably the somatostatin cells, are furnished with long processes ending in clublike swellings in juxtaposition to neighboring cells. Does this provide the morphological basis for cell-to-cell contact and a paracrine mode of signal transmission?

The Gastrin Cell

In several species, such as the cat, dog, and man, the gastrin cells are located in the midzone of the glands, at the transition of the apical straight portion of the glands and the basal convoluted portion (Figure 1) (23). In the rat, gastrin cells are located at the base of the glands. One possible explanation for this very characteristic difference in distribution between species may be that the gastrin cells are located where they can adequately taste the contents of the stomach and still retain a well-protected position

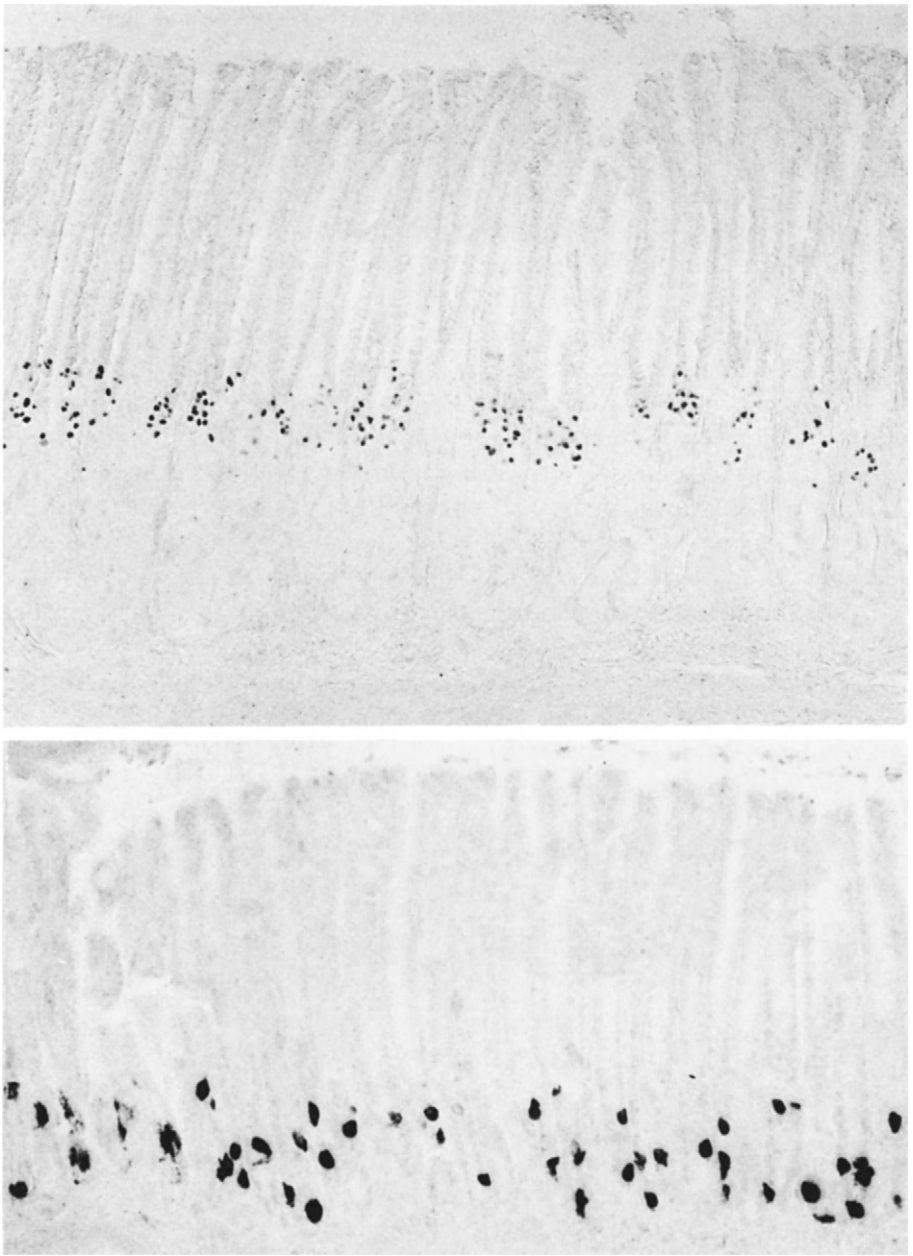

Figure 1. Gastrin cells in the pyloric gland region of a dog (top) and a rat (below). Immunoperoxidase staining. Tranverse sections showing the entire height of the glands. Note the characteristic localization of gastrin cells in a midzone of the pyloric glands in the dog. In the rat the gastrin cells are located at the base of the glands. ×200 (top), ×65 (below).

within the glands. If so, that particular place should be favored for a very specific reason.

We performed a series of studies in which we gave cats and rats various

dyestuffs mixed with the food. At various times after ingestion of the dyes the animals were killed and specimens of the pyloric glands were taken for histological examination. Our findings are illustrated in Figure 2. The dye migrated down to, but never below, the point where the gastrin cells were located. Apparently, the flow of material in the lumen of the pyloric glands is such that this is a strategic position. Perhaps this is the lowest point in the glands where changes in pH and in composition of the juice can be adequately perceived by the gastrin cells.

Is it possible to change the distribution of the gastrin cells by, for instance, permanently changing the antral pH? To test this possibility we performed various types of gastric operations in rats (Figure 3). In one group we eliminated the oxyntic gland area completely (fundectomy); in another group we excluded the antrum from continuity with the gut so that no food passed through it (antrum exclusion); a third operation involved the transposition of the antrum with its vascular supply intact to the transverse colon (antrocolic transposition). In all these three groups the spatial relationship between the oxyntic glands and the pyloric glands had been interrupted and acid no longer passed into the antrum. In a fourth group we performed bilateral truncal

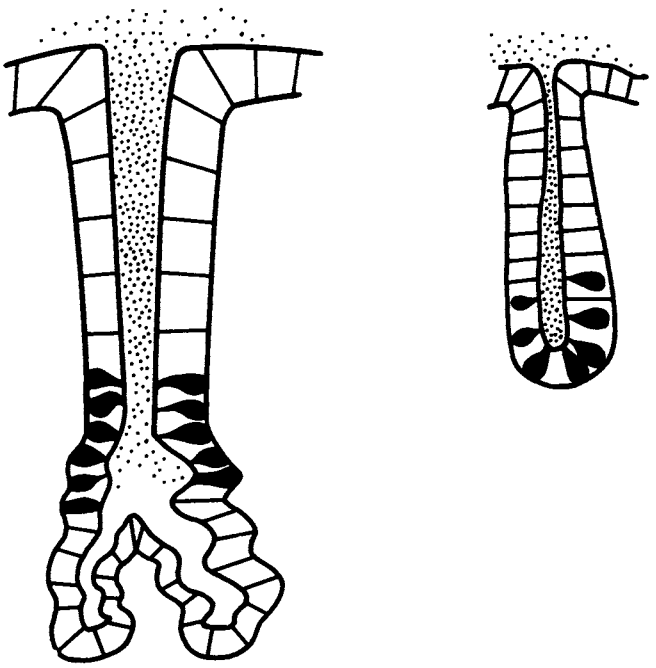

Figure 2. Drawing indicating the distribution of dye (methylene blue) in the pyloric glands of cats (left) or rats (right) 15–45 min after ingestion of food mixed with the dye. Tissue specimens were taken from the wall of the antrum, frozen, freeze-dried, and fixed with gaseous formaldehyde. Paraffin sections were mounted in Entellan and examined in a light microscope.

vagotomy, which is known to lower acid output (9, 10). Another group of rats was subjected to portacaval shunting, which increased basal acid secretion (4). Figure 4 shows antral gastrin cells demonstrated by immunoperoxidase staining in a control rat and after fundectomy, antrum exclusion, or antrocolic transposition. It is apparent that these various operations affected both the topographic distribution and the number of gastrin cells. In the fundectomized rat, the antrum-excluded rat, and the rat with antrocolic transposition, a greater proportion of the gastrin cells was found higher up in the glands than is normally the case.

Figure 5 summarizes our findings on the altered distribution of gastrin cells in the glands. The gastrin cells tend to climb upward in the pyloric glands when secretory products from the oxyntic glands no longer reach them. The reasons for this are unknown. The change in antral pH and/or the altered composition of the gastric juice might be responsible. Also, the density of gastrin cells was affected as illustrated in Figure 6. In fundectomized rats the cell density was increased ($p < 0.001$) whereas in portacaval-shunted rats the density was reduced. The serum gastrin concentrations were affected by the operations, being very dramatically increased after fundectomy ($p < 0.001$) and in fasted animals also after antrum exclusion and vagal denervation ($p < 0.001$) (2, 14) (Figure 7). After antrocolic transposition the serum gastrin level was the same as after antrum exclusion. In freely fed shunted animals the serum gastrin concentrations were lower than in the controls ($p < 0.001$) (Figure 7). Also the concentration of gastrin in antral mucosa was greatly affected by the various operations (Figure 8). Fasting lowered the gastrin concentration in unoperated and portacaval-shunted rats but not in the other

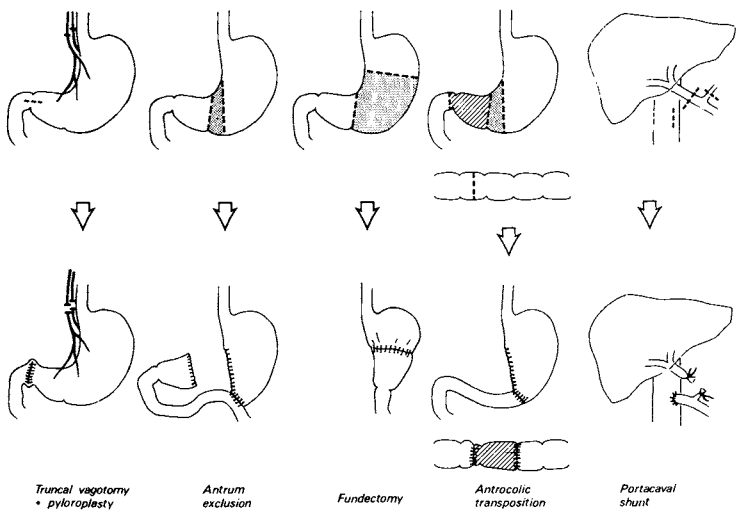

Figure 3. Schematic presentation of the surgical procedures employed. In antrum exclusion and antrocolic transposition, the antral portion was excised in such a manner that no parietal cells remained in the transitional zone.

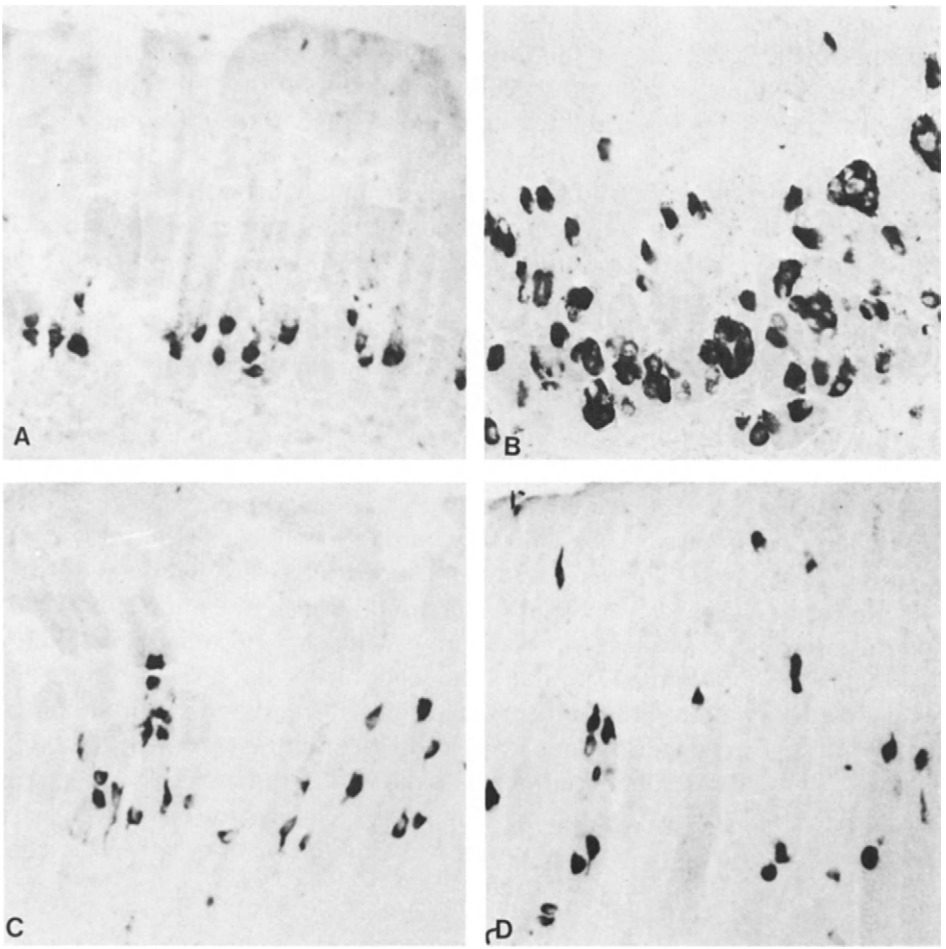

Figure 4. Gastrin cells in the pyloric gland area of (A) a control rat and of rats subjected to (B) fundectomy, (C) antrum exclusion, or (D) antrocolic transposition. Immunoperoxidase staining. ×200. The rats were killed 6–10 weeks after the operations and after a 48-h fast. Note that the gastrin cell density was increased following fundectomy and unchanged following antrum exclusion and antrocolic transposition. Note also that gastrin cells were found not only at the base of the pyloric glands but also higher up following fundectomy and in particular following antrocolic transposition.

experimental groups. Antrum exclusion and antrocolic transposition greatly reduced the gastrin concentration ($p < 0.001$), regardless of the prandial state. The gastrin concentration was largely unaffected by fundectomy but greatly reduced by combined fundectomy and antrum exclusion ($p < 0.001$). These alterations reflected changes in the gastrin cell density and/or changes in the amount of gastrin per cell. Obviously, fundectomy brings about a powerful stimulation of the gastrin cells. As shown previously, the serum gastrin concentration was conspicuously high (Figure 7), conceivably reflecting a high rate of gastrin release. Second, the gastrin cell density was

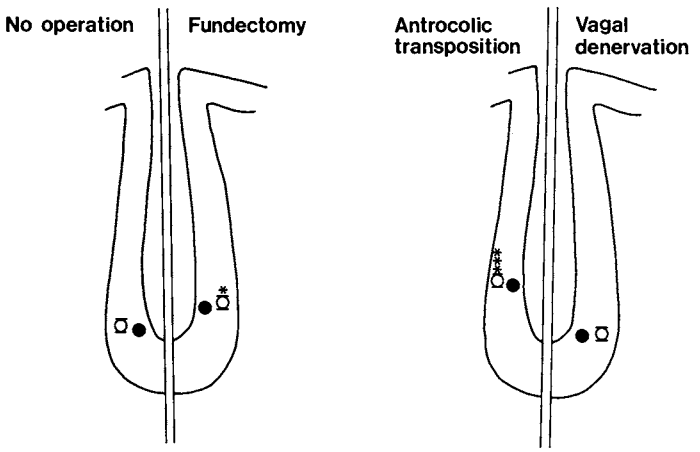

Figure 5. A schematic illustration of the topographic distribution of gastrin cells in the pyloric glands of control rats and rats subjected to fundectomy, antrocolic transposition, or vagal denervation. The rats were killed 6–10 weeks after the operation and after a 48-h fast. The location of each individual gastrin cell was defined by the distance between the cell and the glandular base divided by the height of the gland. Median values are indicated by filled circles, mean values by open circles. At least five animals in each group and at least 300 cells in each group. Vertical bars indicate S.E.M. (*) $p < 0.05$; (***), $p < 0.001$.

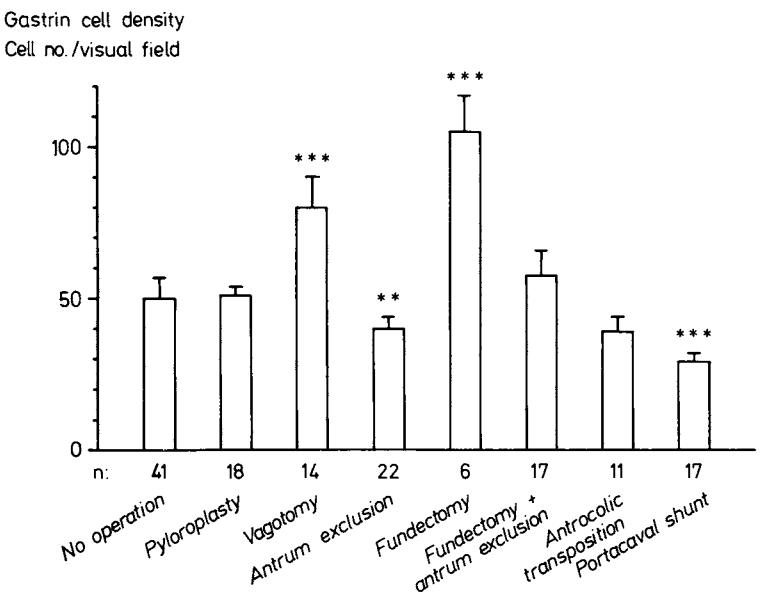

Figure 6. Antral gastrin cell count (expressed as number of cells per visual field) following the various operations (2). The rats were killed 6–10 weeks after the operation and after a 48-h fast. Vertical bars give S.E.M. (n = number of rats). (**) for $0.001 < p < 0.01$ and (***) for $p < 0.001$ refer to the difference between operated and unoperated rats.

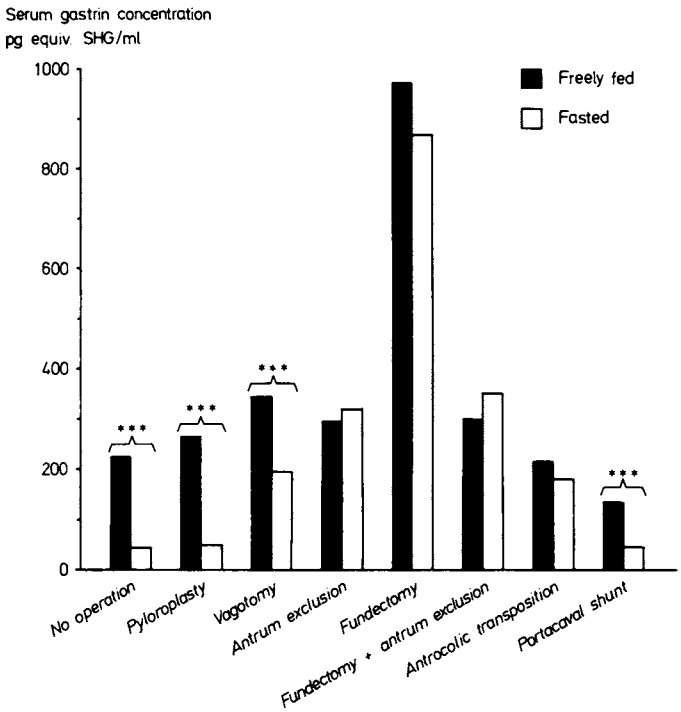

Figure 7. Serum gastrin concentrations 6–10 weeks after the operations. At sacrifice the rats were either fed freely or fasted for 48 h. Each value is the mean of 4 to 50 determinations. Student's t-test was used to establish significance of differences between fasted and freely fed rats within each experimental group. Elevation of antral pH seems to be a strong stimulus for gastrin release. Following operations that prevented the flow of gastric juice into the antrum, the serum gastrin concentration was increased and independent of the prandial state.

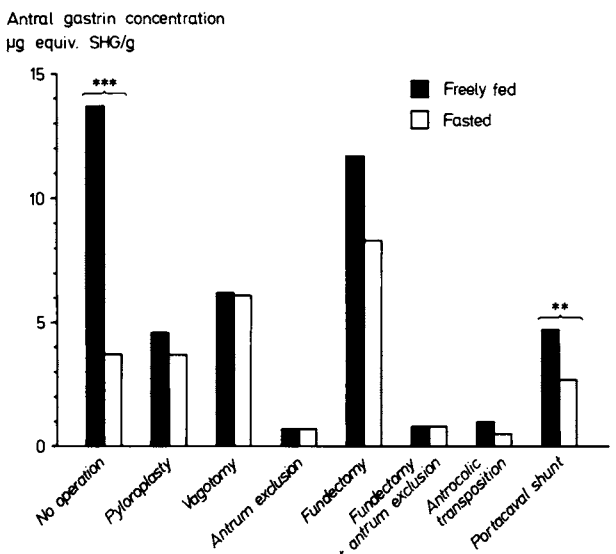

Figure 8. Gastrin concentration in the mucosa of the pyloric gland area 6–10 weeks after the operations. At sacrifice the rats were either fed freely or fasted for 48 h. Each value is the mean of 4 to 43 determinations. Student's t-test was used to establish significant difference between fasted and freely fed rats within each experimental group. A high antral gastrin concentration probably reflects either a high rate of gastrin synthesis and/or a low rate of gastrin release; vice versa for a low gastrin concentration.

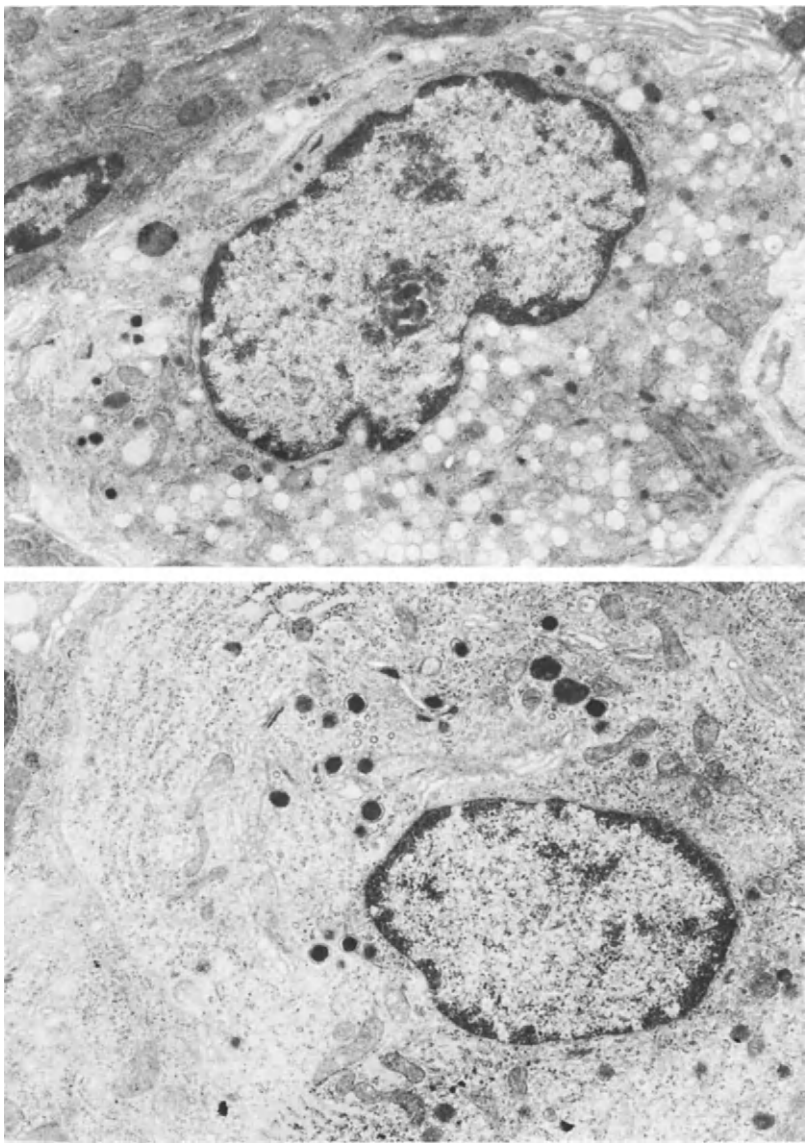

Figure 9. Electron micrographs of gastrin cells from unoperated rat (top) and fundectomized rat (bottom), selected to illustrate the effects of fundectomy. Note the reduced granularity. Note also that highly electron-dense granules occur in greater proportion following fundectomy, that the endoplasmic reticulum is increased, and that the Golgi area is enlarged. ×12,000.

greatly increased (Figures 4, 6), reflecting a trophic influence as well. Figure 9 shows the ultrastructure of a gastrin cell from a fundectomized rat. It displays an increased endoplasmic reticulum and an enlarged Golgi area compared with gastrin cells of unoperated rats. In addition, the number of granules was lowered and the proportion of electron-dense (dark) granules was increased following fundectomy (2). Figure 10 summarizes our morphometric findings on gastrin cells from fundectomized rats.

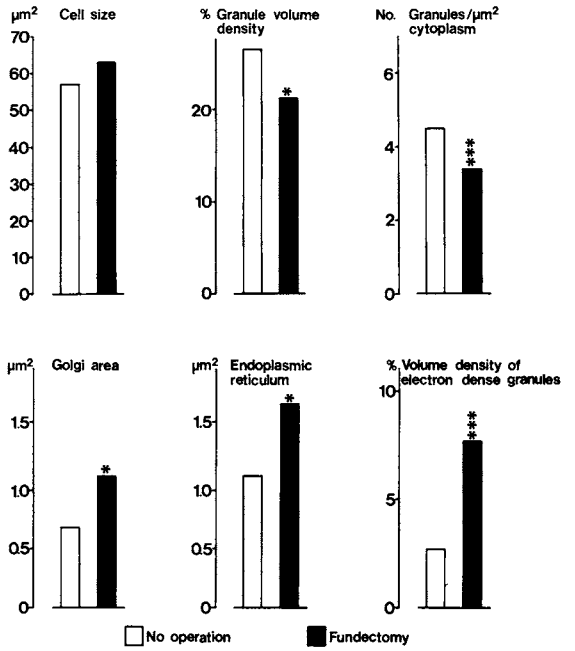

Figure 10. Effect of fundectomy on the ultrastructure of gastrin cells. The rats were killed 6–10 weeks after the operation and after a 48-h fast. The cells were increased in size but the increase was not statistically significant. The granule volume density (i.e., the proportion of the cytoplasm occupied by granules) was reduced as was the number of granules per unit cytoplasm. The Golgi area was enlarged, the endoplasmic reticulum was increased and the proportion of electron-dense granules was greatly increased. The number of cells analyzed was 38 for the control group and 60 for the fundectomy group (2).

What is the stimulus that activates the gastrin cells in fundectomized rats and causes proliferation as well? The antral pH is raised, but obviously that is not enough, since the pH is probably raised just as much in the antrum-excluded rats, where no gastrin cell proliferation occurred. However, in the fundectomized rat, food passes through the antrum. Hence the food might be important. In rats with the antrum transposed to the colon there was high pH and there was food material passing over the pyloric glands too (albeit highly digested). Nonetheless there was no gastrin cell proliferation in these rats (Figure 6). Apparently, therefore, there was some stimulus present in the gastric luminal content that was missing in the intestinal luminal content. But could it not be a blood-borne suppressor of the gastrin cells originating from the fundus and eliminated by the fundectomy? Probably not, because if both fundectomy and antrum exclusion were performed there was no proliferation of the gastrin cells (Figure 6).

The ECL Cell and the A-like Cell

The major endocrine cell populations in the oxyntic mucosa of the rat's stomach are the ECL and the A-like cells, both of which are believed to

produce and store histamine (11) (for a different point of view see refs. 21, 22) (Figure 11). Both of them are probably peptide hormone-producing (15), although the hormones produced are still unknown. The ECL cells represent 60–65% and the A-like cells 25–30% of the endocrine cells in this area (17). The production of histamine is catalyzed by the enzyme histidine decarboxylase. In a series of papers we have argued that the gastric histidine decarboxylase activity reflects the functional activities of these two cell populations (3, 14, 16, 17, 18). In the fasted rat the enzyme activity is low; it is raised after feeding. At the same time the histamine content of these cells is

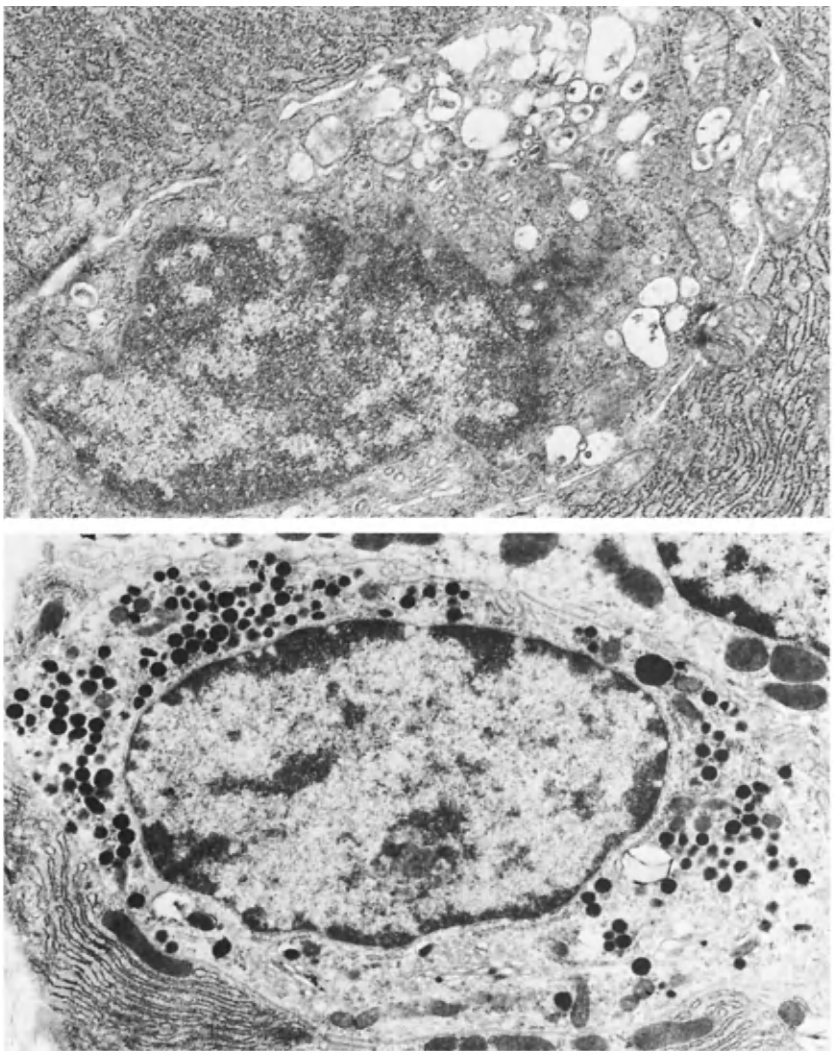

Figure 11. An ECL cell (top; ×12,000) and an A-like cell (bottom; ×10,000) in the rat oxyntic mucosa. Note the vesicular-type cytoplasmic granules that are characteristic of the ECL cells and the typical round, electron-dense, membrane-enclosed granules of the A-like cell.

lowered, reflecting mobilization of histamine (and peptide hormones?) from the cells (15, 19). Gastrin and pentagastrin produce the same effects, and so do insulin and carbachol. On removal of the endogenous gastrin store by antrectomy, only pentagastrin and gastrin retain their capacity to activate the enzyme and lower the histamine concentration (9). Therefore we believe that all stimuli that activate the ECL cells and the A-like cells do so with gastrin as an intermediate (9, 13, 14, 15).

What happens to the ECL cells and the A-like cells under conditions of extreme stimulation, i.e., hypergastrinemia, and of no stimulation at all, i.e., hypogastrinemia? First hypergastrinemia: Nephrectomy quickly resulted in a very high serum gastrin concentration, probably because the kidneys are important for the elimination of gastrin (6). Figure 12 illustrates the situation 48 h after nephrectomy. The serum gastrin concentration was high and the histidine decarboxylase activity was very high too. Both the ECL cells and the A-like cells were enlarged and partially degranulated, which we interpret as signs of activation (Figure 13). The gastrin cells responded inversely in that they were reduced in size and displayed an increased granule volume density (7). What happens if the hypergastrinemia is maintained over several weeks? Antrum exclusion raised the serum gastrin concentration compared with unoperated rats (2, 3). This is evident if the fasting levels are compared (Figure 7). Antrum exclusion also raised the number of histamine-storing endocrine cells in the oxyntic mucosa (Figure 14), and its histamine content and histidine decarboxylase activity (3). Both the ECL cells and the A-like cells were increased in number and size and there was a reduction in granularity as well (Figure 15).

What happens after antrectomy, when the main source of endogenous gastrin is eliminated and feeding no longer increases the serum gastrin concentration? The effects on the ECL cells and A-like cells are illustrated in

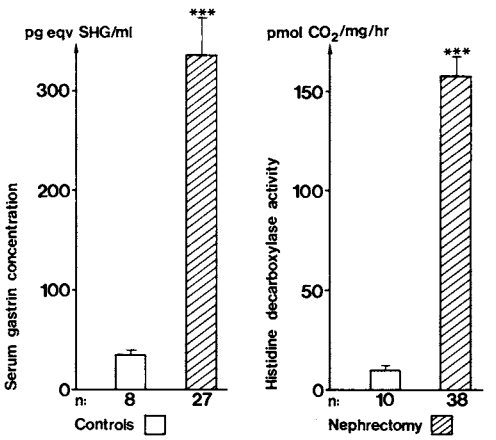

Figure 12. Effects of nephrectomy on serum gastrin concentration and gastric histidine decarboxylase activity. The rats were killed 48 h after bilateral removal of the kidneys; they were fasted for the same period of time (6). Vertical bars give S.E.M. (***) for $p < 0.001$.

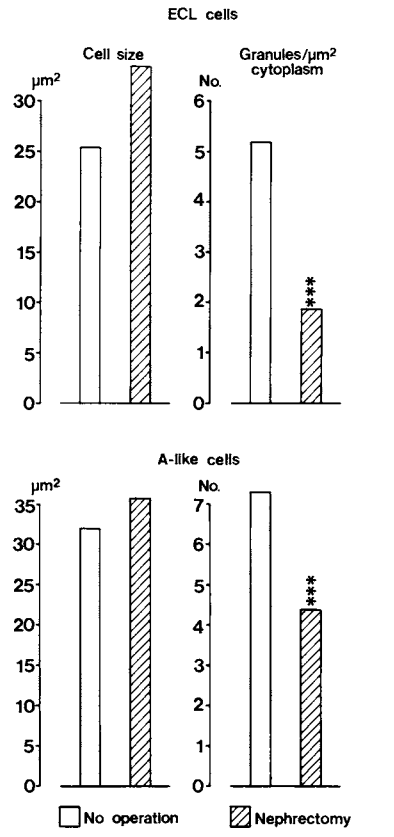

Figure 13. Effects of nephrectomy on the ultrastructure of the ECL cells and the A-like cells. The rats were killed 48 h after bilateral removal of the kidneys. Both the ECL cells and the A-like cells were increased in size (as judged from estimation of their cell profile areas) and had a reduced number of granules per unit cytoplasm. The number of cells analyzed was 46 ECL cells and 45 A-like cells in the control group and 53 ECL cells and 38 A-like cells in the nephrectomy group (7).

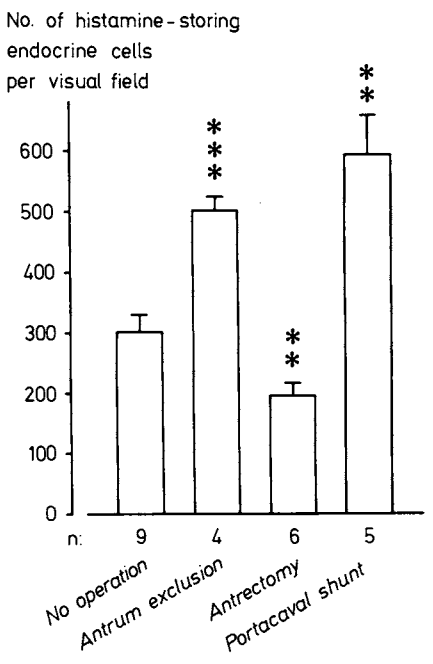

Figure 14. Number of histamine-storing endocrine cells (per visual field) in the oxyntic mucosa of control rats and of rats subjected to antrum exclusion, antrectomy, or portacaval shunt. The animals were killed 3–4 weeks (antrectomy) or 6–10 weeks (antrum exclusion and portacaval shunt) after surgery. The cells were demonstrated by their *o*-phthaldialdehyde-induced fluorescence (15, 17). Vertical bars give S.E.M. n = number of animals. (**) for $0.001 < p < 0.01$ and (***) for $p < 0.001$.

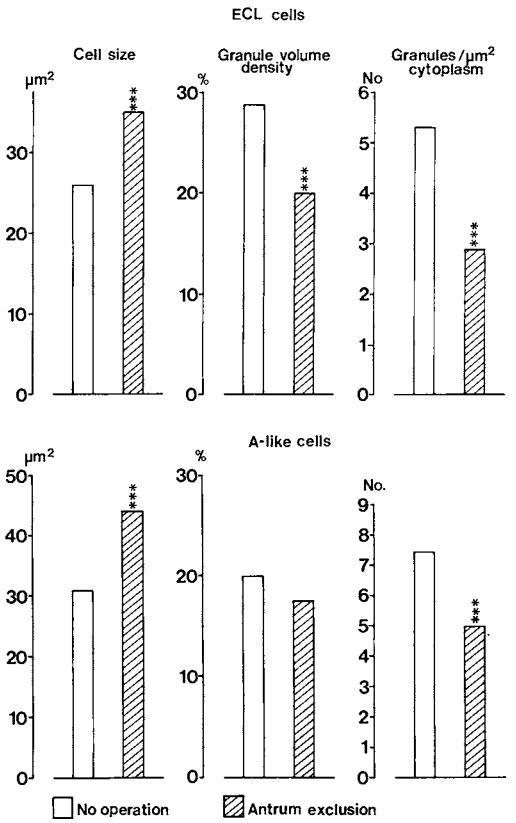

Figure 15. Effects of antrum exclusion on the ultrastructure of the ECL cells and the A-like cells. The rats were killed 6–10 weeks after the operation and after a 48-h fast. The profile areas of both the ECL cells and the A-like cells were increased in size and both cell types exhibited a reduced number of granules per unit cytoplasm. The number of cells analyzed were 55 ECL cells and 50 A-like cells in the control group and 58 ECL cells and 39 A-like cells in the antrum excluded group (3). *** for $p < 0.001$.

Figure 14, which shows that the gastric endocrine cells were greatly reduced in number. Antrectomy had effects also at the ultrastructural level. Both the ECL cells and the A-like cells were reduced in size and had a reduced number of granules (Figure 16) (17).

Portacaval shunting had some unexpected consequences. The ECL cells, but not the A-like cells, became much more numerous (17) (Figure 14). In fact, the number of ECL cells was so large that it was not unusual to find them in clusters of three to four cells. Also, at the ultrastructural level the ECL cells were greatly affected: Cell size was increased and granularity was reduced; in addition, the granules were enlarged. In contrast, the A-like cells were not affected at all (Figure 17) (17). This is the first instance that we have found of the two cell types, the ECL cells and the A-like cells, not responding in parallel, and we still do not know which agent is responsible for the hyperplasia of the ECL cells after portacaval shunting. If gastrin is at all

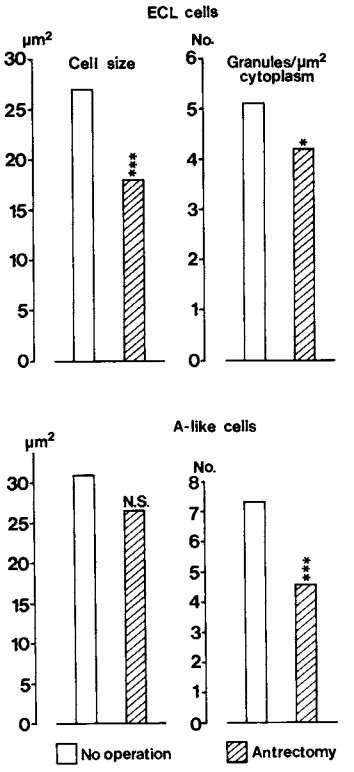

Figure 16. Effects of antrectomy on the size and granularity of the ECL cells and the A-like cells. The rats were killed 3–4 weeks after surgery and after a 48-h fast. The ECL cells were greatly diminished in size. The number of granules per unit cytoplasm was reduced in both the ECL cells and the A-like cells. (For details see 17.) (***) for $p < 0.001$.

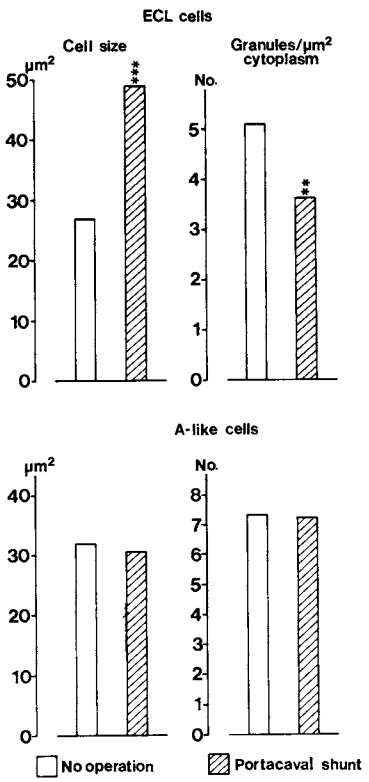

Figure 17. Effects of portacaval shunt on the size and granularity of the ECL cells and the A-like cells. Note that the ECL cells were greatly increased in size and had a reduced number of granules per unit cytoplasm, whereas the A-like cells were unaffected. (For details see 17.) (**) for $0.001 < p < 0.01$, (***) for $p < 0.001$.

responsible we have to postulate that the response of the ECL cells to gastrin is greatly enhanced, since the serum gastrin levels were not raised after portacaval shunting (4, 14, 17).

The Somatostatin Cell

The somatostatin cells of the gastric mucosa displayed some peculiar features. The somatostatin cells in both the oxyntic and the pyloric mucosa of the rat were characterized by long, slender processes that projected from the cell body to reach neighboring cells (Figure 18) (1, 8, 12, 20). The processes ended in thickened, clublike swellings. In the pyloric glands the somatostatin cells were located together with the gastrin cells at the base of the glands. In the oxyntic glands the somatostatin cells were fairly evenly distributed throughout the gland, perhaps with some predominance in the middle third, where the parietal cells are numerous. The cells contacted by the somatostatin cells were identified in the following way: The gastrin cells in the pyloric glands and the parietal cells in the oxyntic glands were suspected targets. We therefore stained the antrum first for gastrin by immunoperoxidase and then for somatostatin by immunofluorescence. As a result, the somatostatin cells became fluorescent, whereas the gastrin cells appeared dark in the fluorescence microscope because of the peroxidase staining (1). In the oxyntic gland area the parietal cells were first stained for carbonic anhydrase and the somatostatin cells were then visualized by

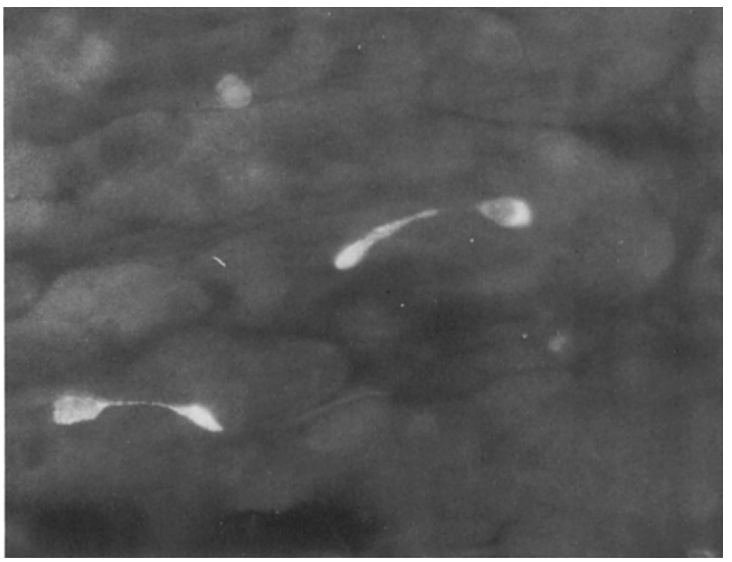

Figure 18. Two somatostatin cells in the oxyntic gland area of the rat, demonstrated by immunofluorescence. Note that the somatostatin cells send out long, slender processes ending in clublike swellings that seem to reach neighboring cells in a manner suggestive of a paracrine mode of signal transmission. Among the cells contacted by the somatostatin cells in the oxyntic mucosa are the parietal cells (1). ×500.

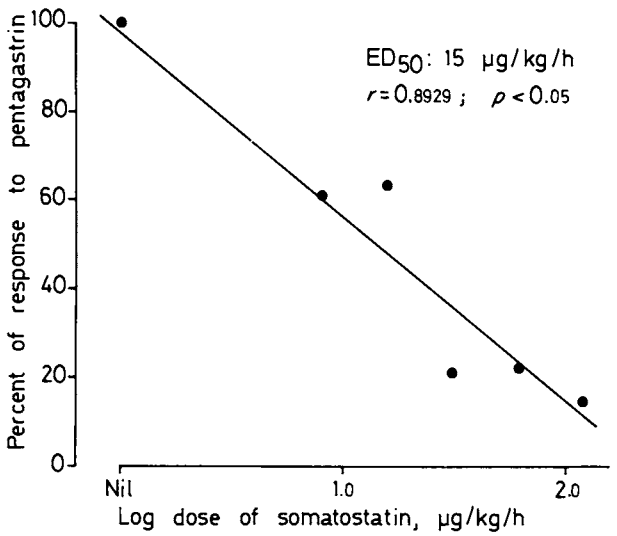

Figure 19. Effect of different doses of somatostatin on the maximal gastric acid response to a standard dose of pentagastrin. Both somatostatin and pentagastrin were given intravenously (pentagastrin in a dose of 10 µg/kg/h) to conscious fistula rats. The ED_{50} for somatostatin was calculated to be 15 µg/kg/h.

TABLE 2
Effect of Somatostatin on Pentagastrin-Induced Mobilization of Gastric Histamine and Activation of Histidine Decarboxylase[a]

	Histamine conc. (µg/g)		Histidine decarboxylase activity (pmol CO_2/mg/hr)	
Treatment	Saline	Somatostatin (60 µg/kg/h)	Saline	Somatostatin (60 µg/kg/h)
Saline	59 ± 1.1 (16)	73 ± 5.1+(9)	4.5 ± 0.6 (16)	4.3 ± 0.9 (10)
Pentagastrin	×××	+++	×××	
0.25 mg/kg	43 ± 2.5 (15)	66 ± 6.8 (5)	21.6 ± 2.5 (15)	14.3± 4. (5)
Pentagastrin	×××	××+++	×××	×××
0.5 mg/kg	42 ± 1.3 (13)	56 ± 2.7 (13)	19.7 ± 1.4 (13)	16.5 ± 2.4 (13)
Pentagastrin	×××	××+	×××	×××
1 mg/kg	40 ± 4.2 (14)	52 ± 3.5 (12)	20.8 ± 1.4 (14)	19.1 ± 1.3 (10)

[a]Mean ± S.E.M. (n). Pretreatment with saline or somatostatin was by the intravenous route (2 ml/h) for 3 h. Treatment with saline or pentagastrin was by the subcutaneous route 1 h before the iv infusion was stopped and the animals killed. Statistical analysis by Student's t-test. + or × for 0.01, $p < 0.05$. ++ or ×× for 0.001, $p < 0.01$; and +++ or ××× for $p < 0.001$. The symbol + denotes the difference between saline-treated and somatostatin-treated rats (within one horizontal row). The symbol × denotes the difference between saline-treated and pentagastrin-treated rats (within one vertical row).

immunofluorescence. As a result, the somatostatin cells became fluorescent and the parietal cells appeared dark (1). The results do not permit us to say that all parietal cells or all gastrin cells are in direct contact with somatostatin cells, nor can we exclude the possibility that there are still other target cells in the gastric mucosa for somatostatin besides the gastrin cells and the parietal cells. What physiological significance does this have? Somatostatin is known to suppress gastrin release regardless of the type of stimulation (1). Further, somatostatin effectively inhibits secretion from the parietal cells (1). This is illustrated in Figure 19 by a dose-response curve showing the effect of somatostatin on pentagastrin-stimulated secretion. But somatostatin was not very effective in inhibiting the effects of pentagastrin on the ECL cells and the A-like cells (Table 2) (1, 5). High doses of somatostatin failed to prevent the activation of histidine decarboxylase and the mobilization of histamine (1, 5) perhaps because ECL cells and A-like cells lack somatostatin receptors. It is obvious therefore that if somatostatin acts locally in a paracrine fashion, it does so by affecting neighboring cells selectively rather than indiscriminately. This selectivity may be achieved by direct cell-to-cell contacts (Figure 20), for instance, through semisynaptic arrangements. Alternatively, only certain cells are supplied with receptors to somatostatin.

In conclusion, it might be pertinent to point out the importance of a morphological basis for an adequate interpretation of what the endocrine cells of the gut do and how they do it.

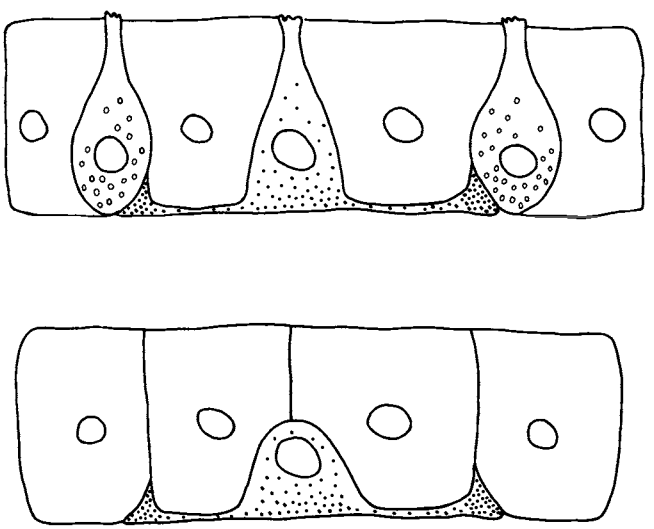

Figure 20. Schematic illustration of the way somatostatin cells (dark granules) may influence gastrin cells (light granules) (top) and parietal cells (bottom). Note that, like the gastrin cells, the somatostatin cells in the pyloric gland area are open (i.e., they possess an apical process that reaches the surface of the epithelial lining), whereas those in the oxyntic glands are closed. Each somatostatin cell seems to give off at least two projections. The clublike swellings at the end of the projections are thought to be the point of communication between the transmitting cell and the target cell.

SUMMARY

Just as the mode of action of most endocrine cells of the gut remains unknown, the mechanisms involved in their activation and deactivation are poorly understood. It is likely that the many diverse peptide hormone-producing cells respond in a similar manner, cytochemically and ultrastructurally, to activating and deactivating stimuli. Once the typical cytochemical and ultrastructural features of activation and deactivation have been identified, predictions can be made as to what physiological functions the many endocrine cell systems of the gut may have.

Endocrine cells are particularly numerous in the gastric mucosa. They are of several different types. Except for the gastrin cells, very little is known of their functional significance. The present study deals with the relationship between function and morphology in the gastrin cells, the ECL and A-like cells, and the somatostatin cells.

The *gastrin cells* display a characteristic topographic distribution in the pyloric glands. They seem to "migrate" upward in the glands when the antral pH is raised. Proliferation of gastrin cells results from a combination of elevated pH and passage of food. If the passage of food is prevented, no proliferation ensues. Removal of the oxyntic gland area strongly stimulates the gastrin cells, reflected in very high serum gastrin concentration and gastrin cell proliferation. Ultrastructurally, the cells respond with reduced granularity, increased proportion of electron-dense granules, increased endoplasmic reticulum, and enlarged Golgi area.

The *ECL* and *A-like cells* are numerous in the oxyntic gland area. There is evidence that the (still unknown) functions of these cells are controlled by circulating gastrin. Long-standing hypergastrinemia results in proliferation of both cell populations; also, both cell types are increased in size. Conversely, long-standing hypogastrinemia results in a reduction in the number and size of the ECL and A-like cells.

The *somatostatin* cells of the gastric mucosa possess long, slender processes that seem to reach neighboring cells. The processes end in thickened, clublike swellings. Among likely target cells for the somatostatin cells are the gastrin cells and the parietal cells but not the ECL and A-like cells. Exogenous somatostatin blocks the release of gastrin and the secretion of HCl but interferes only poorly with the function of the ECL and A-like cells. Somatostatin cells probably act through a paracrine mode of action; they seem to do so by affecting neighboring cells selectively rather than indiscriminately. This selectivity may be achieved by direct cell-to-cell contacts. Alternatively, only certain cells are supplied with receptors to somatostatin.

ACKNOWLEDGMENTS

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Heterogeneity of the D_1 Cell

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INTRODUCTION

The existence of a population of endocrine cells containing *small* secretory granules in the gut and pancreas was recognized as early as 1965 by Munger *et al.* (9), and later by many other workers (2–8,13). These cells were termed D_1 cells in 1972 by Solcia's group (2).

This new cell type, the D_1 cell, was internationally accepted for the first time at Bologna in 1973 (14) and defined as possessing small, round (140–190 nm) argyrophilic, moderately electron-dense secretory granules with a fairly close-fitting membrane and a narrow, almost indistinguishable halo. It was then regarded as a single cell type with unknown peptide product. D_1 cells are present throughout the entire length of the gut and are also found in the pancreas in both islets and exocrine tissue.

The uniformity of the D_1 cells was first challenged in 1977 (15), when the source of human pancreatic polypeptide (PP) was found to be some of the small-granuled cells of the human pancreas. These cells are almost indistinguishable from the remaining small-granuled cells (Figure 1A–D), which retained the name of D_1 cells and became thereafter the fifth endocrine cell type of the human pancreas. Further advances in immunocytochemistry at light- and electron-microscopical level, including the use of region-specific antisera, have permitted the better recognition of a number of other small-granuled endocrine cells, all originally included in the category of the D_1 cell. The identification of the nonenterochromaffin, motilin-containing cells and of gastrin-containing cells of the human intestinal mucosa provides good illustrations.

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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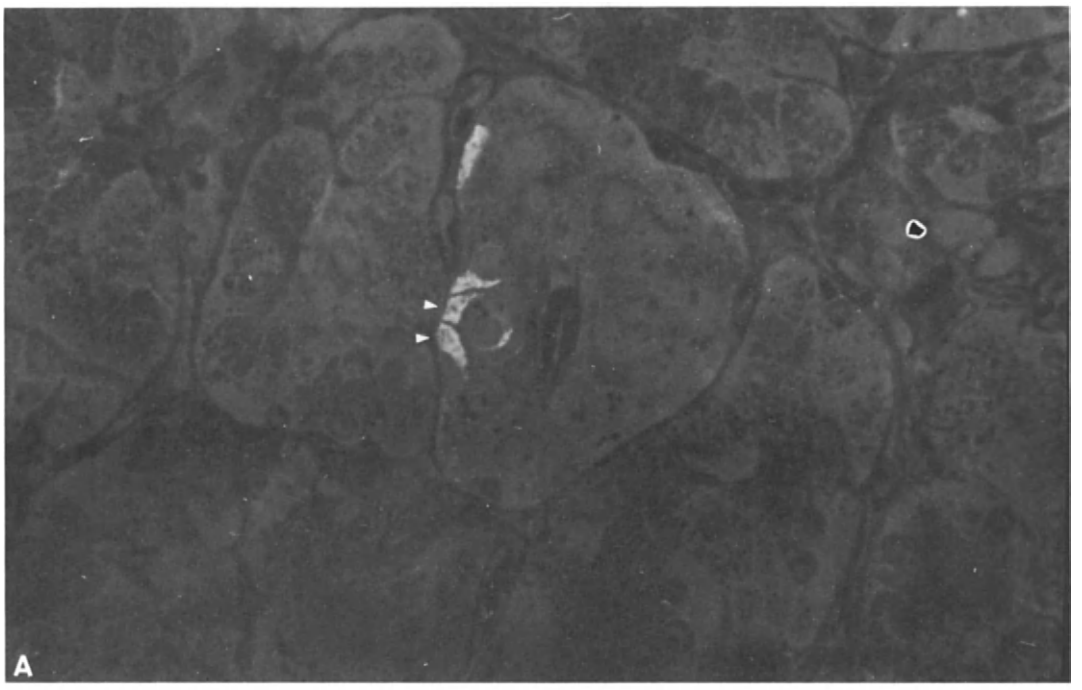
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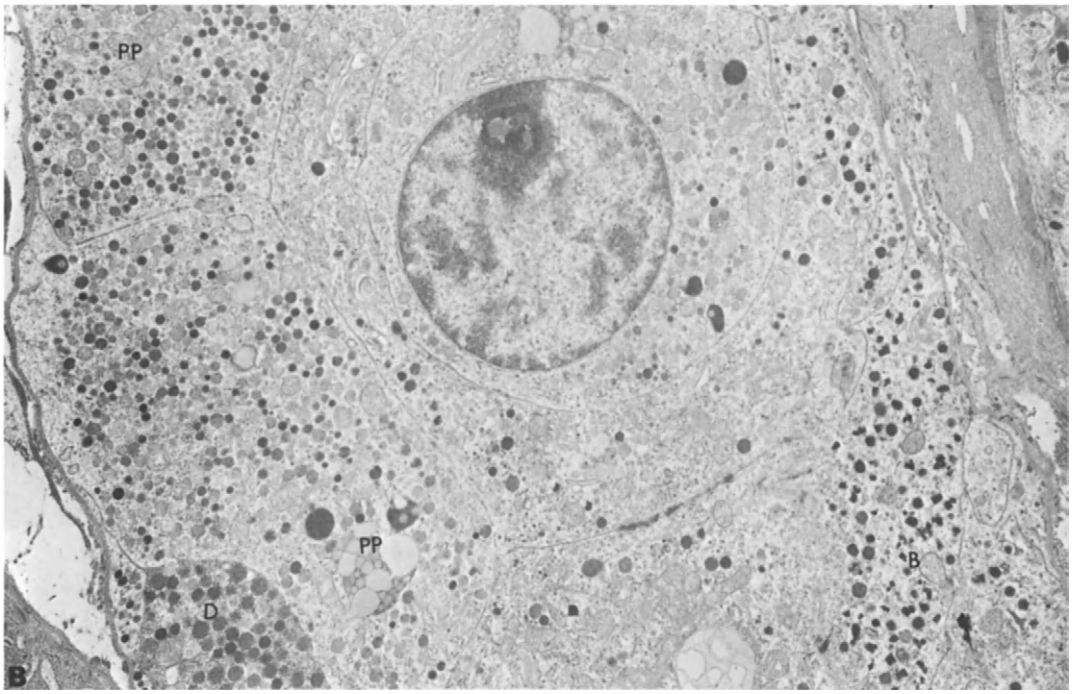
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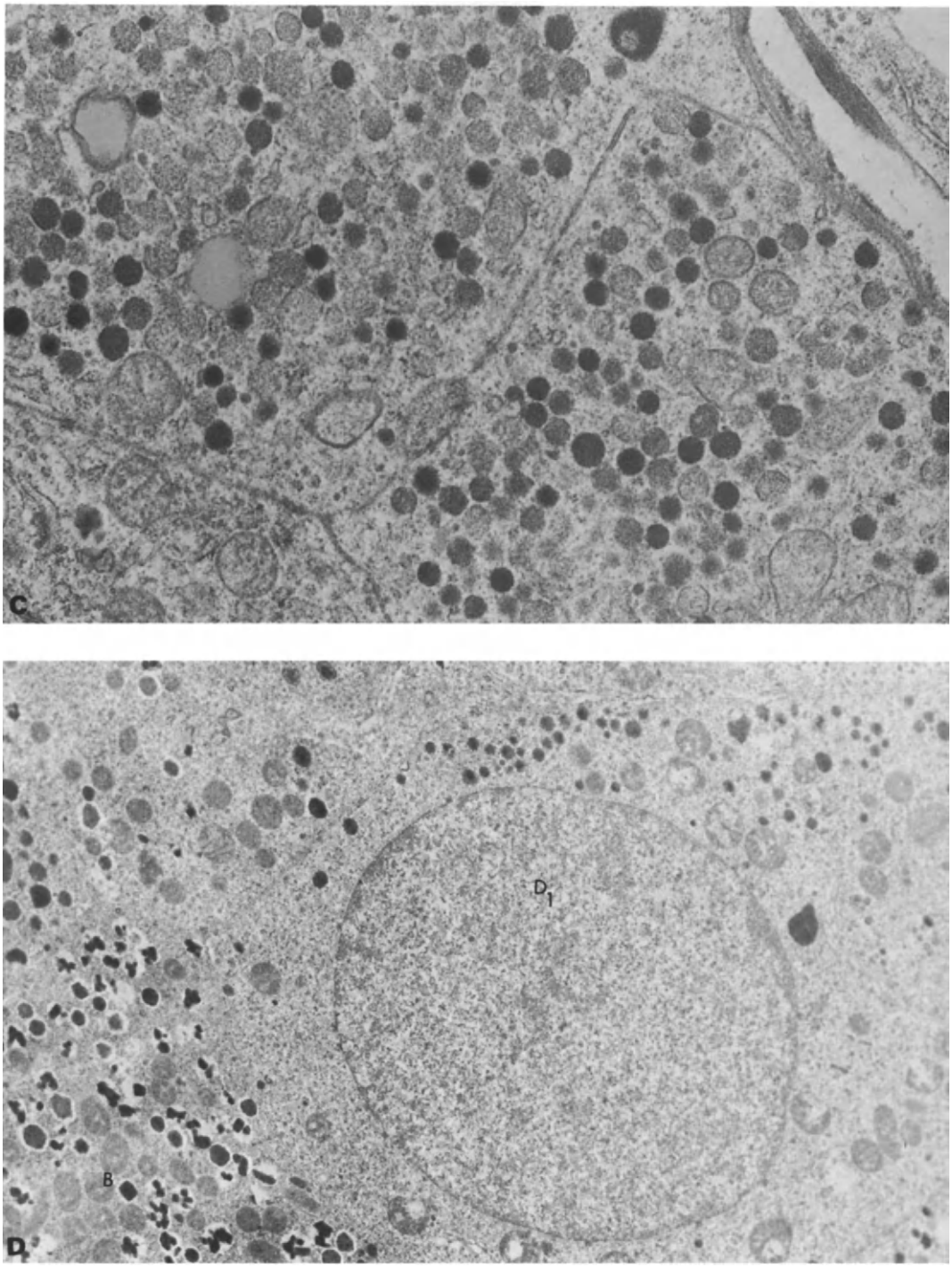
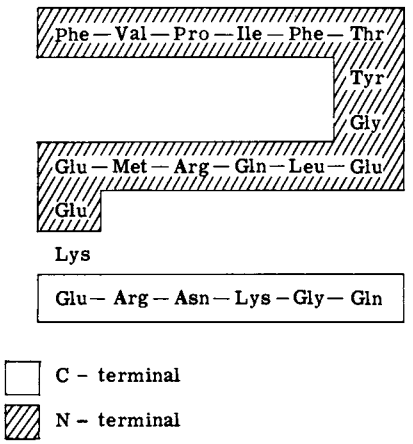


Figure 1. Adult human pancreas (A–C) (body) (36-year-old) fixed in 3% glutaraldehyde followed by 1% osmium tetroxide. (A) Two PP cells (arrows) stained in a 800-nm (semithin) resin-embedded section. ×600. (B) and (C) Ultrastructural identification of their small, round secretory granules. ×1500 and ×25,000, respectively. PP, PP cells; D, D cell; B, B cell. (D) Human pancreas (6 months old) fixed as above. Non-PP immunoreactive small granule cell (D_1). ×17,500. D_1, Small granule cell; B, B cell.



Scheme I. Antiserum specificity to motilin.

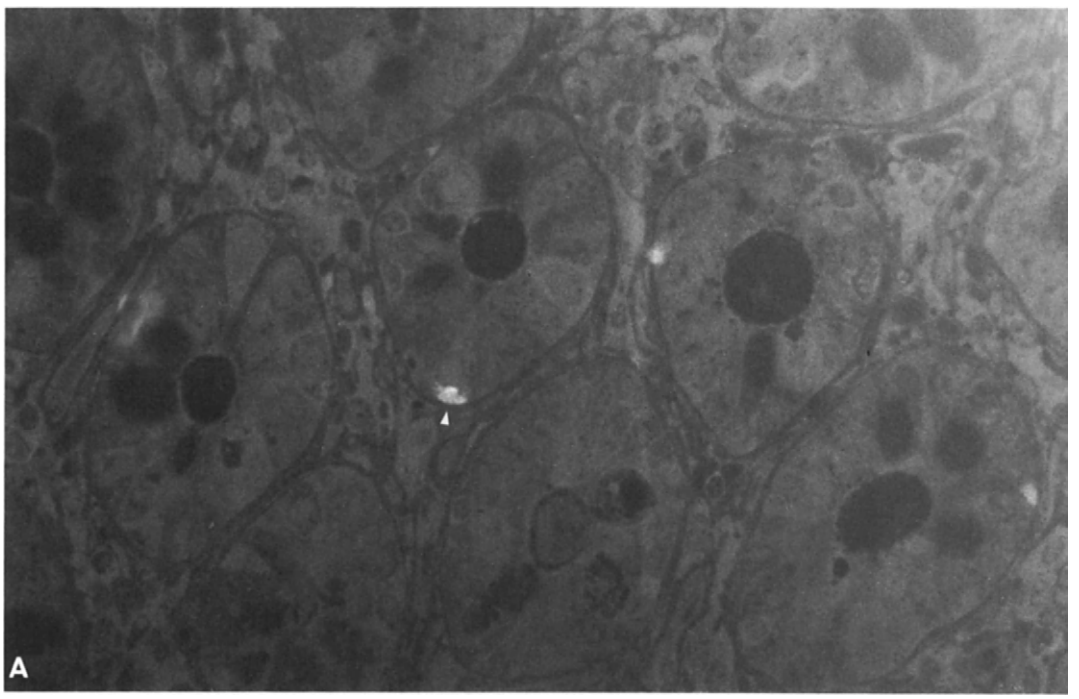


Figure 2. Human duodenal mucosa fixed in a Karnovsky's mixture using 5% purified glutaraldehyde and 2% paraformaldehyde prior to osmication in a 1% solution of osmium tetroxide. Motilin cell (arrow) stained with C-terminally directed antibodies. (A) 800-nm (semithin) section. (Arrow shows motilin cell.) ×600. (B) and (C) 80-nm (thin) section, conventional EM staining showing a motilin cell with numerous round, small electron-dense granules. ×7500 and ×25,000, respectively. G, Granules; MF, microfilaments.

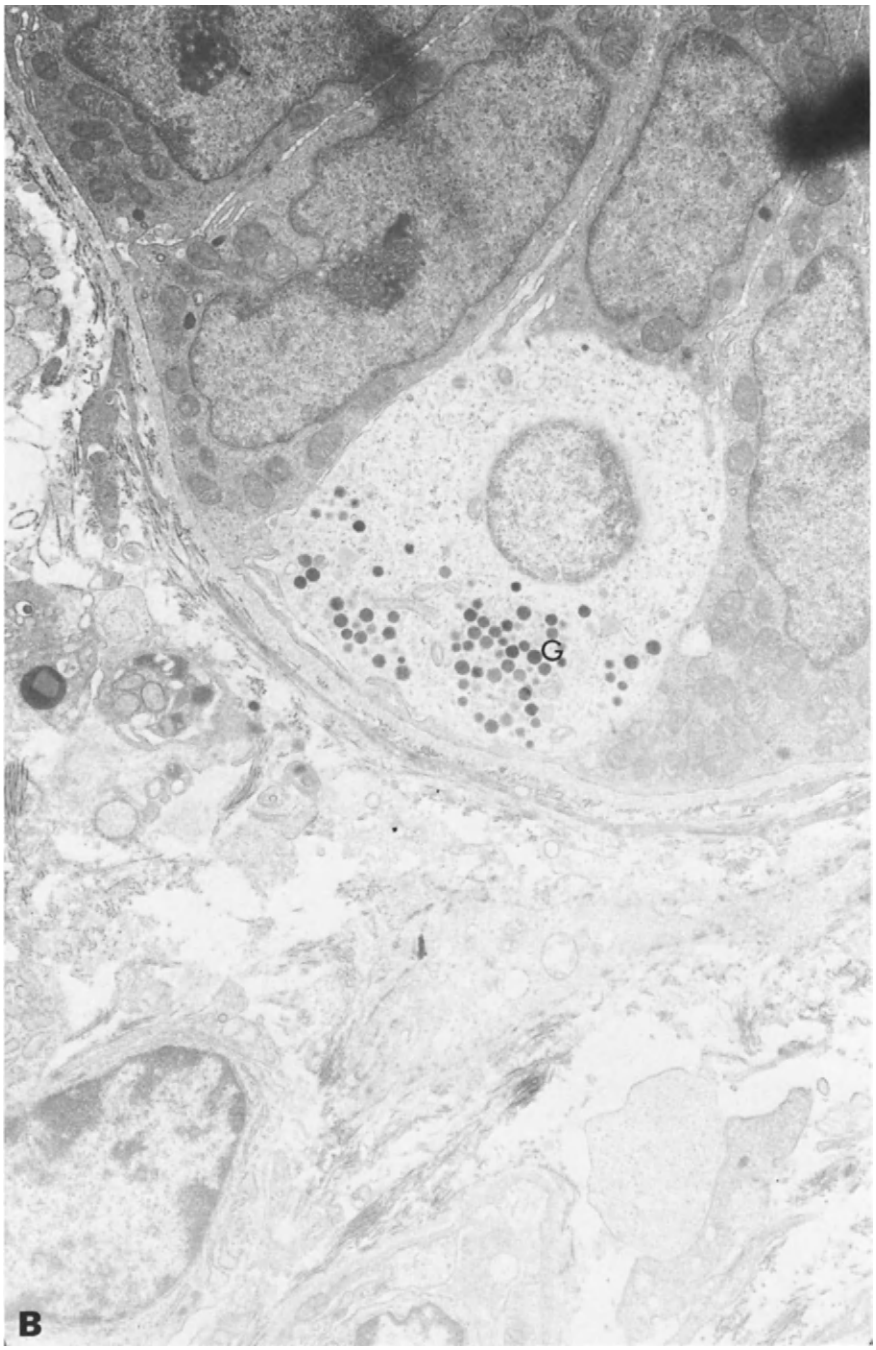


Figure 2 (*continued*).

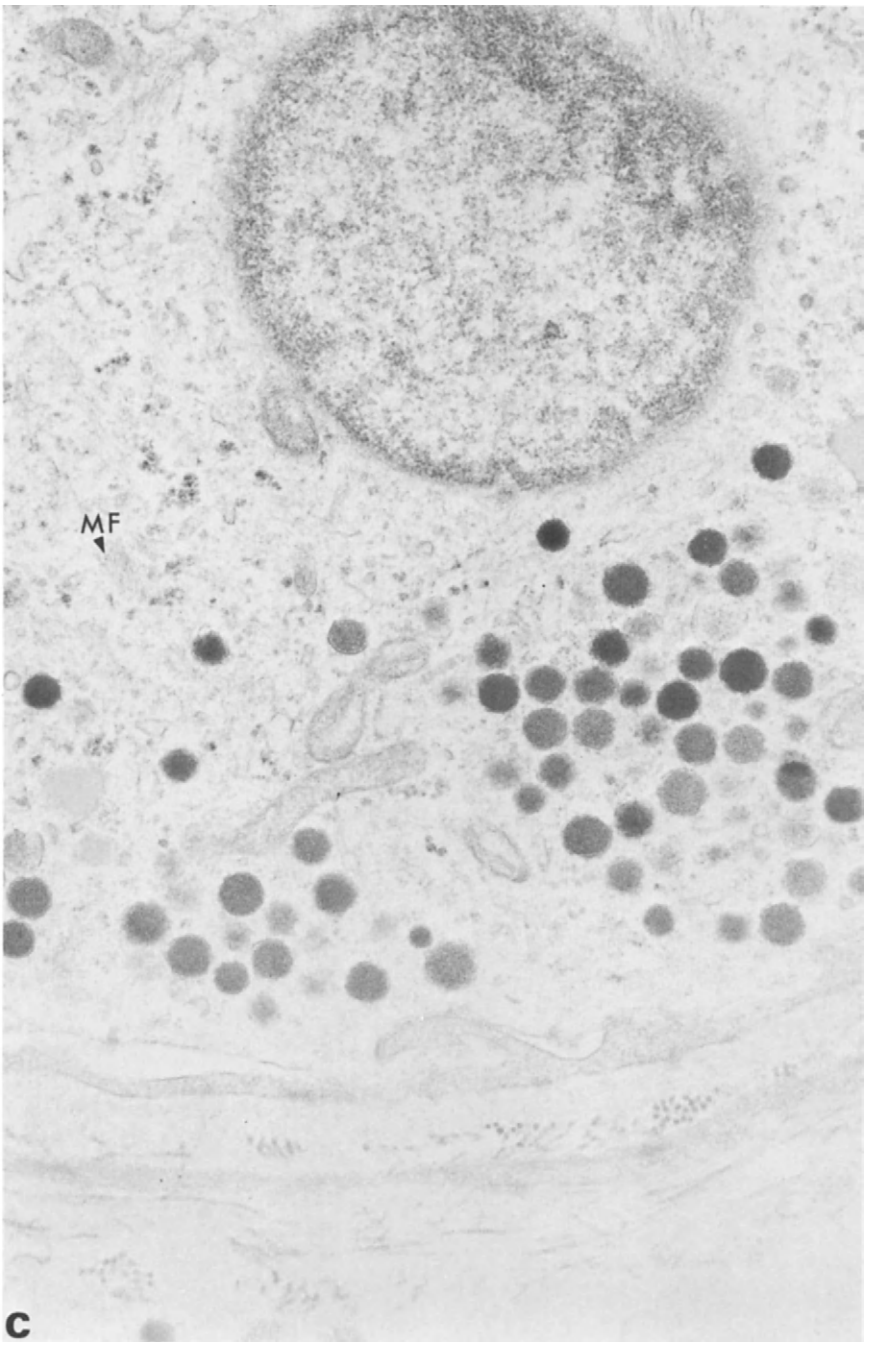


Figure 2 (*continued*).

NONENTEROCHROMAFFIN MOTILIN-CONTAINING CELLS OF THE HUMAN SMALL INTESTINE

Motilin, originally extracted from the porcine intestine, was shown to be produced by the cells responsible for amine precursor uptake and decarboxylation (APUD) of the small intestine (10). Motilin was originally found in both enterochromaffin and nonenterochromaffin cells in proportions that varied according to the species. This puzzling finding has recently been further elucidated by the use of region-specific antisera. These antisera, raised to either natural or synthetic motilin, react to either the C-terminal or the N-terminal part of the motilin molecule (see Scheme I). The nonenterochromaffin motilin cells are detected by both the C and N terminally directed antisera, and in addition, a proportion of enterochromaffin cells are stained by the N-terminal antibodies (11). The serial semithin/thin section method reveals that the nonenterochromaffin, motilin-producing cells correspond to the D_1 small-granuled cells of the small intestine, containing granules with a mean size of 180 nm (160–190 SD ± 24 nm) surrounded by a limiting membrane (Figure 2). Numerous intracytoplasmic microfilaments are often seen.

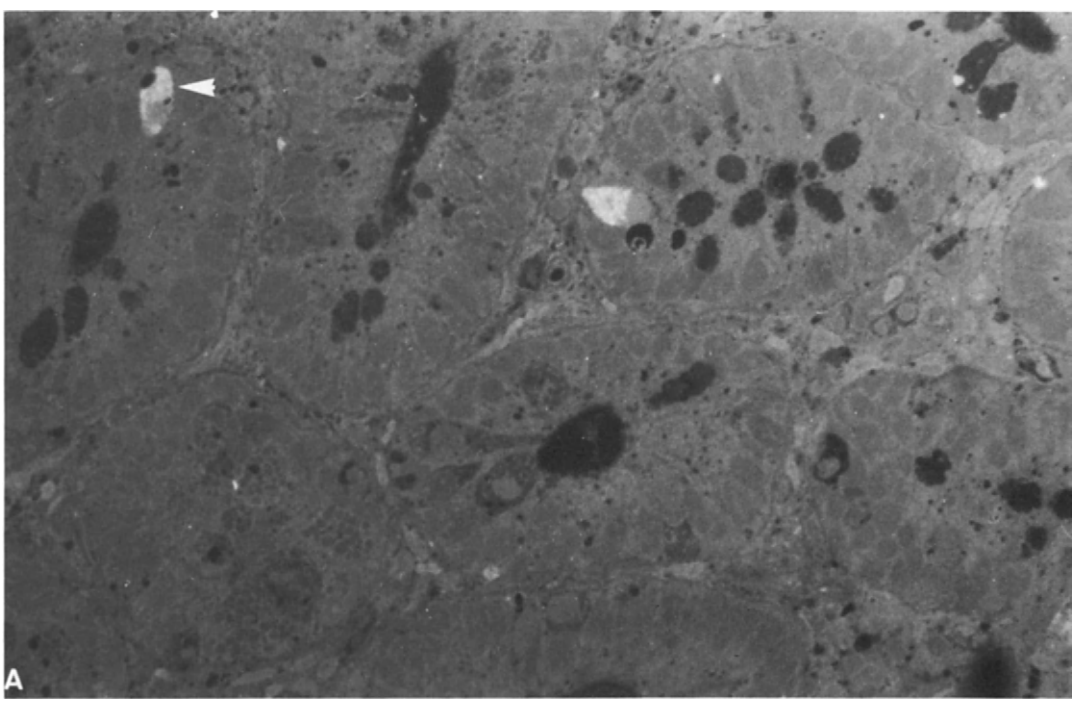


Figure 3. Human duodenum fixed in 2.5% purified glutaraldehyde for 15 min. (A) 800-nm (semithin) section stained with N-terminally directed antibodies to gastrin. Intestinal gastrin cell indicated with arrow. ×600. (B) and (C) 80-nm (thin) section conventionally stained for EM showing one intestinal cell (arrow) with numerous round, small, electron-dense secretory granules, ×2500 and ×21,000, respectively.

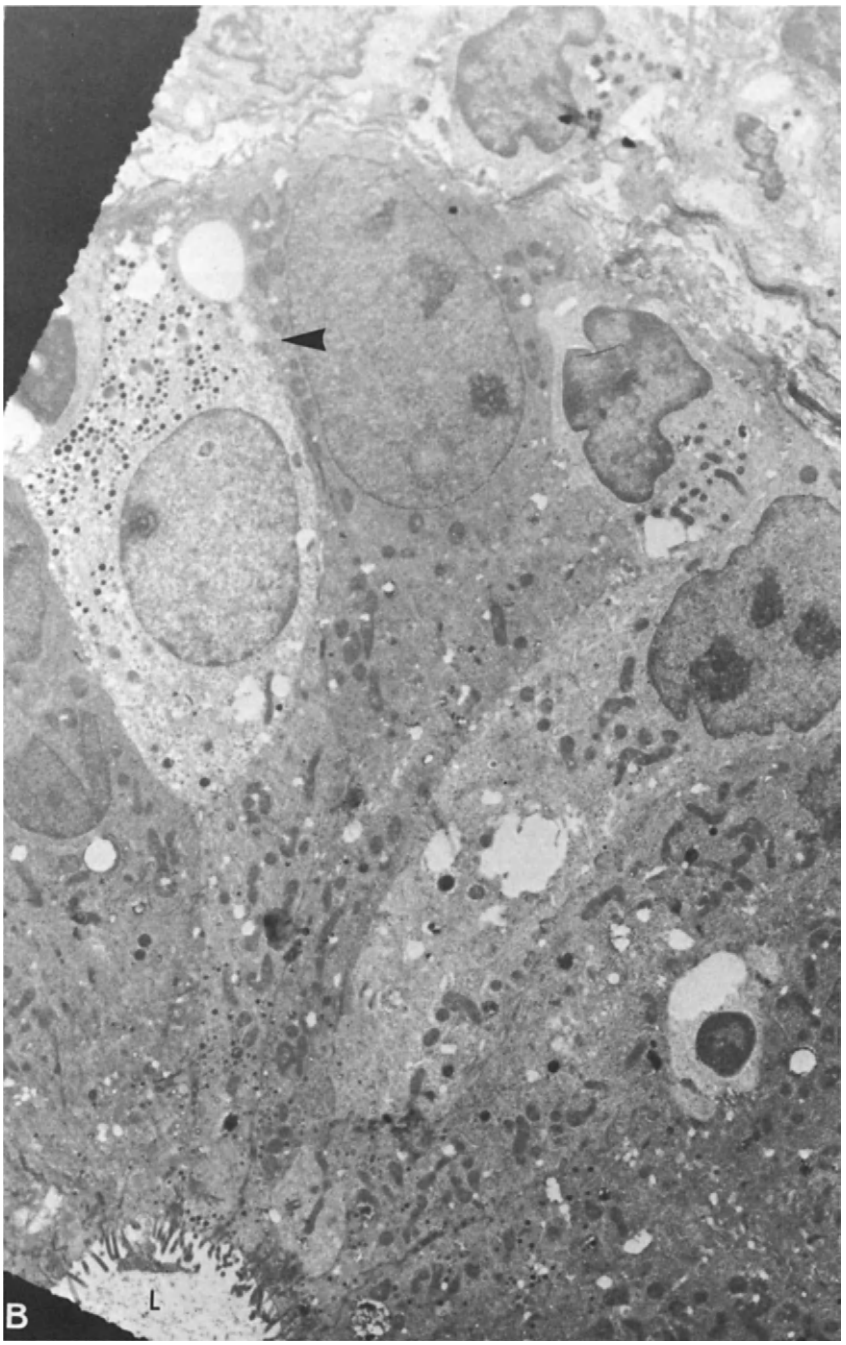


Figure 3 (*continued*).

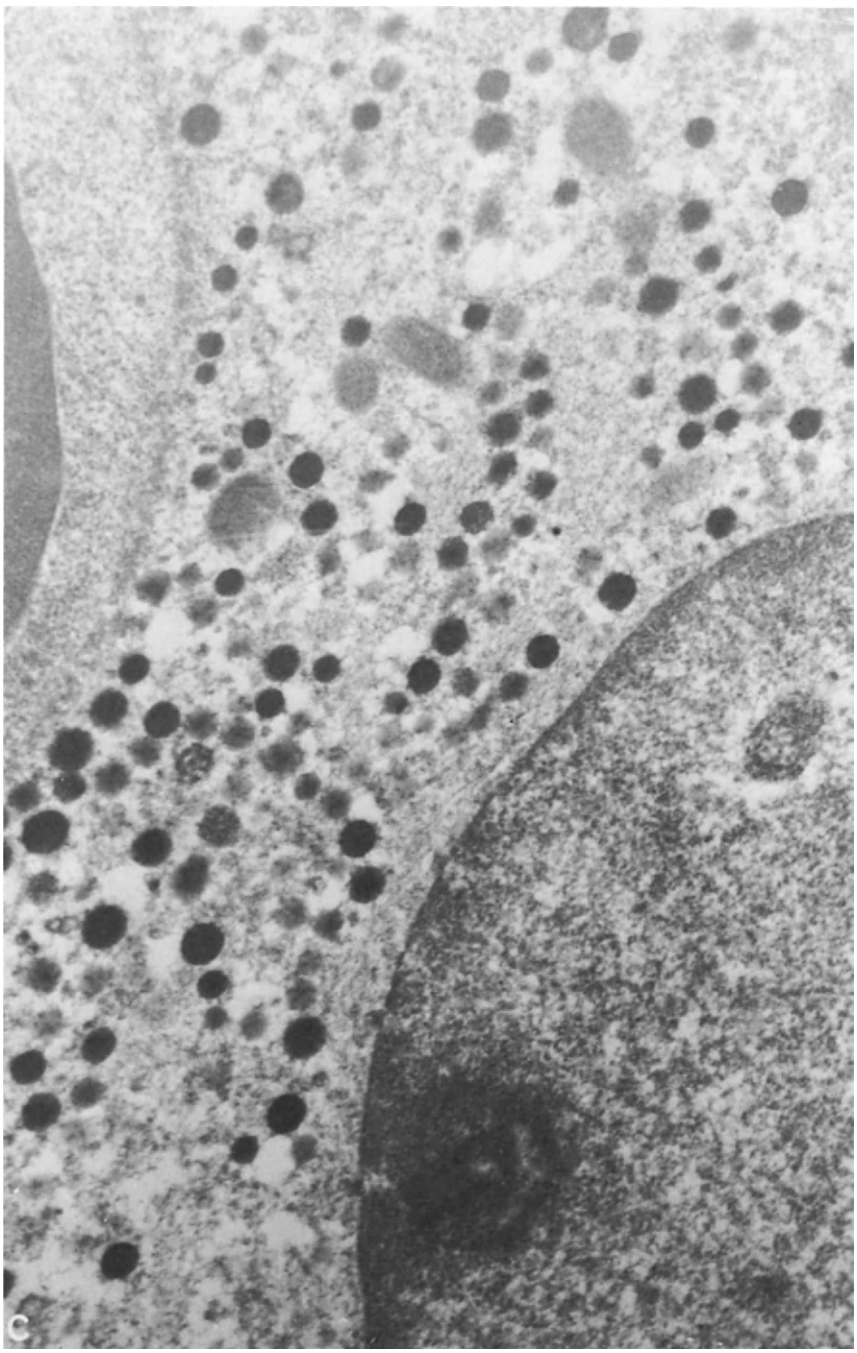


Figure 3 (*continued*).

CELLS CONTAINING GASTRIN OF INTESTINAL ORIGIN

The cell of origin of the gastrin like immunoreactive material extractable from the human small intestine has recently been identified (1). Its independence and distinction from the cholecystokinin (CCK) cells of the same area were established through the use of a range of well-characterized region-specific antisera, including some directed to the N-terminal part of the gastrin molecule and some to the middle part (9-20) of the CCK molecule (12). These intestinal gastrin cells, too, have been shown to possess D_1-type secretory granules with a mean diameter of 190 nm (150-210 SD ± 32 nm) (Figure 3).

CONCLUSIONS

The replacement of the lettering nomenclature for identifiable endocrine cell types by a more functional classification was suggested in 1977 (15). Two major reasons for this were

ADVANCES IN IMMUNOCYTOCHEMISTRY

Advances in immunocytochemistry at light- and electron-microscopical level have led to the recognition of multiple cell types in categories that were previously considered as one. For example, the L cells of the human intestine were subdivided into EG (enteroglucagon) and NT (neurotensin) cells (15) and the small-granuled cells of the human pancreas became two subgroups: the PP cells and the remaining D_1 cells with an as yet unidentified product (15).

SPECIES VARIATION

A more functional terminology is also required because of the pronounced variation of a single cell type between different species. The PP cell of the pancreas again provides a good example. It is now recognized that in humans the PP cell contains smaller secretory granules than those observed in other mammals. In addition, in man the cells responsible for the production of enteroglucagon (glicentin) possess granules of a smaller size than those observed in other species and the cells containing secretin-like immunoreactivity in man vary considerably in size (250 ± 34 nm) from those of other species such as pig (190 ± 28 nm). It is thus clear that further investigations into the identification of gut endocrine cells by their peptide product will demonstrate their ultrastructural appearance, allowing us to subdivide further heterogeneous groups such as the D_1 cells.

ACKNOWLEDGMENTS

This work was generously supported by the Cancer Research Campaign.

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Ultrastructural Identification of TG Cells in Man and Monkey

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Gastrin and cholecystokinin (CCK) share a common COOH-terminal tetrapeptide portion, which is necessary for the biological activities of the two hormones. As a consequence, gastrin and CCK show the same spectrum of physiological actions and differ only with respect to their relative potencies for different target cells. The common COOH-terminal tetrapeptide portion also appears to be highly immunogenic; thus many gastrin and CCK antisera contain COOH-terminus-reactive antibody populations capable of reacting with both hormones.

Availability of gastrin- and CCK-specific antisera devoid of COOH-terminal antibodies has made possible the identification of gastrin and CCK-containing cells in man and in several other species (1–7). Since COOH-terminus-specific antibodies are able to detect both gastrin and CCK, it was initially expected that the sum of COOH-terminus-reactive endocrine cells would equal the sum of gastrin and CCK cells. This was not borne out, however, as we found more COOH-terminus-reactive cells than could be accounted for by gastrin plus CCK cells (7). COOH-terminus-reactive cells, which failed to react with antibodies specific for other gastrin or CCK sequences, were detected in all divisions of the small intestine. These cells were first demonstrated with COOH-terminus-specific antisera raised against gastrin. Hence they were labeled "terminal gastrin" (TG) cells (7). It should

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be noted, however, that, since such antisera do not differentiate between gastrin and CCK, this sequence may be either "gastrin-like" or "CCK-like." The fact that no other gastrin- or CCK-specific sequences can be detected in TG cells suggests three alternative possibilities: (1) TG cells contain levels of gastrin or CCK too low to be immunocytochemically detectable; (2) the tetrapeptide portion detected in these cells may be part of a larger peptide, which in the remainder of its amino acid sequence is different from both gastrin and CCK (such a putative peptide would be expected to represent an as yet unknown third member of the gastrin-CCK family and perhaps to share some of the biological activities of these); (3) the TG cells may store large amounts of the free COOH-terminal tetrapeptide amide or a molecule closely resembling this peptide, formed from an as yet undetected precursor.

Since immunocytochemistry does not provide information about the molecular size of the peptides demonstrated, all three possibilities must be considered. Subsequent radioimmunochemical studies have both suggested (7) and denied (4) the presence of the free COOH-terminal tetrapeptide amide in extracts of the porcine terminal ileum.

In pig and monkey, TG cells were frequent in all parts of the small intestine and were occasionally detected also in the antropyloric mucosa of monkeys. They were fairly well represented in the Brunner's glands of the duodenum and in the terminal ileum (7). In monkeys these two regions are virtually devoid of gastrin and CCK cells—a feature that made possible the ultrastructural identification of the TG cell as a new and morphologically unique gut endocrine cell type (7). It should be emphasized that although TG cells are not particularly numerous in ileum and Brunner's glands in monkeys and pigs, the near total absence of gastrin and CCK-containing cells in these regions facilitated their study by electron immunocytochemistry and radioimmunoanalysis. In some other species, like dog, TG cells seem to be absent from the ileum (Larsson: preliminary observations).

Observations on the ultramorphology of gut endocrine cells in the human intestine have now led to the identification of a novel cell type that resembles the TG cells as identified in monkeys. This cell type is characterized by round, medium-sized (200–300 nm), relatively solid granules with closely applied, evident membrane (Figure 1). The inner texture of the core is homogeneous or finely fibrillar; definite areas of increased or decreased density may be found in it, showing targetoid patterns. A proportion of small, round granules resembling those of intestinal gastrin (IG) cells (1) may also occur in some human TG-like cells. Occasionally, large irregular granules may also be found, admixed with the usual round granules. The presumed human TG cell is scattered in the duodenal crypts, Brunner's glands, and jejunum of adult and fetal specimens, never being numerous at any one site. Pyloric-type gastrin (G) cells with vesicular granules are found regularly in fetal duodenum—both in the crypts and in the Brunner's glands—but very

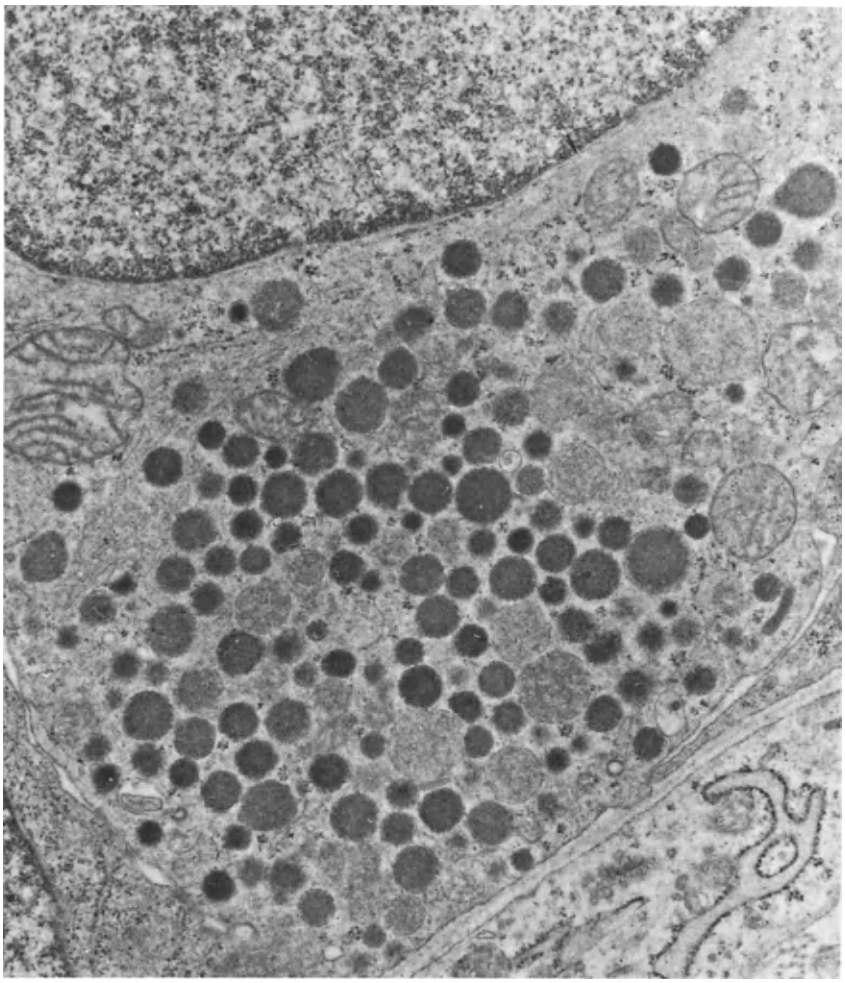

Figure 1. Human fetal duodenum (22 weeks). TG-like cell showing granules more solid than those of G cells and less dense than those of CCK cells. ×28,000.

few are present in adult duodenum. In both fetal and adult duodenum IG cells are fairly well represented.

The notion that the cell type detected in human intestine corresponds to the TG cell is corroborated by studies in human fetuses, where a large number of ultramorphologically distinct COOH-terminus-reactive cells have been identified (5). The properties of some of these cells resemble those of the human TG cell and those of the monkey TG cell (Figures 2 and 3). Other COOH-terminus-reactive cells were identified as G cells, IG cells, CCK cells, and cells with large irregular granules (7), somewhat resembling VL cells of conventional electron microscopy (8). Cells showing mixed patterns intermediate between those of the cells described above were also found (7).

These observations have led us to believe that TG cells occur also in human

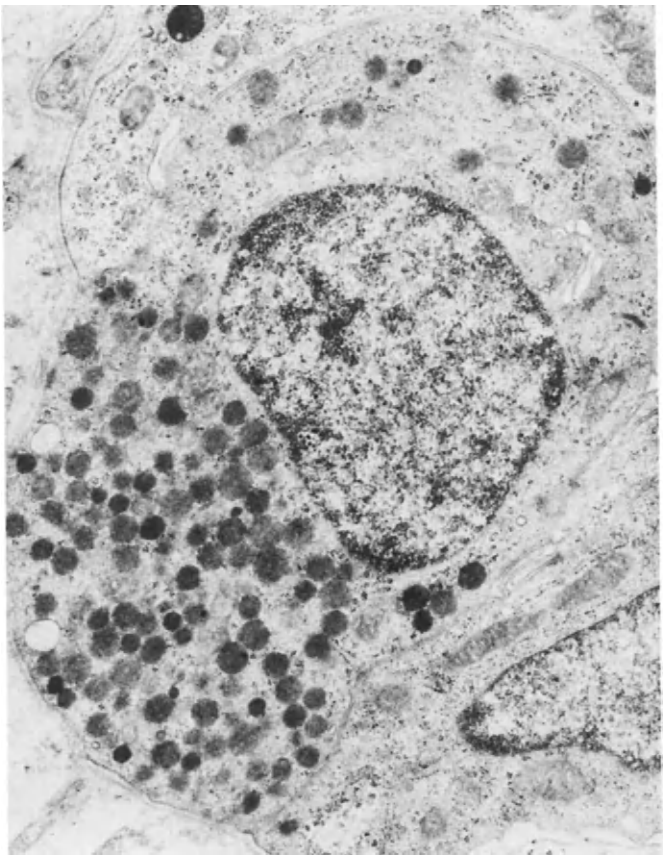

Figure 2. Human fetal duodenum (28 weeks) COOH-terminus-immunoreactive cell displaying a morphology similar to the TG-like cell shown in Figure 1 and to the monkey TG cell shown in Figure 3. ×19,800.

intestinal mucosa. Final confirmation of this supposition will, however, have to await region-specific immunocytochemical studies, of the type carried out in the monkey, documenting that human TG cells also are devoid of gastrin- and CCK-specific regions other than the common COOH-terminal tetrapeptide-like sequence.

The function of the TG cells is unclear. At present, radioimmunoassay data both suggest (7) and deny (4), that TG cells contain the free COOH-terminal tetrapeptide of gastrin/CCK. Region-specific immunocytochemistry is, unfortunately, not able to clarify this issue, since these techniques are not able to give information on the molecular size of immunoreactive molecules. Thus immunocytochemistry has localized a peptide sequence reminiscent of the tetrapeptide in the TG cells. Whether this sequence corresponds to the free COOH-terminal tetrapeptide amide or is part of a larger, yet uncharacterized sequence remains to be clarified by biochemical techniques.

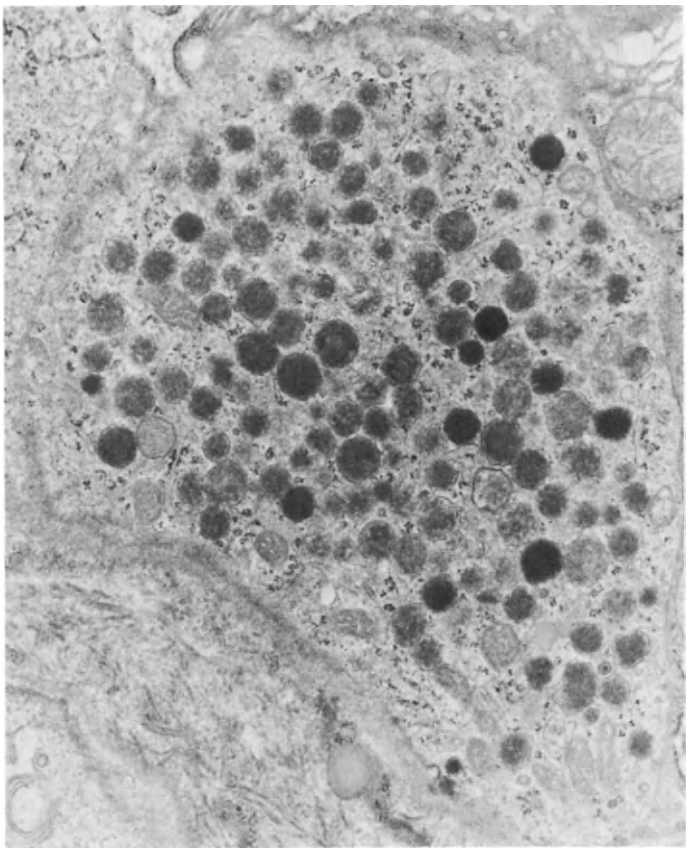

Figure 3. Monkey duodenum, Brunner's gland area. An immunocytochemically identified TG cell process is shown. ×24,500.

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Neurotensin in the Central Nervous System, Gastrointestinal Tract, and Plasma: Its Isolation and Chemical Characterization

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During the course of purification of substance P from bovine hypothalamic extracts, a vasoactive substance was detected in the eluate of an ion-exchange column that clearly separated from the sialogogic activity (Figure 1). It was found that the vasodilatation was associated with a transient hypotension and that these effects were labile to proteolytic digestion. The peptide was isolated by Carraway and Leeman (4) and named neurotensin (NT) because of its presence in neural tissue and its ability to affect blood pressure. The amino acid sequence of this peptide was determined to be <Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH by a combination of Edman degradation and carboxypeptidase treatment of papain-generated fragments of the molecule (5). Synthetic material was prepared (6) and used to generate radioimmunoassays (RIAs) (7) with several region-specific antisera (3). Figure 2 illustrates the binding sites determined for three of the antisera generated. Using these RIAs, the distribution of neurotensin was determined in acid–acetone extracts of rat tissues (8), and equally high concentrations (50–60 pmol/g wet weight) were found in hypothalamus and in full thickness jejuno-ileum. Subsequent purification of immunoreactive neurotensin from bovine small intestine [Kitabgi *et al.* (14)] yielded a peptide identical to hypothalamic neurotensin. We recently had the opportunity, in collaboration with the late Dr. Robert Williams, to study the distribution of neurotensin in fresh postmortem human mucosal scrapings (11). Figure 3 shows that

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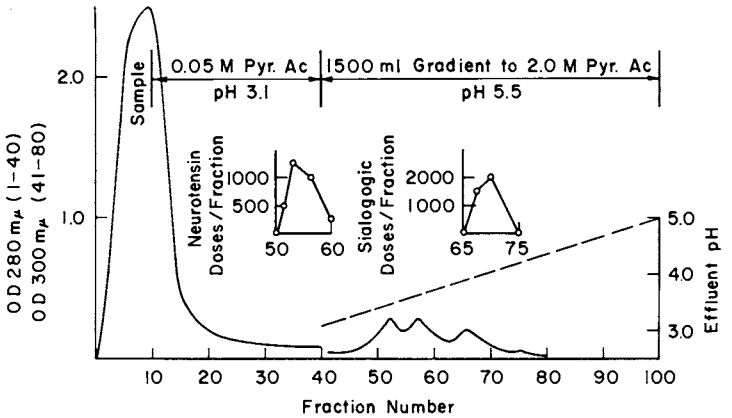

Figure 1. Ion-exchange chromatography on sulfoethyl-Sephadex C-25 of the sialogogic activity extracted from 22 kg of bovine hypothalami, after two gel filtration steps on Sephadex G-25. Column size, 2.2 × 15 cm (resin volume 50 ml); fraction size, 20 ml; column buffer, 0.05 *M* pyridine acetate, pH 3.1. Neurotensin and sialogogic activities, determined by injecting lyophilized aliquots dissolved in 0.85% saline into test rats, were eluted in fractions 50 to 60 and 65 to 75, respectively (see insets). – – –, an estimation of the pH gradient. (Reprinted, with permission, from 4.)

intestinal neurotensin increases in the distal jejunum and ileum, in agreement with findings in other animal species. The immunoreactivity as measured with the three antisera was nearly identical in the distal samples (9–19), but a two- to threefold disparity was observed for measurements with the different antisera on proximal samples, probably because of the presence of small cross-reacting peptide(s) also observed in rat and bovine stomach and duodenum (R. Carraway, unpublished observations).

Sufficient material was collected from scrapings of the distal mucosa to

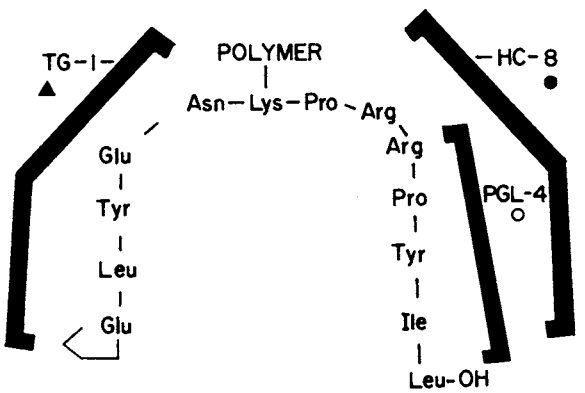

Figure 2. Orientation of NT in immunogens used and average binding sites determined for three of the antisera obtained. Polymer refers to carrier: PGL, antisera obtained with NT-poly(Glu^{60}, Lys^{40}) conjugate; HC, antisera obtained with NT-succinylated hemocyanin conjugate; TG, antisera obtained with NT-bovine thyroglobulin conjugate. (Adapted, with permission, from 3.)

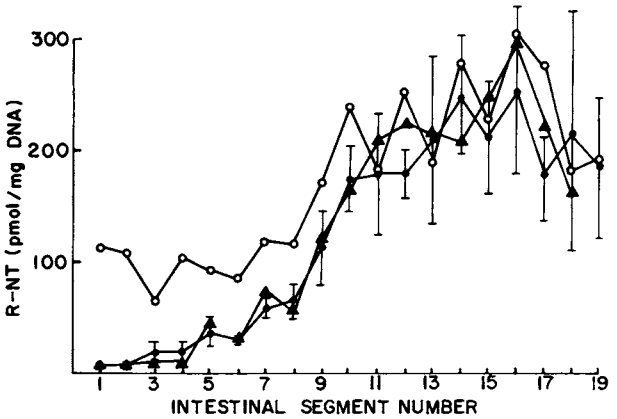

Figure 3. Average measurements of radioimmunoassayable NT (R-NT) at points sampled along the small intestine of four subjects, determined with antisera HC-8 (●), TG-1 (▲), and PGL-4 (○). Error bars indicate standard deviation of HC-8 measurements. The samples consist of the mucosa scraped from 12-cm-long segments of small intestine at approximately 30-cm intervals, extracted by homogenization in 10 ml of cold (−10°C) acetone–0.6 *M* acetic acid, 7:3 (v/v). After centrifugation, the supernatants were lyophilized and dissolved in 1 ml each of phosphate-buffered saline for assay. (Reprinted, with permission, from 11.)

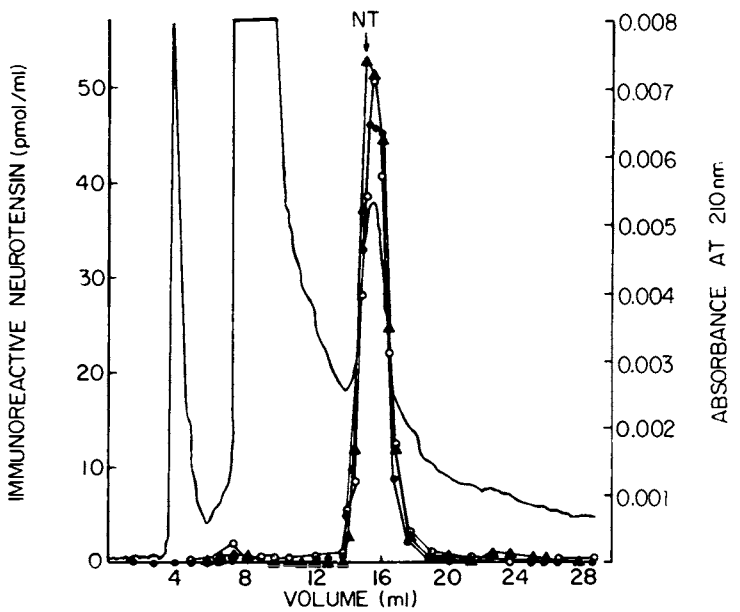

Figure 4. R-NT in fractions eluted from HPLC column after injection of 90 pmol of pure human NT, measured with antisera HC-8 (●), TG-1 (▲), and PGL-4 (○). Sample was injected in 5 μl of 10% pyridine on a 3.9 × 300 mm μ-Bondapak C-18 column from Waters, and eluted with 20% acetonitrile in 0.01 *M* KH_2PO_4, pH 4.0, at a rate of 2 ml per minute. Each 0.4-ml fraction was lyophilized and diluted for assay. Absorbance at 210 nm is recorded at a setting of 0.01 unit full scale and represented by the light, continuous line. Elution peak of bovine NT is indicated with an arrow. (Reprinted, with permission, from 11.)

permit us to isolate human intestinal neurotensin and to determine its amino acid composition. The peptide was found to be chemically and biologically indistinguishable from bovine neurotensin. The pure material ran as a single peak on reverse-phase high-pressure liquid chromatography (HPLC) (Figure 4) and was identical in elution volume with synthetic NT. Figure 5 illustrates that the specific immunoreactivity of human and bovine NT remained relatively constant in every fraction across the peak (as measured with all three antisera), implying that only one substance was contributing to the optical density of the peak. Only a part of the amino acid sequence of human NT could be determined because of the limited amount of pure peptide. However, digestion of human and bovine NT with papain, followed by separation of the peptide fragments on reverse-phase high-pressure liquid chromatography and determination of the amino acid composition of the separated fragments, yielded identical results for the two molecules, suggesting that the sequence is also identical. Demonstration of the identity of human neurotensin and the bovine peptide to which all antisera thus far reported have been raised means that a reliable radioimmunoassay for bovine neurotensin will give accurate measurements of the human peptide as well.

Several groups have demonstrated neurotensin-containing cells scattered throughout the small intestinal epithelium of many mammalian and avian species (13,19,20,23). Figure 6 shows two cells in the guinea pig ileal mucosa stained positively with neurotensin antiserum (HC-8) by the peroxidase-antiperoxidase technique (22). These cells appear to be of the open variety, with apices in contact with the lumen and immunostaining primarily toward the lamina propria. Figure 7 shows the ultrastructure of a

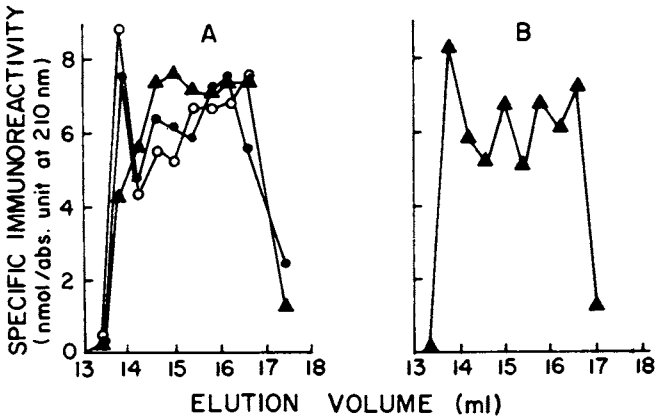

Figure 5. Specific immunoreactivity of NT in peak fractions eluted from HPLC column. This was calculated across the peaks of both human and synthetic NT in nmoles per fraction per absorbance unit. For these calculations the absorbance of each fraction was determined by subtracting the appropriate solvent blank from the value displayed on the strip chart recording. (A) Human NT, as shown in Figure 4. Results are expressed for HC-8 (●), TG-1 (▲), and PGL-4 (○). (B) Bovine NT, as measured with antiserum TG-1. (Reprinted, with permission, from 11.)

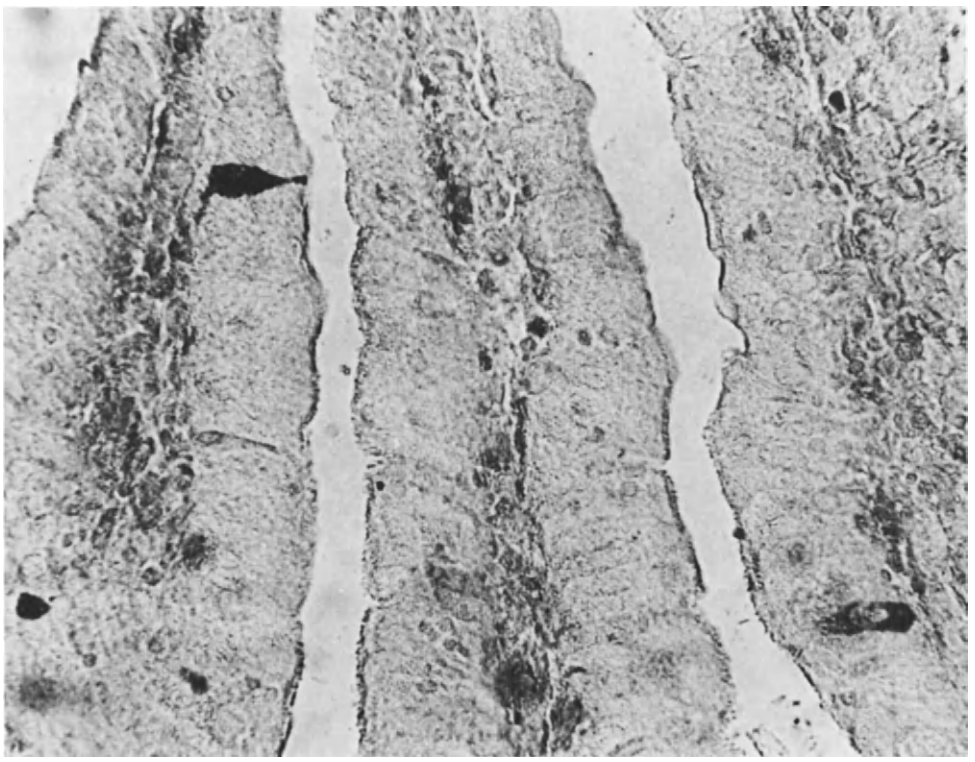

Figure 6. Peroxidase-antiperoxidase staining of guinea pig ileum with antiserum HC-8, after fixation with formaldehyde vapor and vacuum-embedding in paraffin. Two neurotensin-immunoreactive cells are shown, one with an apical process reaching the lumen. ×460. (Reprinted, with permission, from 23.)

large-granuled cell from the primate *Tupaia* that stained in the adjacent semithin section with neurotensin antiserum (12). This work was done in the laboratory of Dr. W. G. Forssman. The dense granules are concentrated toward the base of the cell, but a few are present near the apex, which is in contact with the gut lumen. Its luminal surface is more simplified than that of its neighboring cells.

The orientation of this type of cell and the pattern of peptide-containing nerve fibers in the intestinal ganglia are likely to be important to their functions. Since these functions are presently unknown, one should not think only of anatomy in considering the possibilities. Two examples involving the study of substance P illustrate some of the types of interaction whereby neuropeptides can influence the activity of their targets. When sensory neurons were grown in culture, they were shown to contain substance P and to release it on depolarization with high potassium or electrical stimulation in a calcium-dependent manner (17). Addition of somatostatin to the culture

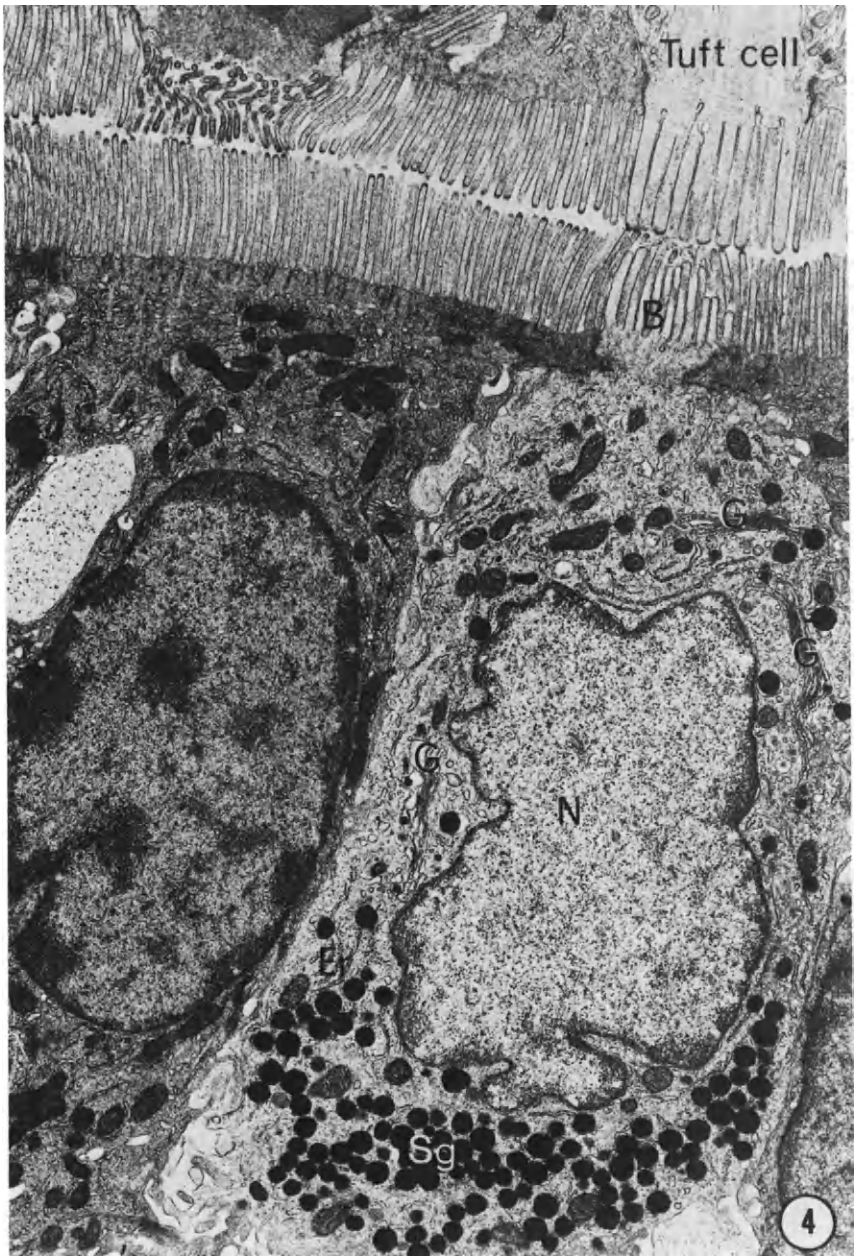

Figure 7. Low magnification of the ileal N cell of the primate *Tupaia belangeri*. The secretory granules (Sg) are clustered mostly at the basal region. Golgi apparatus (G), ergastoplasm (Er), nucleus (N), and the apical brush border (B) are also seen. ×9300. (Reprinted, with permission, from 12.)

medium inhibited the release of substance P. When intracellular recordings were made, it was found that somatostatin had no effect on the resting potential of the sensory neurons but that it caused a significant decrease in

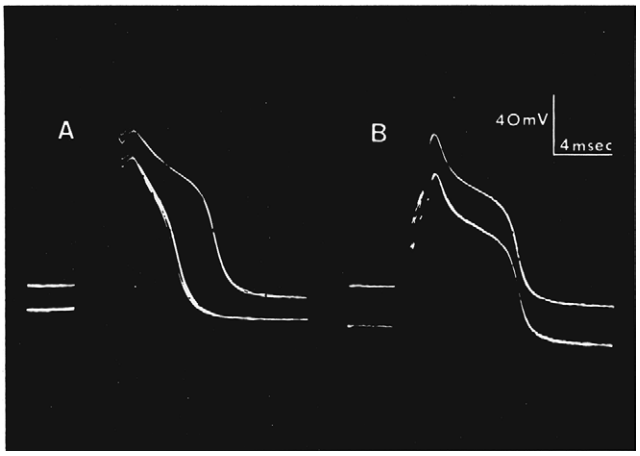

Figure 8. Effects of somatostatin (A) and substance P (B) on dorsal root ganglion cell body action potentials. In each case the upper trace is the control spike; the lower trace is the spike after application of drug. (A) Oscilloscope traces showing the effect of application of 5×10^{-7} *M* somatostatin. (B) Oscilloscope traces showing no effect after application of 10^{-5} *M* substance P.

the duration of the action potential (Figure 8A) (A. Mudge and G. Fischbach, unpublished observations). Although the early phase of the action potential is caused by an inward sodium current, the plateau has been shown to be due in large part to an inward calcium current (18). This decrease in the duration of the plateau implies a decrease in calcium entry. Since calcium is required for the secretion process, this may represent the mechanism by which somatostatin inhibits substance P release from sensory neurons. An implicit assumption is that these effects measured across the membrane of the cell body reflect what is occurring at the nerve terminals. Application of substance P during an action potential had no effect on the duration of the plateau phase (Figure 8B). If this response has physiological meaning, it would imply a role for somatostatin as a presynaptic inhibitory transmitter.

Another possible mode of action of a peptide could be by interacting with another transmitter, thereby modulating the effect of the transmitter. Livett and his colleagues (15) studied the secretion of catecholamines from adrenal medullary paraneurons and found that both somatostatin and substance P produce a marked dose-dependent inhibition of acetylcholine-stimulated catecholamine release. Table 1 provides data obtained by Role and her colleagues (21) studying the effects of substance P and somatostatin on the acetylcholine-stimulated release of catecholamines in freshly prepared guinea pig adrenal medullary cells. Both these peptides inhibited the ACh-mediated secretion but not the secretion following depolarization with high potassium. Further studies have shown that these peptides are effective only in inhibiting those agents that require an interaction with ACh and its nicotinic receptor.

Only a few studies have been done on the role of neurotensin as a possible gastrointestinal hormone. Little is as yet known about what stimuli lead to

TABLE 1
Inhibitory Effect of Substance P and Somatostatin on Catecholamine Secretion by Chromaffin Cells[a]

Condition	[^{3}H]catecholamine secretion (dpm/μg protein)		
	No addition	10 μM SP	10 μM SOM
Control	292 ± 12	311 ± 24	224 ± 16
ACh (100 μM)	1329 ± 60	705 ± 14	738 ± 28
56 mM K^+	498 ± 40	496 ± 25	471 ± 37

[a]ACh, acetylcholine; SP, substance P; SOM, somatostatin.

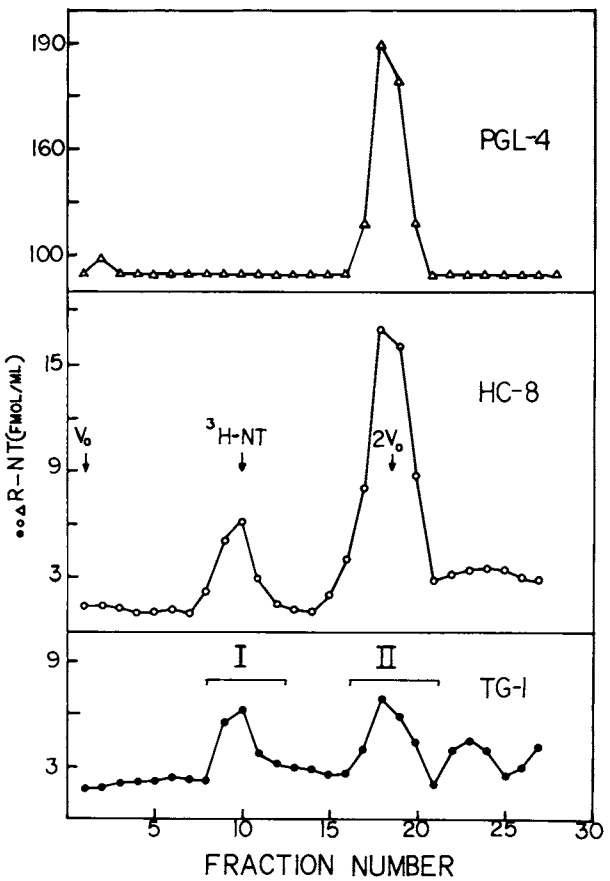

Figure 9. Gel chromatography of bovine plasma extracts on Sephadex G-25. Sample, acid-acetone extract of 1.2 liters bovine plasma dissolved in 200 ml of column buffer; column dimensions, 14 × 123 cm with about 18.5 liter bed volume; column buffer, 0.2 M acetic acid. After the sample was applied, 7 liters of effluent were discarded and then fractions of 500 ml were collected. Multiples of the void volume (V_0) and the elution position for ^{3}H-NT are indicated. Aliquots of each fraction were placed into the radioimmunoassay for NT using the three antisera (PGL-4, HC-8, and TG-1). Recovery, 66%. (Reprinted, with permission, from 9.)

release of NT from the intestinal endocrine-like cells or whether the NT is delivered into the lumen or released into the circulation. Immunoreactive neurotensin has been measured in plasma by several groups (1,2,16) using different antisera; the reported results have been on unextracted plasma. We (9) have examined the character of neurotensin-like peptides present in acid-acetone extracts of animal and human plasma and have demonstrated the presence of multiple substances, only one of which elutes with synthetic NT on Sephadex G-25 chromatography (Figure 9). Material from peak I was purified by ion-exchange chromatography and subjected to reverse-phase HPLC. Figure 10 shows that this material eluted in the position of synthetic NT. The plasma level of the component that is chromatographically and immunochemically indistinguishable from neurotensin is estimated to be 15–25 fmol/ml, which is 30–50% of the measurement obtained on extracts of plasma using antiserum HC-8. Measurements of 200–250 fmol/ml have been obtained in preliminary work with unextracted plasma (using antiserum HC-8), but only 20–40% of this immunoreactivity is retained on extraction. Furthermore, following chromatography of unextracted plasma on Sephadex G-25, a large portion of the immunoreactivity elutes in the void volume. Thus, in our hands, measurement of plasma levels of neurotensin in order to assess a hormonal role for this peptide pose considerable difficulties.

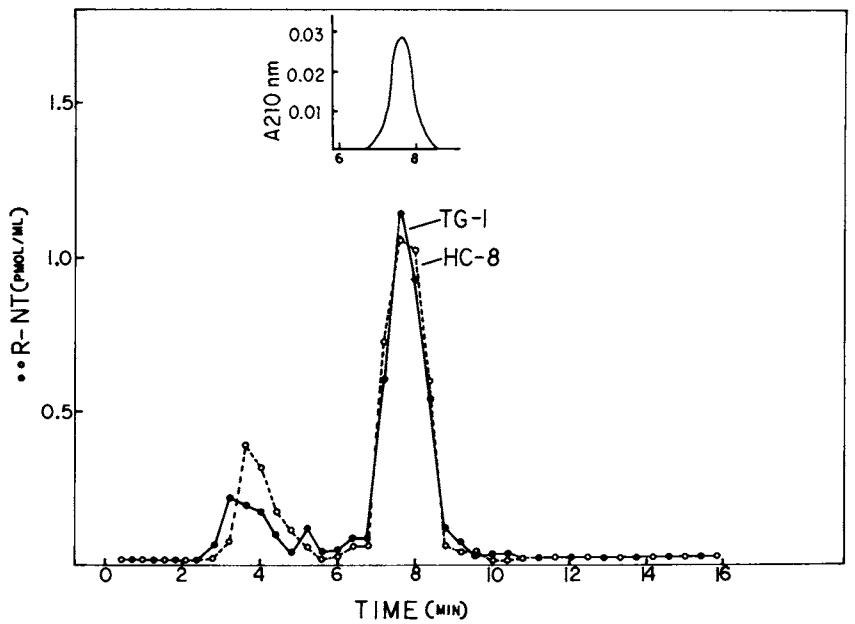

Figure 10. High-pressure liquid chromatography of R-NT from Region I on μ-Bondapak C18. Sample, about 2 pmol of R-NT from the pooled region of immunoreactivity of an ion-exchange column used after the step shown in Figure 9. Sample was applied and eluted in 20% acetonitrile in 0.01 *M* KH_2PO_4, pH 4, run at 2 ml/min. Fractions (0.2 min; 0.4 ml) were collected and aliquots were assayed using the NH_2-terminal antiserum (TG-1) and the COOH-terminal antiserum (HC-8); recovery, 92%. The inset shows the absorbance profile obtained for a sample of synthetic NT (2 nmol) run under identical conditions immediately afterward. (Reprinted, with permission, from 9.)

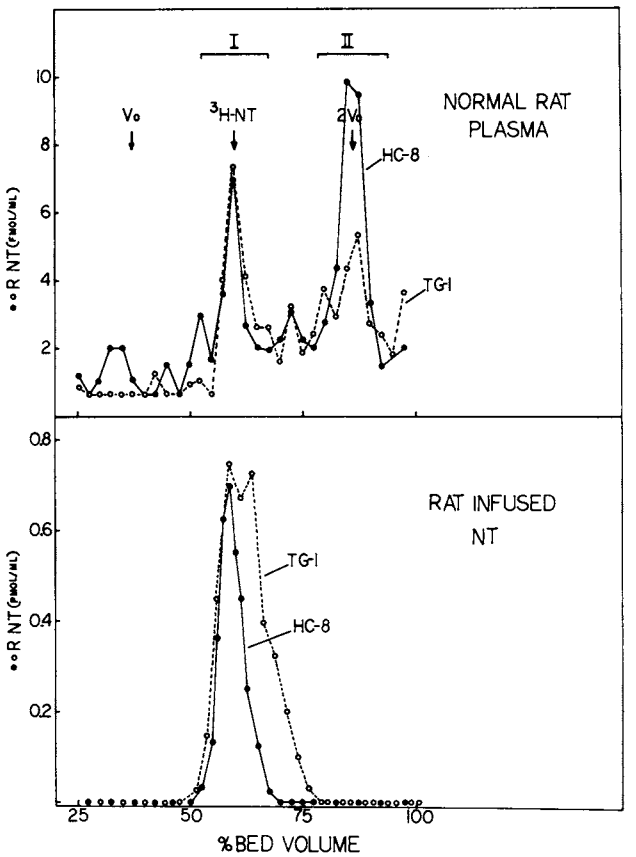

Figure 11. Gel chromatography of rat plasma extracts on Sephadex G-25. (Upper) sample, acid–acetone extract of 160 ml normal rat plasma dissolved in 15 ml of column buffer; column dimensions, 5.2 × 94 cm with about 2-liter bed volume; column buffer, 0.2 *M* acetic acid; recovery, 40%. (Lower) sample, acid–acetone extract of 2 ml plasma from two rats infused with synthetic NT for 30 min at 0.1 nmol/kg/min after a loading dose of 1 nmol/kg. After 30 min the animals were decapitated and trunk blood was collected into heparinized tubes on ice. Because of the vascular effects of NT, only 2–3 ml of blood could be obtained from each rat. Column dimensions, 1.6 × 98 cm with about 200 ml bed volume; column buffer, 0.2 *M* acetic acid; recovery, 87%. Multiples of the void volume (V_0) and the elution position for ^{3}H-NT are indicated. Aliquots of fractions were assayed using the NH_2-terminal antiserum (TG-1) and the COOH-terminal antiserum (HC-8). (Reprinted, with permission, from 9.)

In order to determine whether the neurotensin-related peptides found in plasma extracts represent precursors to or breakdown products of neurotensin, it was infused into rats for 30 min at 0.1 nmol/kg/min. Figure 11 shows the results of Sephadex G-25 chromatography of plasma extracted from such a rat, as well as from normal rats. Infusion of synthetic NT did not result in a generation of the immunoreactive variants normally observed in plasma.

We have recently started to investigate a system that may shed some light on the agents that control the release of neurotensin. Recently, the interesting finding has been made that a cell line from a tranplantable

WAG/Rij rat medullary thyroid carcinoma synthesizes and secretes both calcitonin (10,25) and neurotensin (24). This may provide an excellent system in which to study agents that regulate the secretion of neurotensin.

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Multiple Secretory Messengers in Endocrine Cells and Neurons: ACTH-Related and Opioid Peptides in Gut and Brain

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INTRODUCTION

Biologically active peptides are produced and secreted by a great variety of cell types, including neurons, endocrine cells, and paracrine cells. There is now abundant immunological evidence for the dualistic production of certain peptides by both neurons and endocrine–paracrine cells. In two cases, it has proved possible also to document their amino acid composition or sequence and to show conclusively that the same peptide occurs both in nerves and in endocrine cells (4,6). In submammalian vertebrates it is possible that secretory peptides may be produced by an even larger number of cell types. Thus the cutaneous exocrine glands of certain amphibians are known to produce caerulein-related peptides (3), which also seem to be produced by central neurons and by gastric endocrine-like cells in these species (19). It is likely that the same peptide may subserve different functions, depending on its sites of production. Moreover, recent data indicate that certain endocrine and neuronal cell systems produce more than one type of peptide (reviewed in 15). Although the simultaneous production of multiple secretory products by pancreatic zymogen cells and the coexistence of biogenic monoamines and peptide hormones in certain endocrine cell systems are well known, these new data challenge our concepts of endocrine and neuronal secretion and regulation. Full documentation for the simultaneous production and secretion of multiple peptides by defined cell systems must include

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demonstration of the peptides at the cytochemical level, identification of the peptides at the biochemical level, and demonstration of their biosynthesis and secretion. Such full information has, as yet, been obtained only in the case of the pituitary adrenocorticotropin/α-melanocyte-stimulating hormone/β-endorphin (ACTH/MSH/β-endorphin) cells, in which the secretory peptides are produced by processing of a single precursor (22,23,26). It is possible that other cell systems utilize distinct biosynthetic precursors for producing different secretory messengers. So far, however, information on this point is not available. In an attempt to elucidate mechanisms of synthesis, transport, and secretion in cells producing multiple secretory peptides, we decided to study sites of production of ACTH-related peptides in the brain and gut. In the first section we review findings with central neurons producing ACTH-related and opioid peptides from a single precursor. In the second section we discuss the simultaneous production of ACTH-related, opioid, and gastrin peptides by antropyloric G cells.

ACTH-Related and Opioid Peptides in the Brain

Immunoreactive and bioactive ACTH occurs in several brain areas and has been localized to a system of central nerves (7,9). For the main part these central nerves originate from cell bodies located in, and in the vicinity of, the hypothalamic arcuate nucleus (8,13,30). These nerves react with a variety of region-specific antisera, recognizing both the NH_2-terminal, mid-region, and COOH-terminal portions of ACTH[1–39] (13). The arcuate cell bodies also react with antisera to β-endorphin and β-lipotropin (βLPH) (24). In the pituitary ACTH is synthesized in the form of a 31,000-dalton precursor that also contains the β-LPH and β-endorphin (=β-LPH[61–91]) sequences (22,23,26). Recent data on cultured arcuate cell bodies indicate that the hypothalamus also synthesizes a similar large precursor (21).

Parallel studies have revealed the occurrence of α-melanotropin (α-MSH) in the brain. By immunocytochemistry, α-MSH has been localized to a system of nerves distributed similarly to the ACTH nerves (5). In recent double-staining studies we have shown that the cerebral α-MSH nerves are also ACTH immunoreactive (14). This agrees with the suggestion that peptides containing the ACTH[1–39] sequence may serve as precursors to α-MSH (=N-acetylated and amidated ACTH[1–39]) (27). In addition, a few cerebral ACTH immunoreactive nerves fail to display detectable α-MSH immunoreactivity (14).

Our α-MSH antisera react intensely with cerebral axons and nerve terminals but react poorly with arcuate nerve cell bodies. Following intracisternal injections of colchicine, however, the arcuate somas also react with α-MSH antisera (14). Both in normal and in colchicine-pretreated rats the arcuate somas react strongly with an antiserum to α-endorphin (Melinda; kindly donated by Dr. B. Eipper). This antiserum has been shown to react with both β-endorphin and with the large ACTH-β-endorphin precursor.

It is, thus, likely that in normal rats significant amounts of biosynthetic precursors occur in the arcuate somas. During their anterograde axonal transport, these precursors are continuously processed to smaller end (secretory) products like α-MSH and β-endorphin (Figure 1). Under normal circumstances only very small amounts of end products are localized in the somas. This is well documented by the weak to absent staining of arcuate somas by the α-MSH antisera, which by absorption can be shown not to react with ACTH[1-39] and, hence, are not expected to react with ACTH precursors (14). Interruption of axonal transport with colchicine results in the accumulation of secretory products such as α-MSH in the cell somas. Thus it seems that following its synthesis in the rough endoplasmic reticulum and packaging in secretory granules in the Golgi apparatus, processing of the large ACTH-MSH-β-endorphin precursor continues in the axon.

β-Endorphin binds to opiate receptors in the brain (for review see ref. 20).

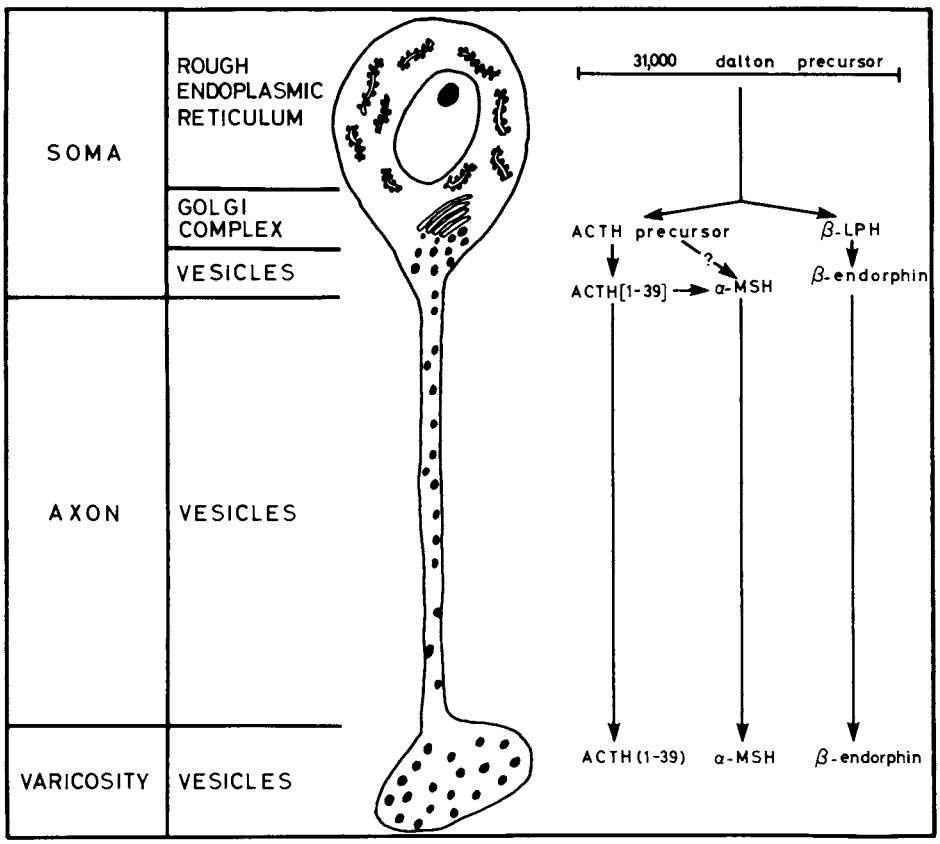

Figure 1. Summary of our ideas about the transport and posttranslational processing of the ACTH(MSH)-endorphin precursor in neurons of the arcuate nucleus of the hypothalamus. These ideas are founded on the demonstration of ACTH-β-endorphin precursor synthesis in the arcuate nucleus (21), on colchicine experiments, and on region-specific immunocytochemistry (14).

Previous data suggest that ACTH may also interact with these or similar receptors (29). Although the NH_2-terminal pentapeptide sequence of β-endorphin is identical with the sequence of Met-enkephalin, there is no evidence for a biosynthetic relationship between these two opioid peptides. By immunocytochemistry it has been possible to show that β-endorphin and enkephalin immunoreactive nerves belong to separate neuronal systems (2). There is, however, a considerable overlap in many of the projections of the enkephalin and ACTH-α-MSH-β-endorphin nerve systems (9,10). It is thus possible that the peptides produced by these neurons may, at least in some areas, act on the same receptors. The possible sites of interaction between ACTH and opiate-related peptides may be even more complex than hitherto conceived. Thus, recently, we have obtained evidence indicating that the two opioid pentapeptides, Met- and Leu-enkephalin, occur in distinct nerve terminals (18). This increases the number of neuronal systems producing opioid peptides to three, including Met-enkephalin, Leu-enkephalin, and ACTH/α-MSH/β-endorphin systems. In addition, at least in the rat, a very small number of arcuate neurons appear to produce ACTH- and β-endorphin-like peptides but not α-MSH (14). The occurrence of multiple systems of nerves producing multiple putative ligands for opiate receptors is matched by recent data suggesting that opiate receptors may be heterogenous (for review see Ref. 28). It seems important to underline that although in some areas overlaps in the distribution of opiate nerves occur, there are also areas in which a more fully differentiated distribution is noted.

ACTH-Related and Opioid Peptides in the Gut

Previously, we have described the occurrence of ACTH-like immunoreactivity in endocrine cells of the antropyloric gland area. The ACTH immunoreactive cells have been identified as gastrin-producing (G) cells (9–13). Recent data have suggested that both the ACTH-like peptides and gastrin are stored in the cytoplasmic granules of feline G cells (17). The G cell content of ACTH-like peptides does not seem directly correlated to the content of gastrin. Thus during ontogeny rat antropyloric G cells start to accumulate gastrin before ACTH immunoreactivity can be demonstrated (11). Also during conditions of fasting and feeding the cellular stores of these peptides appear to vary in a nonparallel manner (12). We do not yet know whether these differences reflect differences in the biosynthesis, secretion, or posttranslational processing of the two peptides. It also remains to be determined whether G cell granules contain varying amounts of the peptides. Hypophysectomy does not affect the antropyloric stores of ACTH-like peptides, suggesting that these may be synthesized locally (9,10).

Region-specific immunocytochemistry, employing a variety of NH_2-terminal, mid-region, and COOH-terminal ACTH[1-39] antisera have indicated that the antropyloric peptide is quite similar to, but probably not identical with, pituitary ACTH[1-39] (13). In preliminary studies we have

failed to obtain staining with endorphin and β-LPH antisera in these cells. In contrast, Met-enkephalin-like immunoreactivity has been reported to occur in G cells (25). Other workers have, however, failed to detect enkephalin staining in G cells (1). Negative immunocytochemical data are, however, always open to question, since so many factors may cause negative outcomes of the staining.

Radioimmunoassay studies have documented that extracts of antropyloric mucosa contain large quantities of ACTH-like immunoreactivity (16). Expressed in ACTH[1-39] equivalents, concentrations measured in human and feline antropyloric mucosae range between one-tenth to one-hundredth the concentrations of gastrin. In other species concentrations equaling those of gastrin have been measured (to be published). After elution by gel chromatography of boiling water extracts of cat or human antropyloric mucosae, two immunoreactive ACTH components are detected (16). The main component elutes in the position of pituitary ACTH[1-39], whereas a quantitatively smaller component elutes in the void volume on Sephadex

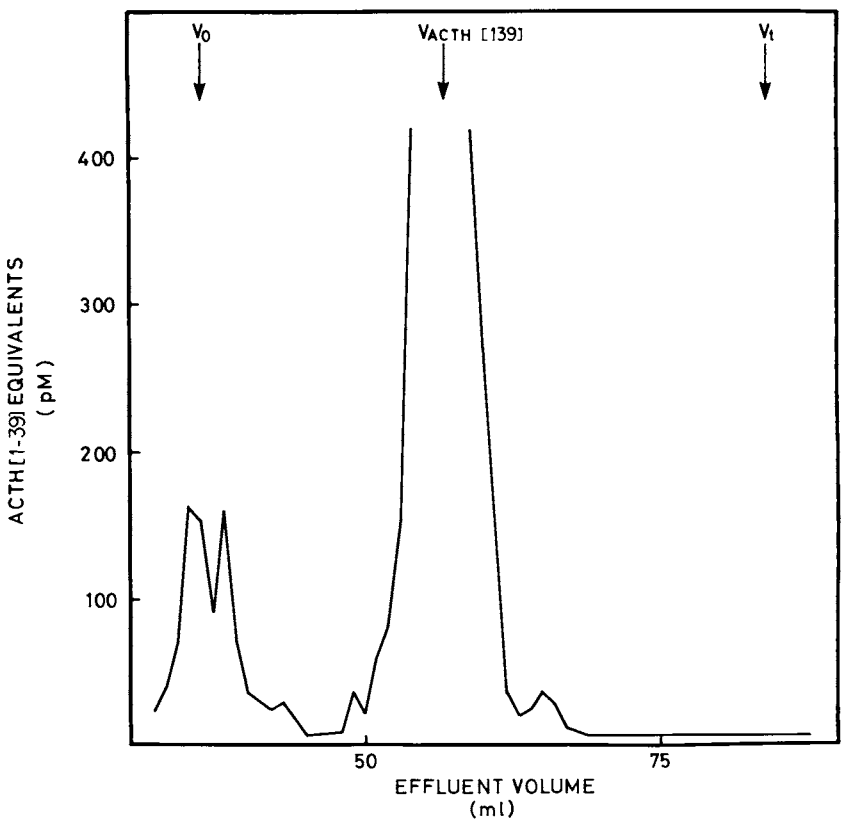

Figure 2. Gel chromatography of boiling water extract of human antropyloric mucosa on Sephadex G-50 SF. The predominant molecular component of antropyloric ACTH immunoreactivity elutes in the position of pituitary ACTH[1-39], whereas a quantitatively smaller amount of immunoreactivity elutes in the void volume. (For details see text and 16.)

G-50 SF columns (Figure 2). In its immunochemical and elution behavior, the main antral component resembles true pituitary ACTH[1-39], although it should be noted that this is not proof of identity of the two peptides. The void volume material either may represent biosynthetic precursor(s) to the main antropyloric ACTH-like component or may be artifactual. We believe in the former possibility, in particular since we have recently obtained evidence for biosynthesis of the two components in cultured antropyloric mucosae.

Thus evidence for the simultaneous production of both an ACTH-like peptide and gastrin by antropyloric G cells is accumulating. The physiological function of the ACTH-like peptide is unknown. The secretion of corticostimulatory substances from the stomach seems unlikely in physiological terms and this together with recent characterization studies makes it possible that the antral peptide is distinct from, although related to, pituitary ACTH[1-39]. Studies on the synthesis, transport, and secretion of this peptide and gastrin are expected to produce valuable information on the functions of multi-messenger cell systems.

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Human Gastroenteropancreatic Endocrine–Paracrine Cells: Santa Monica 1980 Classification

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The identification and classification of the gastroenteropancreatic (GEP) endocrine-paracrine cells was discussed by an ad hoc panel chaired by E. Solcia (Pavia, Italy).

Initial discussion centered on the possibility of introducing a functional classification based on the secretion products of the individual cell types. It was generally agreed, echoing feelings previously expressed by some workers (2,15,24), that such a functional classification is desirable and is now becoming an increasingly realistic goal in light of recent methodological advances in immunocytochemistry at the ultrastructural level. However, a number of problems still remain that preclude the reclassification of the GEP endocrine-paracrine cells on an entirely functional basis at the present time. It must be emphasized that only in very few cases have accurate, region-specific antibodies been employed in immunocytochemical studies of the GEP endocrine-paracrine cells. The fact that a single antiserum reacts with a given cell type indicates, at most, the occurrence of an amino acid sequence of three to eight residues in that cell. Since most gastrointestinal peptides are considerably larger and since many show sequence homologies, appropriate functional classification awaits the use of multiple antibodies recognizing different sequences in different parts of the molecule. Other

*The names of the participants are listed in alphabetical order, with the exception of that of the chairman, Dr. Enrico Solcia.

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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unresolved aspects include the following: (1) Some cells store more than one secretion product. How should such cells be classified? (2) Cells classified on a functional basis should also be characterized ultrastructurally in order that cell types be clearly identified. (3) On the other hand, no secretory product is currently known for some cell types morphologically well characterized. (4) Finally, wide differences among species in ultrastructural appearance and distribution of a number of GEP endocrine-paracrine cell types make it practically impossible at this time to establish an acceptable classification encompassing all animal species. The panel felt that the Lausanne 1977 classification (35) had proved useful; therefore, it appeared desirable to retain its general structure as a basis of an updated classification. Modifications were introduced only for those cell types for which new information about morphological and functional aspects has become available during the last three years. The main changes have taken place in the D_1, EC, and P cells, with a new cell type, the TG cell being added. The other cell types remain essentially as previously described (35) and illustrated (24,34). The updated classification of the human GEP endocrine-paracrine cells is given in Table 1.

As pointed out in the past (23,34), the D_1 cells proved to be a heterogeneous group within which functionally distinct cell types have been identified. A group of cells of unknown function still remains, however, and these are denoted under the general term of D_1 cells. The latter designation has been retained with some hesitation because, in the past, the production of several peptides such as gastric inhibitory polypeptide (GIP), vasoactive intestinal polypeptide (VIP), and pancreatic polypeptide (PP) has been attributed without subsequent confirmation to cells with the D_1-type appearance. Antisera specific for intact G17 and for NH_2-terminal regions of G17 or G34 that do not react with cholecystokinin (CCK), showed immunoreactivity with intestinal cells that possess small, round, electron-dense granules, measuring 170–190 nm in diameter and fitting the D_1 cell description (2,4,31). These intestinal gastrin (IG) cells are structurally different from the CCK-producing I cells and from the pyloric gastrin (G) cells (2). The latter are infrequent in human adult small intestinal mucosa and more common in human fetal duodenal mucosa (19,22,37). Cells resembling IG cells or containing granules typical of both G and IG cells have been demonstrated in gastrinomas (6,13,18,26,38). Cells with the typical D_1 cell morphology have also been described in insulinomas (7) and in VIPomas (5,25).

The TG cell is a recently described cell type found in the small intestinal mucosa of pig, monkey (20), and man (21). The TG cell appears to be morphologically distinct from G as well as IG cells and reacts immunocytochemically with antibodies to the C-terminal tetrapeptide of gastrin–CCK, but not with other gastrin–CCK-specific sequences. This may mean that this sequence is free in the TG cells or that it is part of a larger peptide that has no other amino acid sequences in common with the

TABLE 1
Santa Monica 1980 Classification of the Human GEP Endocrine-Paracrine Cells

Cell type	Pancreas[a]	Stomach		Small intestine		Large intestine	Function
		Oxyntic	Pyloric	Upper	Lower		
A	+	+[b,c]	−	−	−	−	Glucagon
B	+	−	−	−	−	−	Insulin
D	+	+	+	+	+	+	Somatostatin
D_1	+	+	+	+	+	+	Unknown
EC	+[b]	+	+	+	+	+	Serotonin + various peptides
ECL	−	+	−	−	−	−	Unknown (histamine)
G	+[b,c]	−	+	+	−	−	Gastrin
I	−	−	−	+	+	−	Cholecystokinin
IG	−	−	−	+	+	−	Gastrin
K	−	−	−	+	+	−	GIP
L	−	−	−	+	+	+	Glucagon-like immunoreactivity
Mo	−	−	−	+	+	−	Motilin
N	−	−	−	−	+	−	Neurotensin
P	+[c]	+	+	+	−	−	Unknown
PP	+	−	+[b]	−	−	−	Pancreatic polypeptide
S	−	−	−	+	+	−	Secretin
TG	−	−	+[b]	+	+[b]	−	C-terminal gastrin immunoreactivity
X	−	+	+[b]	−	−	−	Unknown

[a]The pancreatic islets of man and other mammals also contain the so-called C cell (11). The existence of these chromophobe, essentially agranular, cells as an independent cell type has not been clearly established as they may represent immature cells originating from small ductules prior to evolving into a well-characterized islet cell type. However, it is important for the pathologist to be aware of their existence, because C cells are increased in some pathologic states and should not be confused with degranulated B cells, which occur in other disease states (11).

[b]In other animals; only exceptionally in man.

[c]In fetus or newborns; only exceptionally in adults.

gastrin-CCK molecules. Conflicting radioimmunoassay studies either propound (20) or deny (10) the existence of the C-terminal tetrapeptide of gastrin-CCK as a freely existing peptide. The isolation of the free N-terminal 1-13 gastrin sequence (9) might argue, on a hypothetical basis, for the existence of the corresponding free C-terminal tetrapeptide. Nevertheless, further studies, including chemical isolation and characterization, are needed for the resolution of this issue.

The availability of antisera against either the C- or the N-terminal sequence of motilin has led to the awareness of the existence of diverse motilin (like)-storing cells. A nonargentaffin cell type, containing small granules of the D_1 cell type, has been immunostained with antibodies directed against both N- and C-terminal peptides of motilin (17,31). The term *Mo* or D_1 (Mo) cell was therefore proposed to designate this nonargentaffin motilin-containing cell of the human small intestine. In addition, a subpopulation of enterochromaffin (EC) cells of the small intestine, previously identified as EC_2 cells (35), is stained with antibodies directed exclusively against the N-terminal sequence of motilin (16,31). It is not clear whether this partial immunoreactivity, the subject of considerable controversy, reflects a stage in the synthesis of the whole motilin molecule or the presence of a different peptide containing the N-terminal motilin sequence in its chemical structure (30).

The EC cells also are a heterogeneous group in regard to their ultrastructural appearance and their peptide secretion products. Motilin (16), substance P (28,29), and enkephalin (1) have been immunocytochemically identified in EC cells. This resulted in the further classification of these cells into subtypes in the past (35). However, as there are wide interspecies differences in morphology and function, it was considered impractical at this time to retain the classification of the EC cells into subtypes. For the time being, EC cells probably should remain as one group characterized as such by their serotonin content, their argentaffinity, and their characteristic ultrastructural appearance.

The P cells, so named because of their morphological resemblance to pulmonary P cells, had been suggested in the past as a possible source of a bombesin-like peptide (32). It has been shown that bombesin-like immunoreactivity occurs in gut endocrine cells of birds (36) and amphibians (27), whereas in mammals so far it has been found only as a neuropeptide. Thus the function of the P cell in man still remains unsettled. Hyperplasia of cells similar to the P cells has been reported in the fundic gastric mucosa of patients with chronic atrophic gastritis (33,34).

Most VIP in the gut is found in nerves. Some mucosal cells have been immunostained with some, but not all, antisera against VIP (22). This VIP-like material in mucosal cells has not been chemically characterized, but it appears to react mainly with antisera against the N-terminal sequence of VIP (3,8). At present, no conclusive combined immunocytochemical-ultra-

structural evidence has been reported that would serve to identify clearly the VIP (like)-containing cell among the known endocrine cell types of the digestive mucosa.

Cells reacting with antibodies to bovine pancreatic polypeptide, and ultrastructurally distinct from pancreatic PP cells, have been demonstrated in the lower intestine of man and other mammals (3,34). These have proved to be L cells, which store both glucagon-like peptides and a PP-like sequence (12,14), therefore casting some doubt on the existence of an independent PP cell in the human intestinal mucosa.

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P-Type Nerves: Purinergic or Peptidergic?

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Nerve fibers of the p-type were first demonstrated in the gut by Baumgarten, Holstein, and Owman in 1970 (7). The term *p-type* was used because the predominating type of vesicles in these nerve fibers resembled those of the neurosecretory nerve fibers in the neurohypophysis that were known to store peptides. Ultrastructurally, p-type nerve fibers differ from cholinergic fibers and adrenergic fibers by a predominance of fairly large electron-dense granular vesicles, sometimes referred to as "large opaque vesicles." In contrast, adrenergic nerve fibers are characterized by the presence of small electron-dense granular vesicles and cholinergic fibers by small electron-lucent vesicles (Figure 1). Subsequently, it has been shown that the p-type nerve fibers of the gut are not a uniform class. Several subpopulations exist, as has been shown by Gabella (54) and Cook and Burnstock (28); they are distinguishable by the size and morphology of their granular vesicles.

The Concept of Purinergic Nerves

In a number of publications Burnstock and co-workers (18, 19, 21, 22) have argued that the p-type nerve fibers are purinergic, i.e., they employ purine nucleotides, adenosine triphosphate (ATP) in particular, as transmitter. The concept is based on the fact that many nerve-mediated effects are non-cholinergic and non-adrenergic in nature (cf. 23,50) and that in several of these cases ATP mimics the effect of nerve stimulation. The presence of non-adrenergic, non-cholinergic nerve fibers is indicated if nervous stimulation of either extrinsic or intrinsic nerve fibers gives responses

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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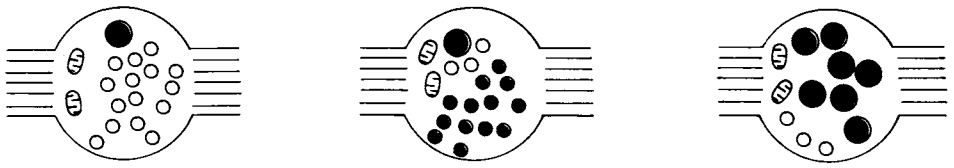


Figure 1. Schematic drawing of the ultrastructure of different types of autonomic nerve endings. Left, cholinergic; center, noradrenergic; right, "p-type." The cholinergic nerve profiles contain vesicles most of which are small and electron-lucent, whereas those of the adrenergic nerve profiles are predominantly small and electron-dense. The p-type nerve profiles are characterized by relative abundance of fairly large granules of varying electron density; they are sometimes referred to as LOV (large opaque vesicles).

remaining after cholinergic or adrenergic blockade or after sympathetic denervation, and if these responses are blocked by a low concentration of tetrodotoxin, which specifically blocks nervous conduction (83). In the early experiments carried out to identify the transmitter in the non-adrenergic, non-cholinergic nerve fibers of the gut, ATP was thought to satisfy best the criteria for a neurotransmitter (18). (1) Both ATP and the enzyme systems that synthesize ATP seem to occur in nerve cells. Tritium-labeled adenosine is taken up by specimens of the gut wall and rapidly converted to ATP (133). Most of the labeled ATP is stored in nerves (see ref. 18). (2) Stimulation of enteric nerve fibers after loading with tritiated adenosine results in release of tritium (86, 133). This release is reduced by tetrodotoxin (133). (3) Smooth muscle is highly sensitive to adenine nucleotides. The response to exogenous ATP resembles that to stimulation of non-cholinergic, non-adrenergic nerves (see, for instance, 33, 103, 104). Other transmitter candidates were rejected as contenders on the grounds that they were either inactive or did not mimic the nerve-mediated responses; that specific blocking drugs for these substances did not affect the nerve-mediated response; or that their action was mediated indirectly through stimulation of nerve fibers and not by a direct effect on smooth muscle (cf. 19).

Burnstock has argued that the purinergic nerves represent the main inhibitory system to the cholinergic excitatory system in the mediation of propulsive gut motility and that purinergic fibers are involved in reflexes that facilitate passage of material through the alimentary canal. Among functions thought to be controlled by purinergic nerve fibers are (1) reflex relaxation of the esophagogastric junction, (2) "receptive relaxation" of the stomach, (3) "descending inhibition" of peristalsis, and (4) reflex relaxation of the internal anal sphincter (cf. 23). There are also excitatory nerve fibers in the small intestine that are non-cholinergic and non-adrenergic. Also these excitatory fibers have been claimed to be purinergic (19). Nerve fibers that are neither adrenergic nor cholinergic have been shown to supply a variety of other organs (cf. 24). ATP mimics some of these nerve-mediated responses, whether inhibitory or excitatory in nature (cf. 14). Whether non-adrenergic, non-cholinergic nerve fibers outside the gut display the ultrastructural

features of p-type nerves is not known. Although the existence of a non-adrenergic, non-cholinergic neuronal system is generally accepted, the proposal that ATP is the transmitter is not (see, for instance, 10, 73, 81, 128).

Neuropeptides in Brain and Periphery

Burnstock's concept has undergone little modification since 1972, when it was first introduced. In the last four or five years, many other putative neurotransmitters have appeared that are competing with ATP for a transmitter role. Peptides in particular have attracted much interest. The number of peptides found in neuronal elements is rapidly increasing. Interestingly, many peptides known or thought to be hormones have been detected in the brain and in nerve fibers in the periphery (cf. 42, 67, 113, 136). Conversely, many brain peptides have been demonstrated in the periphery, in nerves or in endocrine cells, or in both. The distinction between neurons and endocrine cells is becoming increasingly vague. Initial observations concerned substance P in the gut wall (105, 109) and in primary sensory neurons (64, 68, 69), but substance P and other peptides are present in virtually all parts of the autonomic nervous system (cf. 66, 67, 136). Peptide-containing nerves are particularly numerous in the gut wall (see, e.g., 52, 136). Table 1 lists peptides demonstrated in the brain and Table 2, those found to have a neuronal localization in the gut. The following discussion will deal with substance P, vasoactive intestinal peptide (VIP), somatostatin, and enkephalin.

SUBSTANCE P

Substance P was the first putative neurotransmitter peptide. In 1931 von Euler and Gaddum (44) had already demonstrated the presence of substance

TABLE 1
Known or Suspected Peptides in the Brain

ACTH 1-39 (adrenocorticotrophic hormone)	MSH-release-inhibiting factor
ACTH 4-10	Neurophysins
Angiotensin	Neurotensin
Bombesin	Oxytocin
Bradykinin	Pancreatic polypeptide
CCK-8 (cholecystokinin)	pro-γ-MSH
Endorphins	Prolactin
Enkephalins	Secretin
Gastrin-17, gastrin-4	Sleep-inducing peptide
Growth hormone	Somatostatin
Glucagon (gut-type)	Substance P
Insulin	Thyrotropin-releasing hormone
Luteinizing hormone-releasing hormone	Thyroid-stimulating hormone
β-Lipotropin	Vasoactive intestinal peptide
α-MSH (melanophore-stimulating hormone)	Vasopressin

TABLE 2
Neuropeptides in the Gut

Substance P	Bombesin
VIP	Gastrin-4(?)[a]
Enkephalins	Pancreatic polypeptide(?)[a]
Somatostatin	Angiotensin(?)[a]
Neurotensin	Glucagon(?)[a]
CCK-8	ACTH(?)[a]

[a]The immunoreactive peptides labeled with the question mark have not yet been identified unequivocally.

P in extracts of small intestine by its contractile effect on the isolated rabbit jejunum. Substance P was also shown to be present in the brain. The finding of conspicuously large amounts of substance P in the dorsal root of the spinal cord (111) led Lembeck (94) to suggest that substance P is involved in the transmission of sensory information. Substance P was isolated and sequenced by Chang, Leeman, and Niall in 1971 (26) and found to be an undecapeptide. Substance-P-containing nerve fibers are found in most areas of the central nervous system (CNS), although they are comparatively scarce in the cerebral and cerebellar cortices. Areas particularly rich in substance P are the spinal cord (dorsal horns) and the substantia nigra. Substance-P-containing cell bodies occur in several areas of the CNS, including the spinal cord and the brain stem (95). The exact localization of substance P in the gut was clarified only recently when immunocytochemistry revealed it to have a dual localization, in endocrine cells in the gut epithelium and in nerve fibers in the gut wall (105, 109). Neuronal substance P in the gut (105, 109, 136) is found predominantly in relation to smooth muscle, in the myenteric plexus in particular (Figure 2). The distribution of substance P nerve fibers suggests that the main targets are smooth muscle cells and neurons (Table 3). However, substance-P-containing nerve fibers occur also around blood vessels and in certain species in the mucosal layer (137).

VASOACTIVE INTESTINAL PEPTIDE (VIP)

Isolated in 1970 from porcine small intestine (119), VIP is a 28-amino acid peptide, chemically resembling secretin, glucagon, and gastric inhibitory polypeptide (GIP). VIP was originally believed to be a gut hormone. The demonstration that it had a neuronal localization (17, 120) caused a reorientation of research on its physiological role. Radioimmunoassay revealed large amounts of VIP in the brain, with the highest levels in the cortex. VIP neurons are numerous in layers II–IV of the neocortex, where they constitute 1–5% of the cortical cell bodies. Most of the VIP neurons have fusiform cell bodies and dendrites oriented perpendicular to the pial surface, suggesting that they are interneurons. VIP cell bodies are also present in the

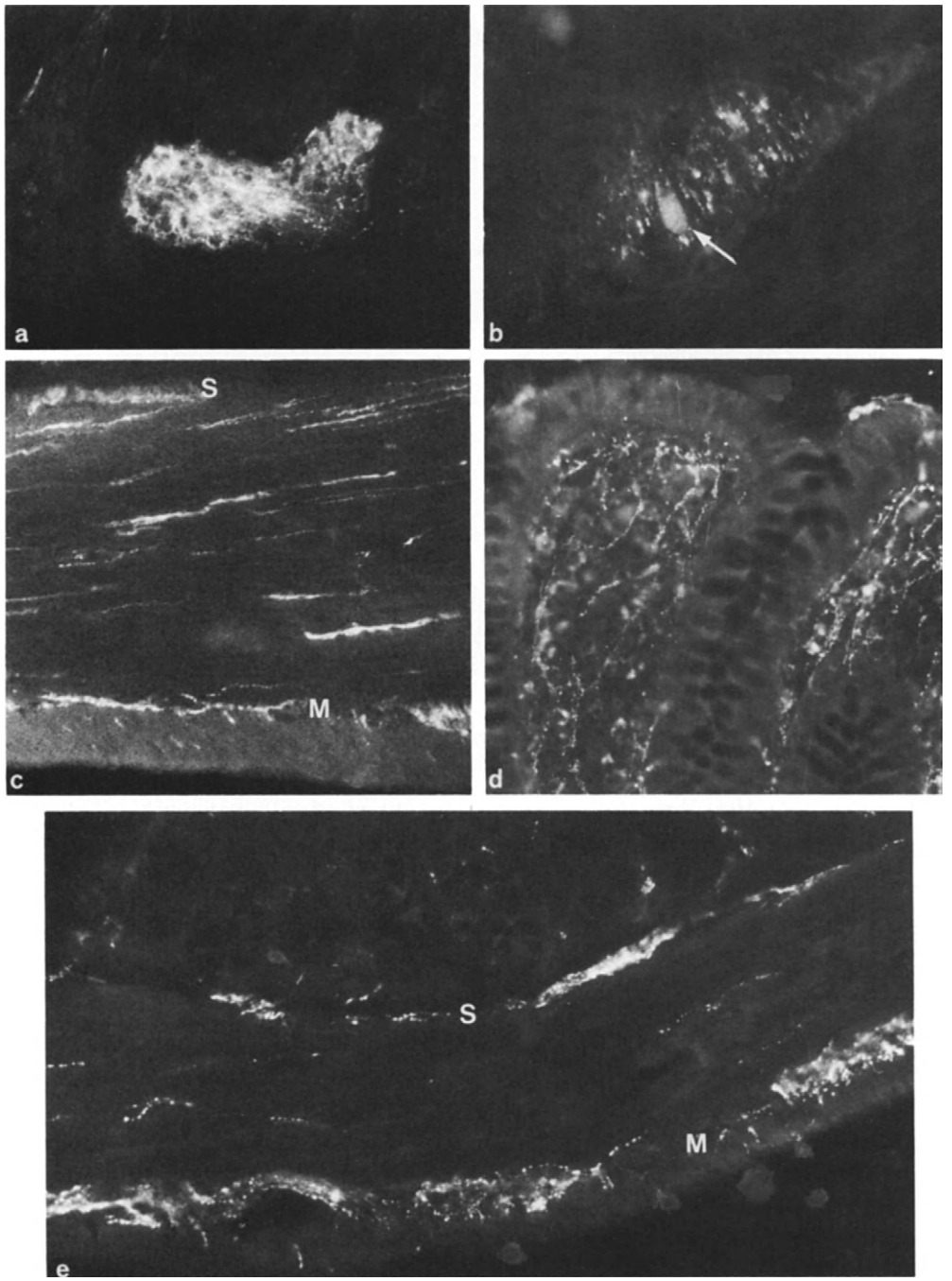


Figure 2. Peptide-containing nerve fibers and nerve cell bodies in the gut wall. (a) Guinea pig colon. Dense accumulations of substance P nerve fibers in the myenteric plexus. Single beaded fibers in the smooth muscle (×300). (b) Cat ileum. Numerous nerve fibers and a single nerve cell body (arrow) displaying enkephalin immunoreactivity in the myenteric plexus (×300). (c) Cat colon. VIP-immunoreactive cell bodies and nerve fibers in the submucous (S) and myenteric (M) plexus and in the smooth muscle (×150). (d) Human ileum. Dense network of VIP-immunoreactive fibers in the core of villi (×300) (e) Mouse colon. Somatostatin-immunoreactive nerve fibers in the myenteric (M) and submucous (S) plexuses. A few single fibers also in the smooth muscle and around the base of crypts (×150).

TABLE 3
Potential Targets for Neuropeptides in the Mammalian Gut

Substance P
Neurons (myenteric plexus)
Smooth muscle
Blood vessels
VIP
Neurons (myenteric and submucosal plexus)
Smooth muscle (especially sphincters)
Glands and surface epithelium
Blood vessels
Somatostatin
Neurons (myenteric and submucosal plexus)
Smooth muscle(?)[a]
Glands and surface epithelium
Enkephalin
Neurons (myenteric plexus)
Smooth muscle(?)[a]

[a]Targets labeled with a question mark denote morphological signs of innervation but lack of detectable effects.

hippocampal complex, amygdala, olfactory bulb, anterior olfactory nuclei, bed nucleus of the stria terminalis, lateral septum, suprachiasmatic nucleus, superior colliculus, and mesencephalic periaqueductal gray. On the whole, the distribution of VIP fibers parallels that of VIP cell bodies, suggesting that they project locally. Some VIP neurons also seem to form long projections, such as descending and transcallosal projections from cortical cells and connections from the amygdala to preoptic, hypothalamic, and basal forebrain area (cf. 96).

VIP neurons are also present in various autonomic ganglia, and VIP-containing nerve fibers occur in nerve trunks and in several peripheral organs (53, 78, 91, 99, 100). VIP-containing nerve fibers occur in all layers of the gut (91). Besides being numerous in the smooth muscle and the intramural plexuses, where VIP cell bodies are also found, VIP nerve fibers are abundant in the mucosa, where they run in the lamina propria often immediately beneath the epithelium (Figure 2). In the plexuses, varicose VIP fibers are seen to ramify around nerve cell bodies. From the distribution it seems that the potential targets for the VIP nerve fibers are epithelial cells, blood vessels, smooth muscle, and neurons (Table 3). Furness and Costa (51) reported that VIP occurs in fibers that originate from cell bodies in the myenteric plexus and project in an anal direction to innervate the circular muscle. This distribution is consistent with VIP neurons being inhibitory neurons forming the final link in the descending inhibitory pathway of the peristaltic reflex.

SOMATOSTATIN

Somatostatin was isolated from bovine hypothalami (12) by virtue of its ability to inhibit the release of growth hormone by the pituitary. It is a cyclic 14-amino acid peptide that has been found to inhibit the release process in various types of endocrine as well as exocrine cells (60). Somatostatin is found in several regions of the brain, notably in the median eminence. Experimental evidence suggests that somatostatin is synthesized in cell bodies of the preoptic and periventricular nuclei and then transported posteriorly (15, 16). Soon after its isolation, somatostatin was identified in endocrine cells of thyroid, pancreas, and gut and in nerve fibers of the digestive tract (6, 37, 63, 98, 110, 114, 117). In the gut wall, somatostatin-containing nerve fibers predominate in the myenteric and submucous plexuses (Table 3) where they are sometimes seen closely applied to nerve cell bodies (Figure 3). Somatostatin fibers regularly occur in the mucosa, where they form a network around intestinal crypts; occasionally they are found also in the smooth muscle.

ENKEPHALIN

Endogenous opioid peptides were isolated from pig brain by Hughes *et al.* (71) and found to consist of two pentapeptides, methionine^5-enkephalin (Met-enkephalin) and leucine^5-enkephalin (Leu-enkephalin). In the brain, enkephalin neurons are widely distributed. Dense accumulations of fibers occur in the dorsal horn of the spinal cord, in layers I and II, hypothalamus, globus pallidus, and parts of the limbic system (41, 65, 80). Enkephalin was also demonstrated by immunocytochemistry in peripheral nerve trunks and ganglia and in nerve fibers in several peripheral organs (cf. 3, 122, 123). In addition, immunoreactive enkephalin was detected in endocrine cells of the

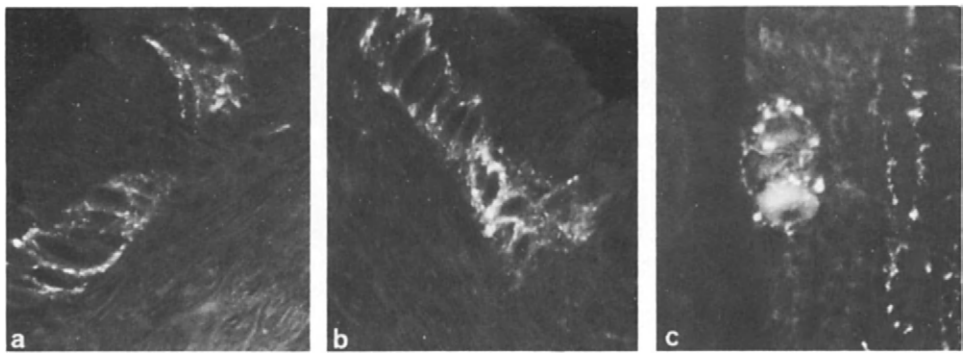


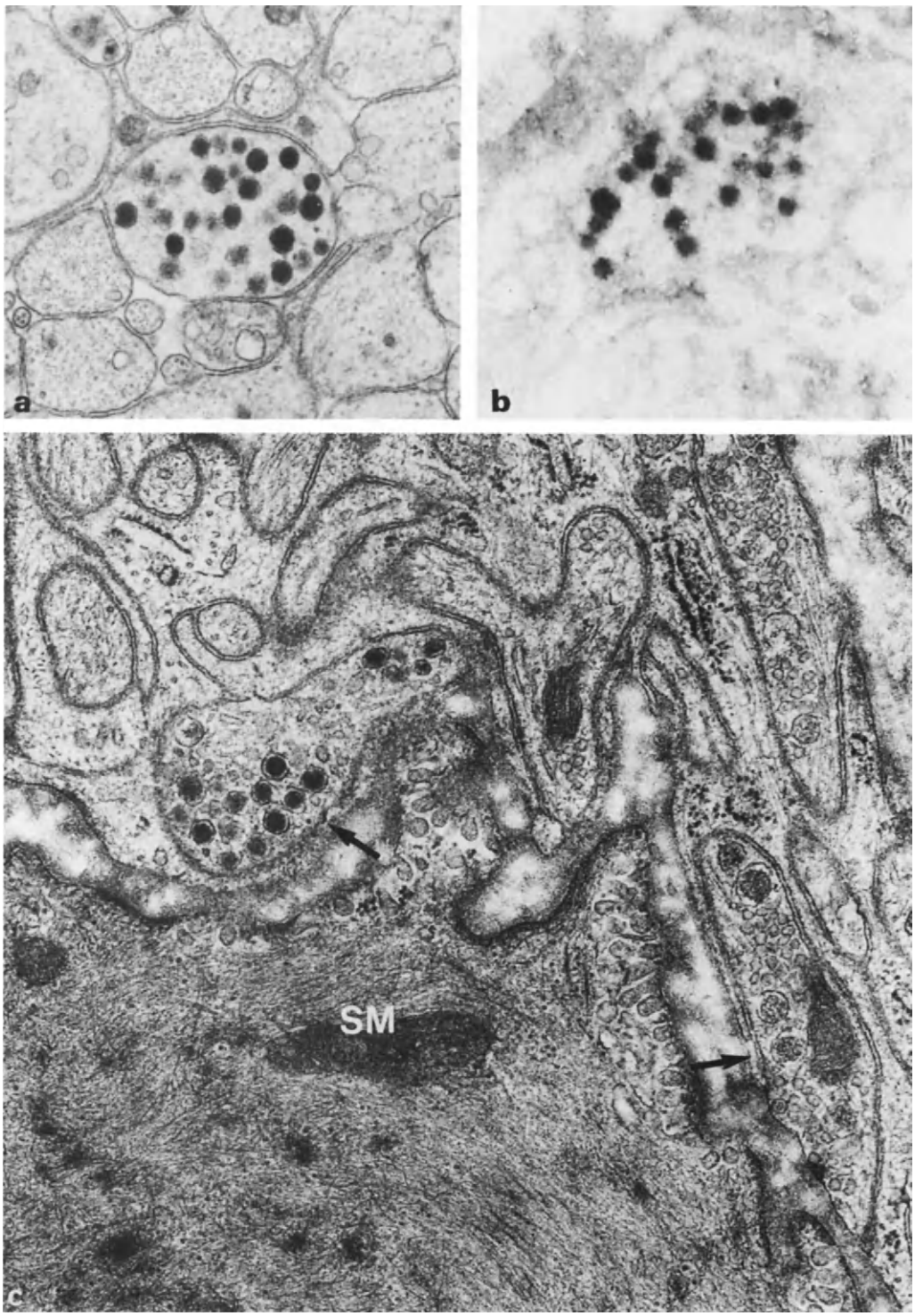
Figure 3. Delicate, varicose peptide-containing nerve fibers ramify around cell bodies in the intramural plexuses. (a) Substance P fibers in myenteric plexus of mouse colon. (b) Enkephalin fibers in the myenteric plexus of mouse colon. (c) VIP fibers in submucous plexus of human ileum. Note that occasionally VIP fibers surround VIP-immunoreactive cell bodies (×300).

gut (4) and in adrenal medulla (122, 124) and carotid body (3). Enkephalin nerve fibers in the gut wall have a topographic distribution very similar to that of substance P fibers, i.e., they predominate in the smooth muscle layer and in the myenteric plexus (Figure 3). Enkephalin-containing cell bodies occur in the myenteric plexus. From the distribution it appears that the potential targets for the enkephalin nerve fibers are smooth muscle cells and neurons (Table 3). Recently, Larsson and co-workers (90) provided evidence for the existence of separate Met-enkephalin and Leu-enkephalin nerve fibers with an allegedly overlapping distribution. In the gut wall, most areas showed great parallelism in the distribution of the two types of nerve fibers. The functional significance of this finding is obscure.

Peptides Occur in P-Type Nerves

Various peptides are now well-established neuronal constituents. However, peptide-containing nerve fibers do not seem to have unique characteristics that set them apart from other types of nerve fibers. Ultrastructural analysis has not revealed any fine structural feature exclusively characteristic of peptide-containing neurons. Even the large "opaque" granular vesicles that are often used to differentiate p-type nerves from other types of nerves may not be a unique feature (see 32); occasionally they are found also in adrenergic nerves. Conversely, it appears unlikely that neuropeptides are invariably stored in p-type nerve fibers. Neuropeptides are widely distributed in the brain, but p-type fibers are found only in the hypothalamus and median eminence. The subcellular localization of the various neuropeptides in the gut wall has been established by immunocytochemistry at the ultrastructural level. All data available from such studies (88, 92, 113, 136) support the view that the peptide-containing neurons are identical with the p-type neurons first described in the gut by Baumgarten *et al.* (7). The terminals of such neurons are characterized by the presence of fairly large granular vesicles (diameter around 120 nm) containing a core of varying, mostly high, electron density. In addition, such neurons usually also harbor small, empty vesicles of the type found in cholinergic neurons. An example of the fine structure of a peptide-containing nerve as revealed by electron microscopic immunostaining is given in Figure 4.

Figure 4. Ultrastructural properties of peptide-containing nerve fibers in the myenteric plexus of the gut wall. Formaldehyde-glutaraldehyde and osmium tetroxide fixation. (a) and (b) Cat pylorus. (a) Routinely stained section. Single p-type nerve profile. (b) Same nerve as that in (a) in an adjacent section stained for VIP using the immunoperoxidase technique of Sternberger (131). Heavy deposits of reaction product over the neurovesicles only. (c) Various types of nerve fibers (arrows) containing large, opaque or dense-cored vesicles running close to smooth muscle cell (SM) in cat esophagus. Approximate distance between nerve and smooth muscle membrane is 150–200 nm. Note the fingerlike invaginations of the plasma membrane of the smooth muscle close to the nerve profiles (×37,000).


a
b
SM
c

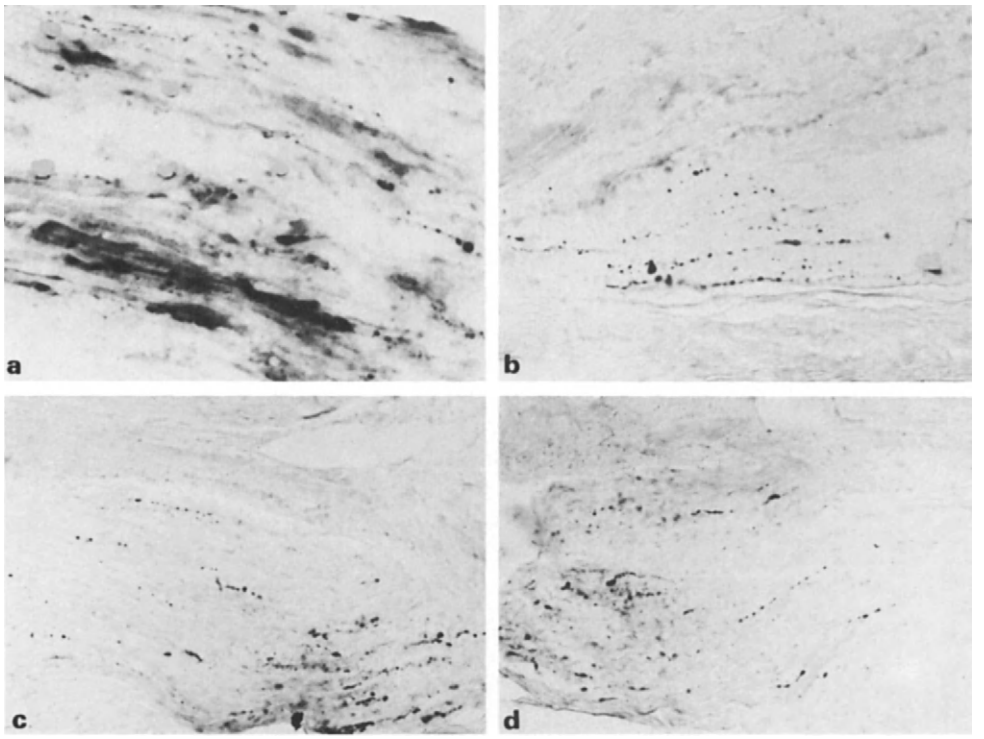


Figure 5. Substance P [(a) and (b)] and VIP [(c) and (d)] immunoreactive nerve fibers in the ligated vagus of the pig. (a) and (c) Accumulation of immunoreactive material above the ligature. (b) and (d) Some accumulation of immunoreactive material also below the ligature (×200).

Origin of Peptide-Containing Nerves in the Gut Wall

Where do the peptidergic nerve fibers originate? Are they intramural or do they derive from extrinsic sources such as the vagus? Figure 5 shows that the vagus is a rich source of peptide-containing nerve fibers (see also 55, 99, 100). On ligation of the porcine vagus nerve, there is a considerable accumulation of neuropeptides, notably substance P and VIP, in swollen nerve fibers both above and below the ligature (101). It is not yet known precisely what proportion, if any, of the nerve supply in the gut is derived from the vagus. Figure 6 illustrates an attempt to study this question (101). Segments of the jejunum in pigs were removed and transplanted back into the animal with one end closed blindly and the other end joined to the abdominal wall by a jejunostomy. In this way all extramural nerve fibers were eliminated. Histochemical examination revealed that the adrenergic nerve supply was lost in the autotransplant, whereas the acetylcholinesterase-positive and peptidergic nerve supplies were unaffected. These results were corroborated also by chemical determination of substance P, the concentration being 5.7

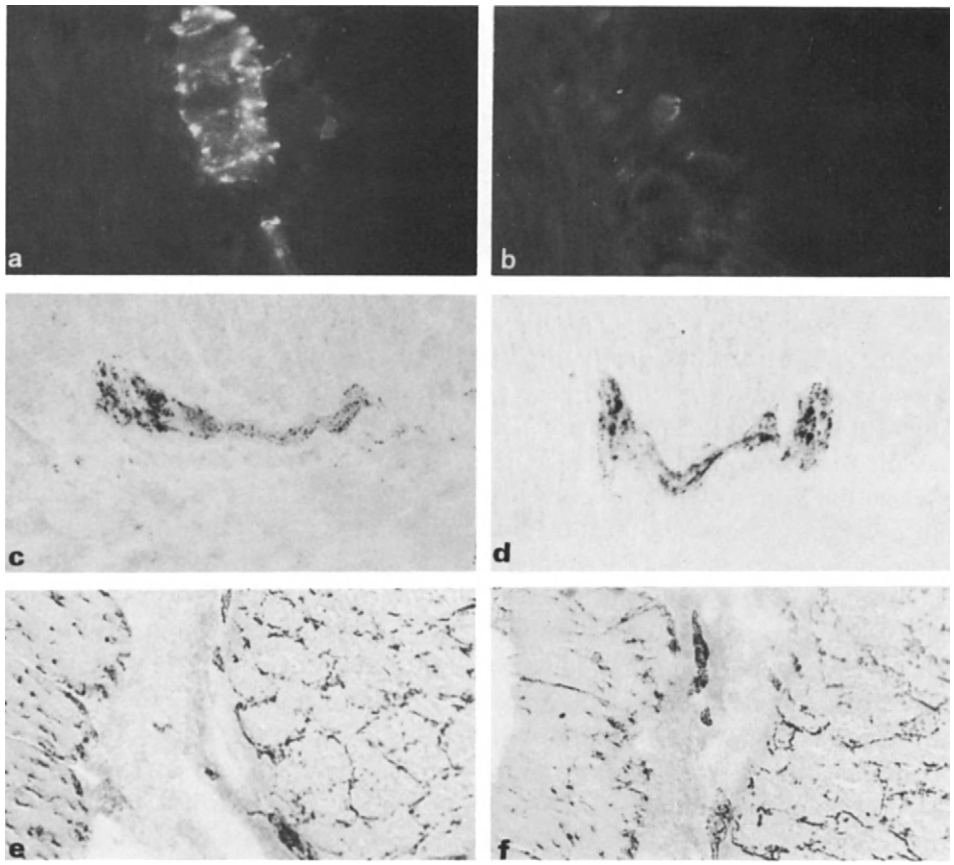


Figure 6. Intramural innervation of intact porcine jejunum (left) and autotransplanted jejunum (right). Top: Adrenergic nerves demonstrated by their formaldehyde-induced fluorescence within the myenteric plexus. After extrinsic denervation no adrenergic nerves were demonstrable (×250). Middle: VIP nerve fibers in the myenteric plexus of intact jejunum and after extrinsic denervation (×300). Bottom: Acetylcholinesterase (AChE)-containing nerve fibers in intact jejunum and after extrinsic denervation. Mucosa to the right (×150). Note seemingly unchanged number of VIP fibers and AChE-containing fibers after extrinsic denervation.

pmol/g ± 2.0 (S.E.M., $n = 5$) in jejunum *in situ* and 7.7 ± 2.2 (S.E.M., $n = 5$) in the autotransplant (101). Taken together, the results suggest that in the porcine gut the contribution of peptide-containing nerve fibers from extrinsic sources such as the vagus is minor despite the fact that the vagal trunks are rich in such nerve fibers. Similar conclusions have been drawn from studies on gut segments grown in tissue culture (121). However, there may be species differences. In a recent study of guinea pig ileum, Costa *et al.* (29) found substance P-containing fibers associated with blood vessels to originate outside the intestine; vascular VIP nerves, on the other hand, were of intrinsic origin (30).

FUNCTIONAL ROLE OF NEUROPEPTIDES

There is a good deal of evidence that the peptide-containing neuronal systems of the gut function within the context of the autonomic nervous system. It is generally believed that the intramural neurons modulate the response to the extrinsic nerve impulse flow and that they are involved in local reflex mechanisms as sensory neurons, interneurons, or motor neurons. The nervous supply of the gut consists principally of two ganglionated plexuses, the myenteric and the submucous. The myenteric plexus is primarily concerned with peristaltic activity, whereas the submucous plexus is thought to control absorptive and secretory functions. The details of how the various neuronal systems in the gut are morphologically and functionally integrated are still lacking. They seem to form a continous communication system that encompasses the entire digestive tract, enabling impulse propagation from its most proximal to its most distal parts. Peptide-containing neurons are also involved in the communication between the myenteric and submucous plexuses; there is evidence for a VIP-containing pathway running from the submucous neurons to innervate neurons in the myenteric plexus as well as for a substance P-containing pathway in the reverse direction (79). Thus the systems of peptide-containing neurons provide the morphological basis for the functional coordination and autonomy of the gut. In the following are summarized data on the actions of the various neuropeptides found in the gut. When the proposed actions of the various peptides are viewed with the distribution of the peptide-containing nerve fibers in mind, it is tempting to argue that the peptides are strong candidates for serving a neurotransmitter role.

SUBSTANCE P

There is little doubt that substance P serves a transmitter role in the CNS. Particularly strong is the evidence that substance P is an excitatory transmitter of primary afferent neurons (108; see also 64, 68, 69). From the distribution of substance P fibers in the periphery it cannot be excluded that they may also be efferent in nature. Potential targets for peripheral substance P nerve fibers as judged by their distribution are listed in Table 3. What actions to look for may be suggested by listing some of the more potent peripheral effects of substance P: vasodilatation, contraction of nonvascular smooth muscle, and stimulation of salivary and pancreatic secretion (Table 4). The original observations by von Euler and Gaddum (44) already indicated that gut smooth muscle was an important target for substance P, and it has been shown that very low concentrations of synthetic substance P cause contraction of gut smooth muscle *in vitro* (13, 116, 139, cf. 25). Studies on the guinea pig ileum have shown substance P to be more potent than, e.g., acetylcholine, histamine, and serotonin (116). Substance P mimics the contractile component of the response to electrical field stimulation of guinea

TABLE 4
Peripheral Effects of Exogenously Applied Peptides

Substance P
Vasodilatation
Contraction of nonvascular smooth muscle
Stimulation of salivary and pancreatic secretion
VIP
Vasodilatation
Relaxation of nonvascular smooth muscle
Stimulation of pancreatic bicarbonate secretion
Stimulation of bile flow
Stimulation of intestinal secretion
Inhibition of gastric acid secretion
Stimulation of insulin and glucagon release
Stimulation of glycogenolysis and lipolysis
Somatostatin
Inhibition of secretion from exocrine glands
Inhibition of secretion from endocrine cells
Inhibition of transmural stimulation of smooth muscle
Enkephalin
Inhibition of transmural stimulation of smooth muscle

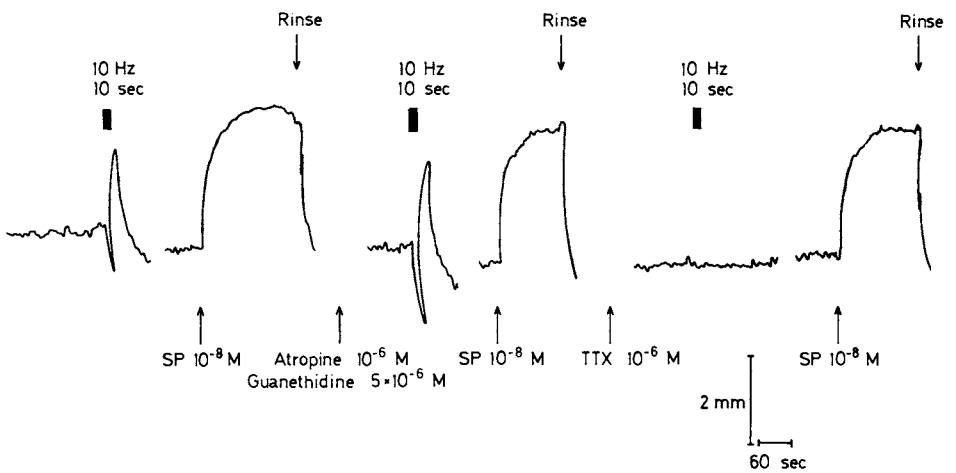


Figure 7. Motor activity of smooth muscle from the isolated cecum of chicken. Electrical field stimulation caused relaxation followed by contraction. Substance P (SP) also contracted the muscle. Cholinergic and adrenergic blockade by atropine and guanethidine had no effect on the response either to electrical stimulation or to substance P. The response to electrical stimulation but not that to substance P was blocked by tetrodotoxin (TTX), which indicates that the motor effects were caused by the nerve stimulation and that the muscle cells responded to substance P directly. The possibility cannot be excluded that SP is the neurotransmitter responsible for the contraction induced by electrical stimulation.

pig taenia coli following cholinergic and adrenergic blockade (92). Figure 7 illustrates the effects of substance P on gut smooth muscle, in this particular case the cecum from chicken (13). Since the effect of substance P was unaffected by tetrodotoxin as well as by cholinergic-, adrenergic-, histaminergic-, and serotoninergic-blocking drugs, the action of substance P on intestinal smooth muscle may be mainly a direct one. Findings *in vivo* (112) and *in vitro* (62) suggest that the actions of substance P on the gut might be twofold: a direct effect on the smooth muscle cells and a neurotropic effect involving stimulation of afferent nerve fibers in the peristaltic reflex arc (Figure 8). Such a neurotropic effect is also suggested by the presence of varicose substance P fibers around most ganglion cells in both the submucous and myenteric plexuses.

Further, substance P is a potent vasodilator (cf. 39). Among the vascular beds sensitive to substance P is the splanchnic area (61). Substance P has also been shown to increase secretion from various exocrine glands (cf. 93, 138).

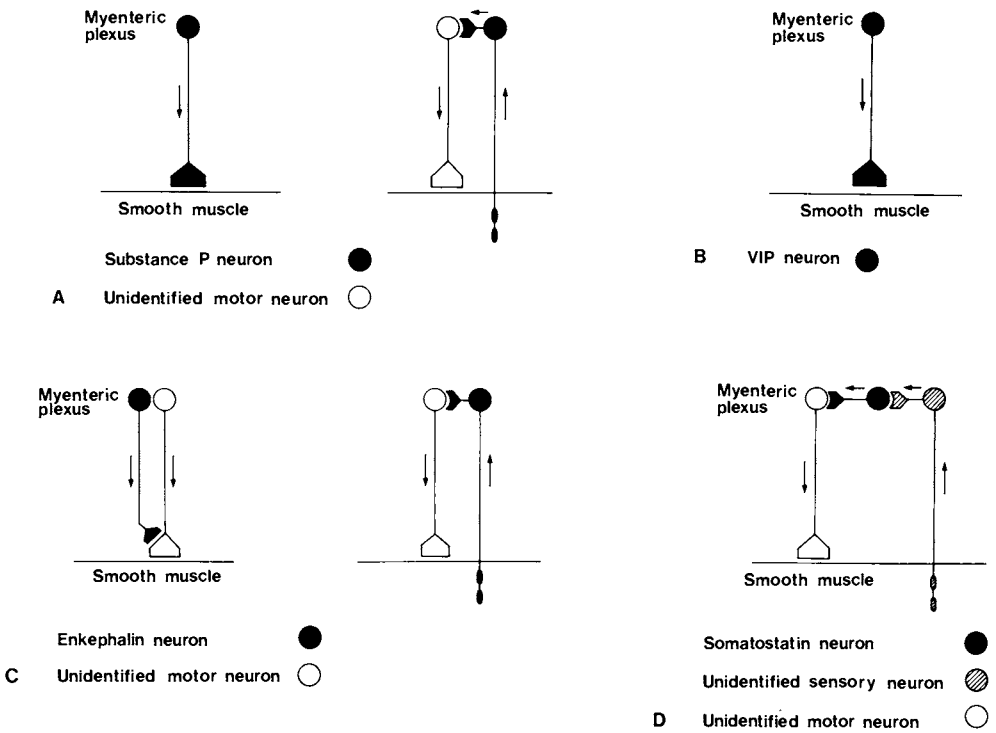


Figure 8. Proposed roles for various types of peptide-containing neurons in the control of motor activity in gut smooth muscle. (A) Substance P neurons are motor neurons (excitatory) and/or sensory neurons (excitatory). (B) VIP neurons seem to be motor neurons (inhibitory). (C) Enkephalin neurons are sensory neurons (inhibitory) or interneurons (inhibitory) acting via axo-axonal mechanisms (which might explain why enkephalin nerve fibers are found in the smooth muscle). (D) Somatostatin neurons are interneurons (inhibitory) in the myenteric plexus.

The fact that substance P-containing nerve fibers are present around blood vessels and in glands such as salivary glands (Sundler *et al.*, to be published) and pancreas (89) suggests that these effects also may be of physiological importance.

VASOACTIVE INTESTINAL PEPTIDE (VIP)

The VIP neurons in the upper respiratory, digestive, and genitourinary tracts seem to innervate blood vessels, smooth muscle, and epithelial cells (1, 91, 134, 135, 140, 141, 143). VIP has very diverse biological actions. The actions are summarized in Table 4 and include relaxation of smooth muscle (especially sphincters), vasodilatation, and augmentation of pancreatic and intestinal secretory activity.

VIP nerves are numerous in salivary glands (143). They are found both around glandular acini and blood vessels and they are particularly numerous beneath the ductal epithelium (Figure 9). In the submandibular gland, VIP cell bodies are found in ganglia located in the hilus region near the main duct. Stimulation of the chorda lingual nerve evokes an atropine-resistant vasodilatation (cf. 8, 56). This effect is mimicked by intra-arterially administered VIP (125). Further, stimulation of the chorda lingual nerve causes release of VIP from salivary glands (Figure 10) (cf. 9, 143).

Exogenous VIP mimics the effects of nerve stimulation on a number of functions in the digestive tract. Such effects include local intestinal vasodilatation (40) and increased pancreatic bicarbonate secretion (49). These effects of nerve stimulation are refractory to cholinergic and adrenergic blockade (Figure 11). A local release of VIP from these organs occurs on nerve stimulation concomitantly with the physiological response to the stimulation (47; cf. 45, 46).

VIP nerve fibers are characteristically numerous in the smooth muscle of sphincters, such as the lower esophageal and pyloric sphincters (2). VIP effectively relaxes both sphincters (38, 102, 115, 141), and the infusion of antibodies to VIP has been found to inhibit relaxation of esophagus following nerve stimulation (59). The model we would like to suggest for VIP neurons in the myenteric plexus of the gut is illustrated in Figure 8, where they are shown as motor neurons. The fact that varicose VIP fibers are numerous beneath the intestinal epithelium and around nerve cell bodies, including VIP-immunoreactive ones, in the myenteric and submucous plexuses and in autonomic ganglia outside the gut suggests additional roles for the VIP neurons, including a neuromodulatory one. In fact, VIP was recently shown to excite neurons of the myenteric plexus (147).

SOMATOSTATIN

The distribution of somatostatin-containing nerve fibers in the myenteric and submucous plexuses of the gut wall suggests a neuromodulatory role; delicate, varicose fibers are seen closely applied to nerve cell bodies within

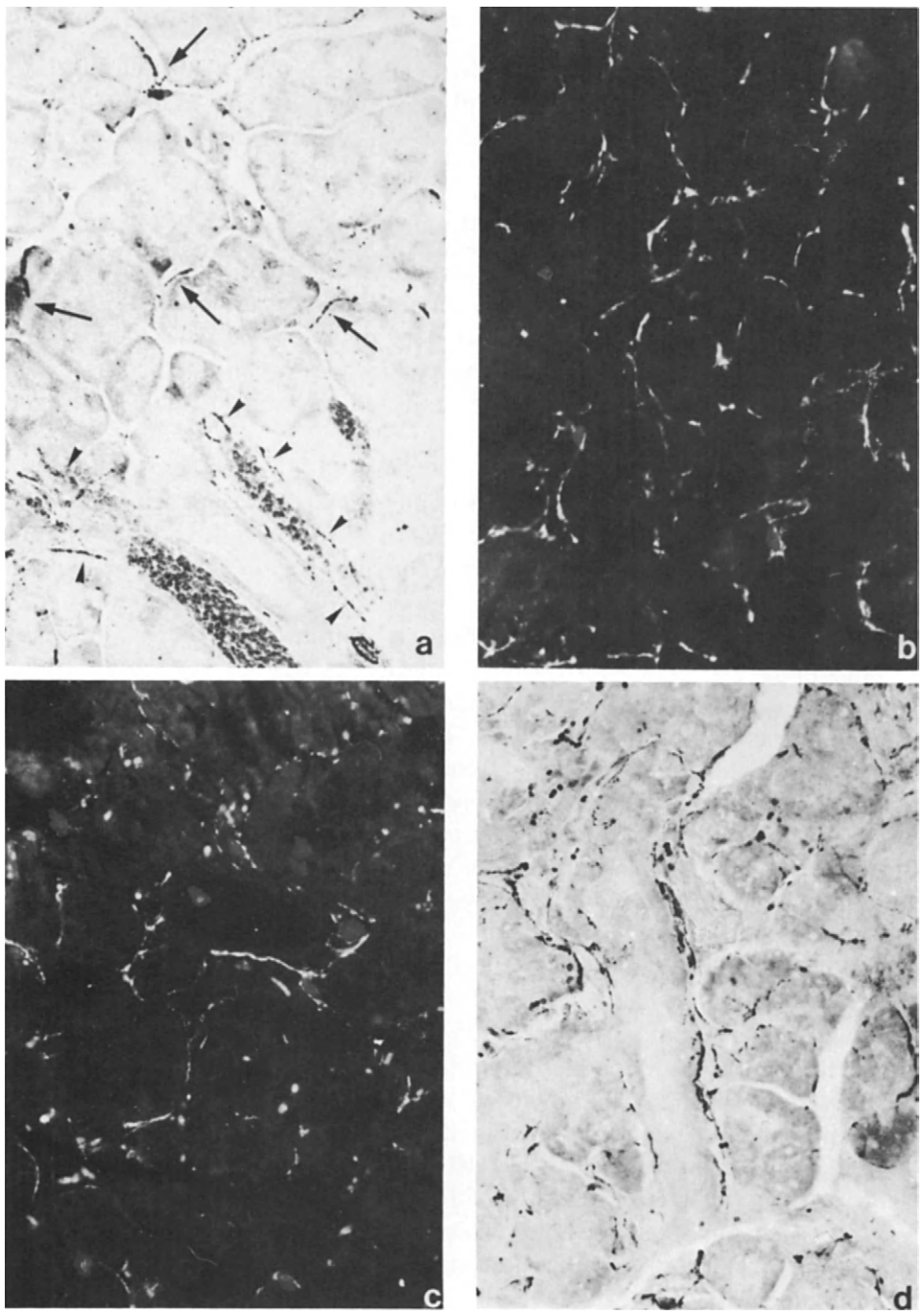


Figure 9. VIP nerve fibers in salivary glands. (a) Rat parotid gland. Few scattered fine varicose VIP nerves in the wall of blood vessels (arrow heads) and close to acini (arrows). (b–d). Cat sublingual (b), submandibular (c), and parotid (d) glands. Rich supply of VIP nerves around acini, salivary ducts, and blood vessels. (a) and (d) PAP staining; (b) and (c) immunofluorescence (×200).

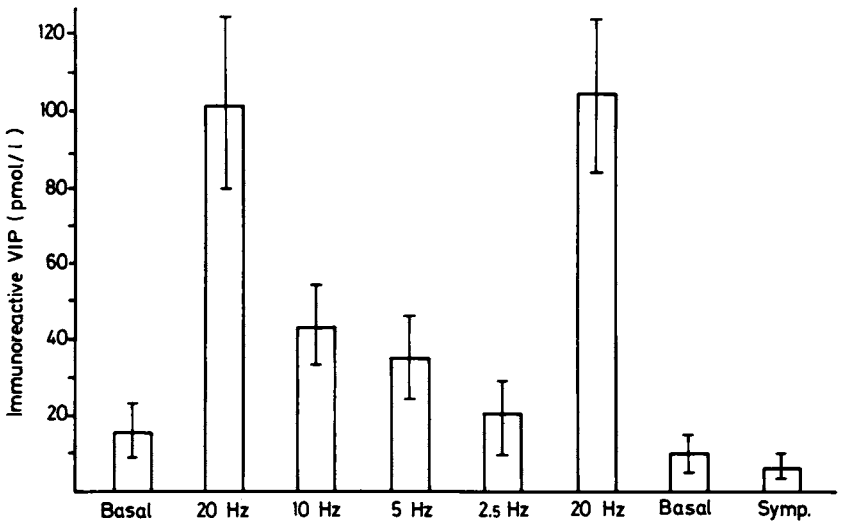


Figure 10. Plasma VIP in venous effluent from the submandibular gland of six cats during stimulation of the chorda lingual nerve or the cervical sympathetic nerve and during unstimulated periods (basal). Vertical bars give S.E.M. The concentration of VIP was increased at stimulation with 20 Hz compared to basal values ($p < 0.01$) and also to values obtained with stimulation at lower frequencies ($p < 0.05$). Stimulation was performed in sequence as indicated in the figure. (For further details see 143.)

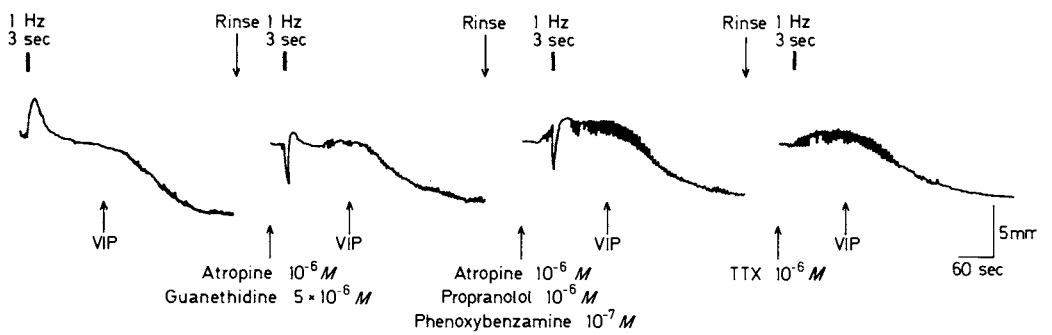


Figure 11. The effect of electrical field stimulation and of VIP (5×10^{-8} M) on the resting tension of the isolated guinea pig taenia coli. Electrical stimulation (1 Hz for 3 sec) evoked contraction, whereas VIP gave relaxation. Following cholinergic and adrenergic blockade the muscle responded with relaxation to both VIP and electrical stimulation. The response to electrical stimulation but not that to VIP was blocked by tetrodotoxin (TTX), which indicates that the motor effects were caused by the nerve stimulation and that the muscle cells responded to VIP directly. The possibility cannot be excluded that VIP is the neurotransmitter responsible for the relaxation induced by electrical stimulation. Note, however, the slow onset of action of VIP compared with the response to electrical stimulation. This may reflect diffusion barriers in the preparation. A neuronal release of VIP giving high local concentrations may give effects along a time scale quite different from that seen after the administration of VIP to the bath.

the plexuses. The biological action of somatostatin seems to involve inhibition of various release processes (see 60), perhaps including inhibition of the release of neurotransmitters (Table 4). So far, electrophysiological and pharmacological experiments have not clarified the role of neuronal somatostatin in the periphery. Studies on the guinea pig taenia coli have shown that somatostatin has no direct myogenic effect (92). Instead somatostatin seems to inhibit the firing of myenteric neurons (146). The somatostatin-containing neurons in the myenteric plexus of the gut wall may thus be interneurons (Figure 8). The presence of somatostatin-containing fibers in the mucosa, where they surround the basal portions of the crypts, suggests additional functions of neuronal somatostatin in the gut.

ENKEPHALIN

Enkephalin nerve fibers are numerous in smooth muscle and in the myenteric plexus. The enkephalins do not seem to have a direct effect on the smooth muscle cells, and it has been suggested that they inhibit transmural

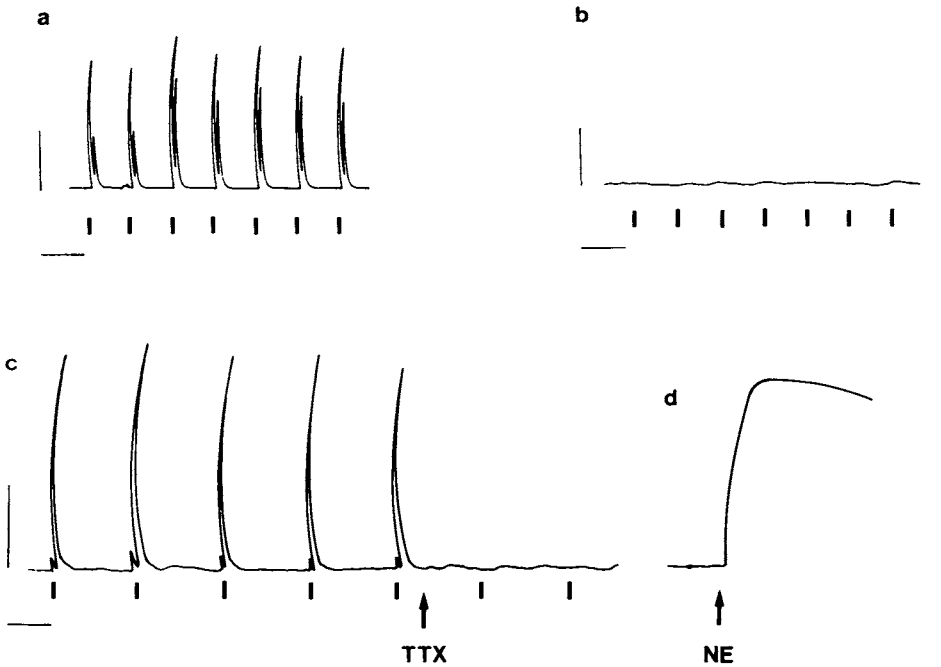


Figure 12. The response to electrical field stimulation of a circular smooth muscle strip taken from the isolated cat esophagus 1 cm above the esophagogastric junction (a). In specimens from a reserpinized cat there were no responses to electrical stimulation (b). Tetrodotoxin (TTX) in the bath was found to block the contractile responses, indicating that they were neurogenic rather than myogenic in nature (c). Norepinephrine (NE, 5×10^{-7} *M*) evoked a contractile response (d). Like the effects of electrical stimulation, the contractile response to norepinephrine was blocked by α-adrenergic blockers (142). Pulse duration: 1 ms; length of stimulation periods: 5 s (indicated by rectangles); frequency: 4 pps; supramaximal voltage. Vertical bar: 1000 dynes, horizontal bar: 1 min.

stimulation of smooth muscle (85) (Table 4). Waterfield *et al.* (144) showed that, like morphine, the enkephalin suppressed electrically evoked contractions of the guinea pig ileum and mouse vas deferens. The effect of enkephalins on electrically evoked inhibitory nonadrenergic nerve responses of the guinea pig taenia coli and stomach was studied by Huizinga and Den Hertog (72). They found that enkephalin did not affect the resting tension or the membrane potential of the smooth muscle (measured by the sucrose-gap method). They concluded that the enkephalins are unlikely to be responsible for the nonadrenergic inhibition evoked by transmural stimulation. Uddman *et al.* (142) studied the motor effect of enkephalin on segments from circular smooth muscle of cat esophagus. Figure 12 shows the response to electrical field stimulation of the lower esophageal sphincter. There was a short-lasting contraction when the electrical stimulation started and another one when the stimulation was ended. Both the on and the off responses were abolished by tetrodotoxin, suggesting that they were neuronal. Neither of them were blocked by atropine or hexamethonium, but they were blocked by an α-blocker, phentolamine, and by guanethidine, which blocks transmitter release in adrenergic nerves, suggesting an adrenergic involvement in the response to electrical stimulation. In specimens taken from reserpinized cats, deprived of norepinephrine, there was no response to electrical stimulation. Norepinephrine per se induced contraction. The enkephalins inhibited the responses to electrical stimulation and this effect was counteracted by naloxone (Figure 13). The contractile effect of norepinephrine was unaffected by enkephalin in the bath. The results suggest that the enkephalin nerve

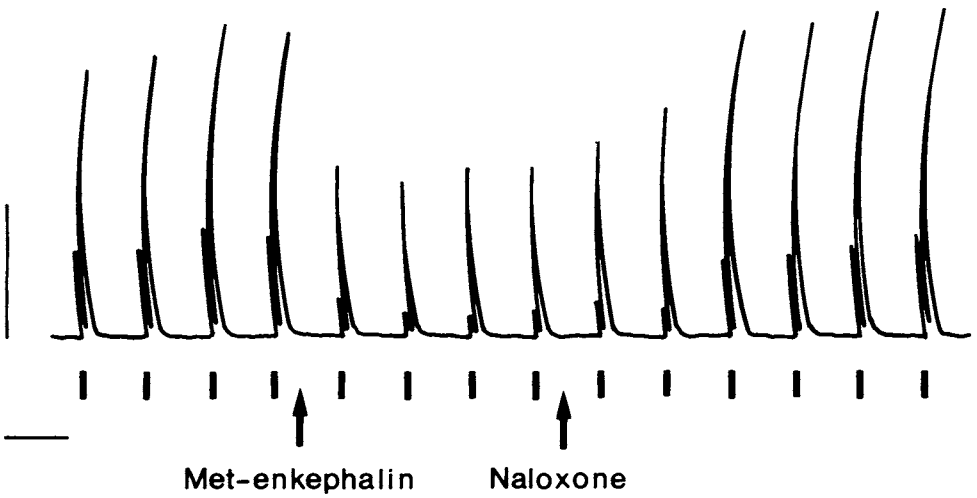


Figure 13. Both Leu- and Met-enkephalin blocked the contractile response of esophageal smooth muscle to electrical stimulation. In this tracing Met-enkephalin-induced (1.7×10^{-7} *M*) inhibition was counteracted by the opiate antagonist naloxone (7.6×10^{-8} *M*). Pulse duration: 0.6 ms; length of stimulation periods: 5 s; frequency: 2 pps; supramaximal voltage. Vertical bar: 5000 dynes; horizontal bar: 1 min.

fibers are involved in the regulation of smooth muscle activity and that the enkephalins act by inhibiting the firing rate of the motor neuron involved, in this case with norepinephrine as the final messenger (142). It has been shown previously that endogenous opiates inhibit the release of other transmitters, catecholamines as well as acetylcholine (43, 84, 144). North *et al.* (106) made intracellular recordings from neurons in the myenteric plexus and found that the enkephalins caused hyperpolarization of somewhat less than half of the neurons, but only if enkephalin was applied to the neurons at some distance from the cell bodies. Figure 8 shows two alternative models that try to explain the fact that enkephalin nerve fibers are numerous in the smooth muscle layer while having no direct effect on the activity of the smooth muscle cells. Either the enkephalin nerve fibers accompany the axons of the motor neuron modulating its functional activity presynaptically by axo-axonal mechanisms (analogous to these recently described in the neurohypophysis (76)) or they are dendrites of sensory neurons inhibiting the firing rate of the motor neurons. The recent finding of separate Met- and Leu-enkephalin-containing neurons in the gut (90) is difficult to understand in view of the fact that Met- and Leu-enkephalin seem to have identical actions. The identity of the immunoreactive material in these two neuronal systems has not yet been fully established. Perhaps one of the two neuronal systems contains components distinct from the conventional enkephalins, as has recently been shown, for instance, in extracts of hypothalamus (70, 82, 130) and adrenal medulla (130, 149). Thus a portion of the enkephalin-immunoreactive nerve fibers in the gut wall may contain a peptide chemically related to, but distinct from, the known enkephalins, and having a different function altogether.

ARE THE NEUROPEPTIDES TRANSMITTERS?

In examining the possible role of peptides as neurotransmitters we must first review what is meant by a neurotransmitter and what criteria should be fulfilled before ascribing such a function to a given substance. Neurotransmitters are stored in nerve endings in high concentration to be released on nerve stimulation, thereby altering the activity of adjacent cells. The criteria for accepting a compound as a neurotransmitter have been discussed by Werman (145) and more recently by Orrego (107). According to these authors the primary criteria are (1) storage in neurovesicles, (2) induced release, and (3) identity of action. A transmitter candidate should be stored in specific cytoplasmic organelles, vesicles, within the nerve terminal. Nerve stimulation should result in a release of the transmitter candidate in proportion to the magnitude of the stimulation. A transmitter candidate should be able to reproduce the postsynaptic effects of the natural transmitter. On the whole, available evidence favors the view that the neuropeptides are transmitters. They are stored in vesicles in nerve terminals (cf. 58) which at least in the gut display the ultrastructural features of p-type nerves (88, 92, 136). They are released by nerve stimulation into the venous

plasma effluent (47, 48). In many instances their effects mimic those of nerve stimulation. Some neuropeptides, however, have effects that are less clear-cut or that differ—for instance, in latency—from those of nerve stimulation (see, for instance, 27). Their identity as "true" neurotransmitters has therefore been questioned. Instead it has been suggested that they are "neuromodulators," modifying the responses to "true" neurotransmitters. This may merely indicate that the neuropeptides affect their targets somewhat differently from the conventional neurotransmitters. If a chemical is confined to neurovesicles, is released on depolarization, and is capable of altering the activity of adjacent cells, it seems justified to use the term *neurotransmitter*. Although there is experimental evidence that at least some of the neuropeptides act as neurotransmitters, all of the neuropeptides have not yet been tested for all these criteria. Admittedly, therefore, the exact physiological role of the neuropeptides is still a matter of speculation. Our present state of ignorance is also due to the lack of drugs that can influence specifically peptide-induced events at synapses.

P-Type Nerves: Peptidergic or Purinergic?

Knowledge of peptidergic neurons is still in its infancy, in contrast to the voluminous literature on the biochemistry, electrophysiology, and morphology of adrenergic and cholinergic nerves. Nevertheless there is growing evidence that the neuropeptides have a transmitter role. What about the nucleotides? As late as 1979 a select group of workers in neurohistochemistry and neurophysiology agreed that ATP is a strong candidate for being the transmitter in "most non-adrenergic, non-cholinergic nerves that directly innervate smooth muscle" (21). As late as 1980, non-cholinergic and non-adrenergic transmission in the guinea pig taenia coli was discussed in terms of purine nucleotides exclusively, with no reference being made to peptide-containing nerves (77). Nonetheless, there are several arguments against the concept of purinergic nerves: (1) Rather than being confined to the neurovesicles of p-type nerves, ATP has a ubiquitous occurrence in the body. It is not inconceivable that ATP occurs in the granules of all types of peptide hormone-producing endocrine cells and in the vesicles of all types of nerves including cholinergic and adrenergic. ATP is located together with acetylcholine (ACh) in synaptic vesicles of cholinergic nerves supplying the electric organ of torpedine rays (11, 36, 74, 75, 148, 150), and it has been shown that ATP is released together with ACh from the phrenic nerves in the rat diaphragm (126, 127). ATP is also released together with catecholamines from adrenal medullary vesicles in perfused adrenal glands (34, 35, 132), and it seems likely that ATP is released together with norepinephrine from adrenergic nerves (57, 86, 129, 133). A ubiquitous occurrence of a chemical appears poorly matched with an important, specialized role in neurotransmission. (2) Further, the possibility cannot be excluded that the release of ATP on nerve stimulation is from muscle cells, or

possibly from all kinds of autonomic nerves, rather than from "purinergic" nerves exclusively (86, 87, 118). (3) Finally, the responses to stimulation of non-cholinergic, non-adrenergic nerve fibers are many and varied. ATP is not capable of reproducing all these diverse responses (see, for instance, 31, 97), and ATP is actually claimed to be a poor relaxant of smooth muscle, on a molar basis, compared with norepinephrine (128). The urinary bladder responds with contraction to stimulation of non-cholinergic and non-adrenergic nerves (5). ATP has been proposed as the transmitter (24). Recently, however, it was shown that ATP inhibits the contraction induced by nerve stimulation (31). Analogues and metabolites of ATP had the same effect. These findings do not support the concept of purinergic transmission.

The recent immunochemical and immunocytochemical demonstration that several biologically active peptides occur within nerve cell bodies and nerve fibers of the gut wall (and in other peripheral organs) has stimulated the idea that the non-adrenergic, non-cholinergic nerves of the gut wall are "peptidergic" rather than "purinergic" (10, 73). A number of physiological observations have supported this view. In fact, at present, the neuropeptides seem to be stronger contenders for a neurotransmitter role than the purine nucleotides on the following grounds: (1) Besides fulfilling major criteria for neurotransmitters, the neuropeptides (unlike ATP) seem to be exclusively located in neurovesicles of specific populations of nerves. (2) Each peptide has a characteristic bioactivity profile; together they display a wide range of biological actions. Hence the system of peptide-containing neurons in the periphery should possess a high degree of specificity—ensured by the characteristic distribution pattern of the various peptide-containing nerves and by the specific actions of the neuropeptides—and a high degree of versatility—ensured by the fact that the many neuropeptides offer such a great variety of bioactivity profiles.

The information transmitted from one nerve cell to another nerve cell or to an effector cell may turn out to be much more detailed and complex than hitherto recognized. Perhaps the belief that neurotransmitters act in only two ways—by simple yes or no signals to excite or to inhibit the postsynaptic cell—is an oversimplification. The amount of information that can be stored in peptides is much greater than that stored in traditional transmitters, such as catecholamines, 5-hydroxytryptamine, and acetylcholine. Accordingly, the complexity of the information conveyed by peptides may be at a different level altogether from the simple commands presented by, for instance, amines and choline esters. The situation is made even more complex by the fact that each neuron may release an array of bioactive chemicals; several messenger peptides may well be stored and released together as fragments resulting from the proteolytic processing of a large precursor molecule; in addition, there is evidence that neuropeptides at times are stored together with amines (cf. 67). The possibility that several bioactive chemicals occur jointly in a neuron suggests the existence of multitransmitter systems. The

"one neuron, one transmitter" concept, also referred to as Dale's principle, may therefore turn out to be untenable in the future (for a discussion see 20). However, our present knowledge does not exclude the interpretation that, of the released material, only one single molecular species represents the principal transmitter, whereas the rest are merely co-transmitters (see 107) with no or subordinate roles in neurotransmission.

SUMMARY

P-type nerves is a term originally used by electron microscopists to classify a population of peripheral autonomic nerves that is distinct from adrenergic and cholinergic ones. Ultrastructurally, p-type nerves contain predominantly large, more or less dense-cored vesicles or granules of a type seen in peptide-storing neurosecretory cells and fibers of the hypothalamus and neurohypophysis. A number of electrophysiological and pharmacological observations support the concept of autonomic nerves that are distinct from adrenergic and cholinergic ones. Nucleotides, notably ATP, have been suggested to serve a neurotransmitter role in non-cholinergic, non-adrenergic inhibitory nervous mechanisms; the mass of evidence accumulated, albeit circumstantial, is impressive. Hence *p* in *p-type nerves* has long stood for *purinergic*. With the recognition that a variety of peptides are neuronal constituents not only in the brain but also in the periphery, the term *p-type nerves* is gradually beginning to take on its original meaning—*peptidergic nerves*.

Available evidence suggests that the vast majority of peptide-containing nerve fibers in the gut wall are intramural in origin. Conceivably, they are links in short reflex arcs, exert a modulating influence on the extrinsic nervous impulse flow, or convey information to the central nervous system. How the various component parts of the intramural nervous system are organized and integrated remains largely unknown. The characteristic distribution of the various neuropeptides in the gut wall probably gives clues as to specific target cells. In addition, the bioactivity spectra of the peptides provide useful information on their possible functional roles. There is mounting evidence that the neuropeptides are neurotransmitters.

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Polarity and Projections of Peptide-Containing Neurons in the Guinea Pig Small Intestine

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INTRODUCTION

A number of mechanisms may contribute to the preferential propulsion of the contents of the intestine from oral to anal. Among these are the peristaltic reflexes whose basic properties (the initiation of excitation above, and inhibition below a point of distension, or mucosal irritation) were described around the turn of the century (5,6,7,46,52). The existence of these reflexes implies that there is a polarity built into some of the neuronal circuits in the intestine, but the large numbers of neurons and nerve processes in the enteric plexuses have meant that there has been little possibility of studying the presumed polarity with conventional histological methods. Only very recently has there been the opportunity to map the distributions and to determine the projections and polarities of defined subpopulations of enteric neurons. This has come about with the discoveries of a variety of different peptides in enteric nerves (Table 1; for reviews see 33,61). By knowing the polarity and projections of particular peptide-containing nerves, potential roles for each group can be proposed. But the nerves within the wall of the gastrointestinal tract are involved in controlling more than just motility. They are certainly involved in modulating blood flow (e.g., 8,27), and from their distribution it seems likely that they control a number of functions of the intestinal mucosa, for example, water and electrolyte transport, absorption of nutrients, secretion from endocrine and paracrine cells, and perhaps even the maturation and turnover of mucosal epithelial cells. Moreover, some of the nerve fibers are the peripheral processes of sensory neurons whose cell

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IN THE DIGESTIVE SYSTEM

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TABLE 1
List of Peptides Present in Intestinal Neurons

Peptide	References
Substance P	17, 19, 28, 33, 54, 57, 60, 61, 65, 66
Somatostatin	20, 21, 33, 40, 60, 61
VIP	2, 18, 24, 33, 34, 36, 47, 49, 60, 61, 65, 67
Enkephalins	1, 26, 33, 35, 50, 58, 60, 61
CCK,[a] C-terminal fragment	48, 59, 61
Neurotensin	61
Angiotensin	36
Bombesin	9, 24
Pancreatic polypeptide	51

[a]Cholecystokinin.

bodies are extrinsic to the intestine. Different functions seem to be controlled through separate, though perhaps interlinked, reflex pathways. It is therefore possible that the one peptide is contained in different sets of enteric neurons. An accurate description of the projections of the peptide neurons is therefore indispensable if their roles are to be ascertained. Knowledge of differences between one part of the gastrointestinal tract and another is also likely to provide important clues.

In this presentation immunohistochemical studies using antisera directed against substance P, somatostatin, vasoactive intestinal polypeptide (VIP), and enkephalin are compared. Most of the studies have been made in the guinea pig small intestine and full descriptions are published elsewhere (17,18,19,20,21,34,35).

Materials and Methods

Guinea pigs were killed by a blow on the head and bled out. Segments from the mid-part of the small intestine were removed and processed for the immunohistochemical demonstration of peptides in whole mounts (16).

Some of the guinea pigs were anesthetized with ether and extrinsic and intrinsic nerve pathways were lesioned, as has been previously described (20,31,34,35). The animals were allowed to survive for two or more days after operation. Extrinsic denervation of segments of intestine, usually 8–10 cm long, was achieved by crushing the nerves running through the mesentery with watchmakers' forceps. Two operations were performed to lesion nerve pathways within the wall of the intestine, a myotomy—in which the outer muscle layers, including the myenteric plexus, were cut through around the full circumference of the intestine—and a myectomy—in which two circumferential cuts through the outer layers were made up to 1 cm apart and

the longitudinal muscle with the underlying myenteric plexus was removed from between the cuts.

To determine the polarity of neurons, nerve pathways in isolated tissues were crushed and the tissues maintained in oxygenated Krebs' solution at 37°C for 3–5 h, which is sufficient time for an accumulation of peptides transported along nerve processes to occur adjacent to the injury.

Some guinea pigs were injected with colchicine (1–10 mg/kg, intraperitoneal, 18–24 h before sacrifice) to increase peptide concentrations in cell bodies so that they could be more readily detected. Guinea pigs were also injected with 6-hydroxydopamine (250 mg/kg, intraperitoneal or subcutaneous, 1 and 8 days before sacrifice) to cause the degeneration of noradrenergic axons.

The whole mounts were examined in a Leitz Ortholux fluorescence microscope fitted for epiillumination.

Observations and Comments

Comparison of the distributions of nerve cell bodies and axons showing substance P, somatostatin, VIP, and enkephalin-like immunoreactivity (Table 2) suggests that each population of cell bodies and axons is separate, although the coexistence of one or more of the peptides in certain subpopulations cannot be entirely eliminated by such comparisons. Schultzberg *et al.* (61) stained sections of intestine sequentially for different peptides and found no evidence for the common presence of any of these four peptides in nerve cell bodies. However, some cells in the submucous ganglia that were reactive for somatostatin also showed cholecystokinin-like immunoreactivity. To demonstrate that the immunoreactivity is due to the authentic peptide and not to another substance with similar amino acid groupings, ideally the peptides should be extracted and their amino acid sequences determined. This has not been done, but other evidence suggests that in each case the authentic peptide is contained in the nerves. Extracts from the outer layers of the guinea pig small intestine, which contain immunoreactive neurons but not other cell types that are immunoreactive, contain a peptide that is indistinguishable from authentic substance P in its pharmacological properties and sensitivity to peptidases (29). Somatostatin-like material extracted from the nerve plexuses of the guinea pig small intestine has the same retention time as authentic somatostatin on reverse-phase high-performance liquid chromatography (J. B. Furness, R. Eskay, M. J. Brownstein, L. E. Eiden, and M. Costa, unpublished). VIP isolated from the guinea pig intestine has not been characterized, but VIP-like immunoreactivity in intestinal nerves of other species (dog, hog, human, and rat) is due to a number of components, among which is the authentic 28 amino acid peptide (22,23,49). Other components may be precursor molecules and subfragments of VIP. Enkephalin-like immunoreactivity is

TABLE 2
Localization of Neuronal Peptides in Guinea Pig Ileum[a]

Peptide	Presence in varicose axons					Polarity of axons in myenteric plexus		Proportion of all neurons[c]	
	Myenteric plexus	Submucous plexus	Circular muscle[b]	Mucosa	Arterioles	Directed orally	Directed anally	Myenteric plexus	Submucous plexus
Substance P	X	X	X	X	X	X	X	3.5%	11.3%
Somatostatin	X	X	—	X	rare	—	X	4.7%	17.4%
VIP	X	X	X	X	rare	—	X	2.4%	42.3%
Enkephalins	X	X	X	—	—	X	X	24.5%	0

[a]X, present; —, absent.
[b]Including the deep muscular plexus.
[c]From figures in ref. 33.

found in nerves, not in other cell types, in the guinea pig small intestine. When extracts are made, both Leu- and Met-enkephalin can be identified by chromatographic separation and bioassay (41). Enkephalins released in response to nerve stimulation have been identified by chromatographic separation and radioimmunoassay (63).

Thus in the case of the four peptides dealt with in the present work it is justified in assuming that the authentic peptide is contained in the neurons and that it could be potentially released as a neurotransmitter.

SUBSTANCE P NEURONS

Axons containing substance P are contained in all layers of the guinea pig small intestine (17,61). In the myenteric plexus varicose axons form dense networks that surround all the nerve cell bodies of the ganglia. The density of innervation of the myenteric ganglia by substance P axons is greater than that for any of the other three peptides. Lesion studies indicate that these pericellular networks originate from intrinsic nerve cell bodies, most, perhaps all, of which are in the myenteric plexus, although the possibility that some axons originate from cell bodies in the submucosa has not been eliminated (19). In fact, this possibility is difficult to test with the techniques so far employed. Most of the varicose axons appear to arise from cell bodies in the same or adjacent ganglia, that is, the pathways run for only short distances up and down the intestine, probably 1 mm or less in most cases. Axons project both from oral to anal and anal to oral in the plexus. Substance P axons also run in the tertiary component of the myenteric plexus, which is a meshwork of fine nerve bundles lying between the ganglia whose significance is not known (33). Most probably all of the axons in the tertiary plexus originate from cell bodies in adjacent myenteric ganglia.

Substance P axons also supply a rich innervation of the circular muscle layer, including the deep muscular plexus that lies among the innermost muscle cells, adjacent to the submucosa. These axons arise from cell bodies in the overlying ganglia of the myenteric plexus (19). It is not known if myenteric nerve cells that send processes to the ganglia also project to the circular muscle or if there are two separate populations of neurons.

In the submucosa, varicose substance P axons are found around the nerve cell bodies of the ganglia and axons also form a network around submucous arteries. The ganglia are innervated from two sources: Some of the varicosities in the ganglia disappear if extrinsic nerves are cut and other varicosities are no longer seen if the myenteric plexus at the same level is removed. After combined extrinsic denervation and myectomy, none of the varicose substance P axons in the submucous ganglia survive (19). The axons surrounding the submucosal arteries disappear after extrinsic denervation. They are therefore like noradrenergic axons in having an extrinsic source and forming a network around the arteries. However, although the noradrenergic axons can no longer be visualized in the submucous plexus from animals

pretreated with 6-hydroxydopamine, this treatment has no effect on the immunohistochemical demonstration of substance P axons.

The rich supply of substance P fibers to the mucosa is not affected by extrinsic denervation. There is some loss of fibers after myectomy, indicating that some axons arise from myenteric neurons, but the majority seem to originate from cell bodies in the submucosa.

There is evidence that noncholinergic excitatory responses of the longitudinal muscle when intrinsic nerves are stimulated are due to the release of substance P. Earlier studies of these responses indicated that the excitatory substance was not acetylcholine, histamine, 5-hydroxytryptamine or a prostaglandin (3). It has now been found that the responses are abolished when receptors for substance P are desensitized (28,29). The desensitization does not affect transmission from other nerve types, nor does it affect responses to unrelated agonists. Pharmacological experiments indicate that substance P neurons are in both orally and anally directed pathways (29), which is consistent with the projections found in immunohistochemical studies (19). There is also evidence that the substance P neurons receive cholinergic inputs (30). Substance P, applied in the bathing solution or through a microelectrode, excites neurons in the myenteric plexus (44), and it is therefore likely that substance P released from the numerous axons around the cell bodies of myenteric neurons is also excitatory.

SOMATOSTATIN NEURONS

The varicose processes of enteric somatostatin neurons ramify in the myenteric and submucous ganglia, but, unlike axons containing substance P, enkephalin, and VIP, somatostatin axons do not supply the circular muscle coat. It was therefore suggested that the somatostatin neurons might be interneurons in the enteric plexuses (21). Somatostatin axons are also found in the submucosa. Extrinsic denervation causes little or no change in the number or distribution of somatostatin axons, although there is evidence of some somatostatin axons projecting to the intestine through the mesentery (20).

Lesion studies indicate that axons arising from somatostatin nerve cells in the myenteric plexus project in an anal direction, but none project from anal to oral in the guinea pig small intestine (20). All the endings around myenteric neurons originate from more orally placed somatostatin neurons in the myenteric plexus. There is an uneven distribution of varicose axons in the ganglia, some of the axons forming basketlike arrangements about a small proportion of the cell bodies and other axons forming a sparse meshwork throughout the ganglia. About two-thirds of the basketline formations were around nerve cells that themselves contained somatostatin, whereas the remainder were around nonreactive cells. This indicates that some of the somatostatin neurons are arranged in anally directed series (35). From the lesion experiments, it has been possible to estimate the average length of the

somatostatin neurons as 8–12 mm (20). On the distal side of a lesion, there are very few or no surviving varicose axons for about 5 mm, which provides an estimate of the shortest pathways.

Somatostatin axons in the submucous ganglia and in the mucosa appear to arise from cell bodies in the submucosa.

In low concentrations *in vitro*, somatostatin has two actions on intestinal motility: It inhibits the output of acetylcholine and hence diminishes the excitatory effects of cholinergic neurons and it stimulates the enteric inhibitory neurons (14,32,39). *In vivo* it inhibits propulsion along the intestine and delays gastric emptying (42,56) and at high concentrations *in vitro* it antagonizes both the ascending excitatory and descending inhibitory components of the peristaltic reflex (32). The polarity and connections of the somatostatin neurons in the myenteric plexus have led to the speculation that it is contained in interneurons in descending reflex pathways, and the observation that somatostatin excites enteric inhibitory neurons suggests that somatostatin interneurons are a link in the descending inhibitory reflex (20,35). Inhibition of this reflex, by high concentrations of somatostatin, could be a result of receptor desensitization.

The functions of the somatostatin-containing axons in the submucosa and mucosa are unknown. Somatostatin enhances sodium and chloride absorption and inhibits bicarbonate secretion in the small intestine (43), and it is thus possible that somatostatin released from mucosal axons could have a role in electrolyte transport.

VIP NEURONS

VIP-containing nerve processes supply the myenteric and submucous ganglia, the circular smooth muscle, and the mucosa of the guinea pig ileum (18,61).

It has been suggested that VIP could be the transmitter released by the enteric inhibitory nerves, which are the final neurons in the descending inhibitory reflex (15) and in vagal inhibitory pathways to the stomach (27). However, it has also been proposed that these nerves utilize ATP or a related purine nucleotide as a transmitter and on this basis they have been called purinergic (12,13). Among evidence that VIP might be the transmitter is the observation that VIP is released from the feline stomach when the enteric inhibitory nerves are stimulated electrically or by eliciting a reflex relaxation of the stomach (27). VIP mimics the gastric relaxation that occurs when the nerves are stimulated electrically or reflexly (25). Relaxations of the lower esophageal sphincter caused by VIP and those caused by electrical stimulation of the enteric inhibitory nerves were both inhibited by the infusion of antisera directed against VIP (38).

Studies of the projections of VIP neurons, which have been made by lesioning pathways in the small intestine, indicate that VIP-containing nerve cell bodies in the myenteric plexus send axons in an anal direction (34). Some

of the axons enter and run within the circular muscle coat, parallel to the muscle bundles. The shortest of the descending VIP pathways is about 0.25 mm long. Such a projection of anally directed neurons from the myenteric plexus to the circular muscle would be expected for the enteric inhibitory neurons (34).

It is clear that VIP-containing neurons are likely to be involved in a number of different enteric pathways. Cell bodies containing VIP are particularly numerous in the submucosa—in the guinea pig small intestine they constitute about 45% of all neurons (18). The rich supply of VIP axons in the mucosa probably arises from these cells. VIP causes a copious secretion of water and electrolytes from the mucosa (4,25,64), and it has been suggested that VIP nerves might stimulate secretion (37). It has also been suggested that VIP could be a transmitter released by enteric vasodilator nerves (27).

ENKEPHALIN NEURONS

It is necessary to be cautious in evaluating the role or roles of enkephalin neurons in the intestine because of the quite striking differences between species in the effects of drugs with opiate activity (10,11,45,53,55,62). The few brief comments made here refer to the guinea pig ileum. In this part of intestine, we have found that a large proportion of the nerve cell bodies in the myenteric plexus, about 25%, have enkephalin-like immunoreactivity (33). Varicose axons were found in the myenteric plexus, where they formed a sparse network around the nerve cells of the ganglia, in the circular muscle and deep muscular plexus (35). Axons in the myenteric plexus projected in both oral and anal directions. No nerve cell bodies were found in the submucous plexus. Axons were very rare in the submucosa and none were found in the mucosa. This distribution suggests that enkephalin-containing nerves in the guinea pig small intestine are involved in the control of motility but do not contribute directly to the control of other functions such as blood flow or secretion.

In the guinea pig small intestine agonists acting at opiate receptors suppress the output of acetylcholine and it is presumably through this mechanism that they inhibit peristalsis (45). The arrangement of the axons suggests that the antagonism of acetylcholine release might be through a prejunctional axo-axonal interaction at the cholinergic nerve endings in the circular muscle. Consideration of some pharmacological observations also leads to the conclusion that enkephalins and other opiate agonists cause a prejunctional inhibition of release from the final cholinergic neurons that go to the muscle (55). It is even possible that enkephalins are contained in the same axons as acetylcholine and when released act back on the endings to limit further secretion. Electrophysiological studies show that a component of enkephalin's action could be to hyperpolarize myenteric nerve cells and thus lower their excitability (55).

Discussion

In the five or so years since peptides were first demonstrated in intestinal neurons by immunohistochemical methods, an impressive amount of information concerning the presence, identification, and pharmacological actions of these neuronal peptides has been gathered. In spite of this, speculations concerning the role of any of the nine peptides that appear to be in intestinal nerves must be very tentative.

Immunohistochemical studies, such as those described and commented on earlier, indicate that at least some of the peptides are in distinct populations of neurons with well-defined projections and that in two cases the neurons in the myenteric plexus project in only one direction, as some neurons might be anticipated to do (see the introduction). There is insufficient information to state with confidence that any of the peptides are neurotransmitters, although this role seems likely for substance P and for VIP and possibly for somatostatin and enkephalins. However, it does seem clear that the peptides act as markers for distinct groups of neurons, and by studying these neurons we may learn much about neuronal circuitry in the intestine.

As was anticipated in the introduction, in a number of cases the same peptide seems to be associated with neurons in pathways subserving different functions. For example, the VIP neurons that project from the myenteric plexus to the circular muscle almost certainly have a role that is quite distinct from that of VIP neurons projecting from the submucosa to the mucosa. Likewise, different roles can be anticipated for substance P neurons that supply myenteric ganglia and those supplying submucous arterioles.

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Immunochemical Characterization of Peptides in Endocrine Cells and Nerves with Particular Reference to Gastrin and Cholecystokinin

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INTRODUCTION

The importance of the hormones gastrin and cholecystokinin (CCK) in the regulation of gastric function and of pancreatic and gall bladder function, respectively, has long been recognized. However, two recent developments indicate that the physiological role of these substances is more complex than previously thought. First, both gastrin and CCK have been shown to exist in several molecular forms that differ in their biological properties and in their distributions. Second, it has been reported that both substances occur not only in gut endocrine cells but also in neurons of the gut and of the brain and so may have neuroregulatory as well as hormonal functions. The implications of these findings are not confined to the study of gastrin and CCK alone, since parallel studies on other peptides, e.g., vasoactive intestinal polypeptide (VIP), somatostatin, and bombesin, indicate that molecular heterogeneity and a dual distribution in neurons and endocrine cells are likely to be relatively widespread occurrences. Immunochemical methods of analysis, notably radioimmunoassay (RIA) and immunohistochemistry, have proved to be particularly valuable in studies of the distribution, identity, and interrelationships of various molecular forms of gastrin and CCK. We propose to discuss here some of the recent evidence that has been obtained when these methods have been applied to studies on the nature and origins of different forms of gastrin and CCK in the mammalian gastrointestinal tract. The problems and issues raised by the study of gastrin and CCK are not

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unique and the data discussed are also likely to be relevant in the study of other neuro- and hormonal peptides.

Region-Specific Gastrin and CCK Antisera

COOH-TERMINAL-SPECIFIC ANTISERA

The methods available for the generation and characterization of antisera to gastrin and CCK have received considerable attention recently (4,16,17). A high proportion of the antisera raised to gastrin and CCK contain antibodies specific for the COOH-terminal region common to both peptides. Such antisera are useful in that they are directed toward the part of the two substances that is essential for biological activity, namely, their common COOH-terminal pentapeptide sequence -Gly-Trp-Met-Asp-Phe-NH_2 (abbreviated to G5). These antisera do not therefore cross-react with biologically inactive NH_2-terminal fragments. There is, however, a disadvantage in the use of COOH-terminal-specific antisera, since not surprisingly they frequently cross-react with both gastrin and CCK and so lack the specificity needed for detection of either substance alone. The importance of this point can be illustrated by the fact that the gastrin-like immunoreactivity in brain that was originally reported by Vanderhaeghen *et al.* (32) has since been shown to be due to cross-reacting cholecystokinins, particularly the COOH-terminal octapeptide of CCK (CCK8) (3,10,22,26).

In RIA the specificity of COOH-terminally directed antisera varies markedly. Some examples are illustrated in Figure 1. One of these antisera (L112) was raised to the COOH-terminal tetrapeptide (G4) coupled to thyroglobulin and cross-reacts in RIA virtually equally with G4, CCK8, and heptadecapeptide gastrin (G17); the antigenic determinant for this antiserum therefore lies exclusively in the G4 sequence. A second antiserum (L48), raised to CCK8 coupled to bovine serum albumin, cross-reacts in RIA almost equally with CCK8 and G17, but immunochemical potency of G4 and G5 is about 50 times lower (5). This antiserum, therefore, requires additional

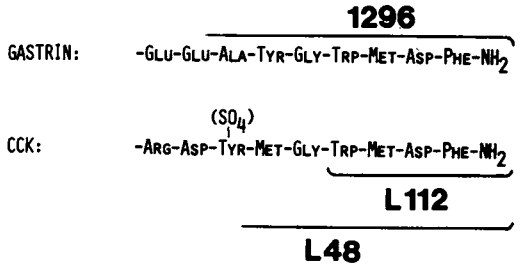

Figure 1. Representation of the antigenic determinants for three COOH-terminal specific antisera, L48, L112, and 1296. (For further details see 5 and 8.) Rabbit antiserum L112 was raised to synthetic G4 coupled to thyroglobulin by glutaraldehyde using immunization schedules already described (5). In radioimmunoassays using G17 label, antiserum L112 shows equal immunochemical potency with G17, G4, and CCK.

NH_2-terminal extensions to G4 but does not discriminate between -Glu-Ala-Tyr- in gastrin, and -Asp-Tyr(SO^4)-Met- in CCK. In contrast, a third antiserum (1296) cross-reacts in RIA about 20 times better with G17 compared with CCK8 and therefore is highly specific for the gastrin sequence immediately preceding the terminal tetrapeptide (8). Conversely, other COOH-terminal-specific antisera may discriminate in favor of CCK in the sequence preceding the tetrapeptide. With both 1296 and L48 the NH_2-terminal 1-13 fragment of G17, and G17 lacking the COOH-terminal amide, are inactive. Thus with these antisera the tetrapeptide sequence, including the COOH-terminal amide, is necessary but not sufficient for full immunochemical activity. It is apparent that in RIA gastrin and CCK, COOH-terminal immunoreactivities can be separately estimated by the use of two or more antisera. However, in immunocytochemistry all COOH-terminal-specific antisera will reveal both gastrin and CCK-like peptides, since this method is essentially noncompetitive and even ligands with relatively low binding energy are still localized. The specific localization of gastrin and CCK in immunocytochemistry therefore requires the use of one or more antisera specific for the unshared mid- and NH_2-terminal sequences of these molecules.

NH_2-TERMINAL-SPECIFIC ANTISERA

Antibodies specific for the mid- or NH_2-terminal regions of gastrin and CCK-related peptides have been obtained by immunizing rabbits with porcine 33 residue (CCK33) and 39 residue (CCK39) CCK, and with G17 and 34 residue gastrin (G34) (2,11,22,25). However, such antisera also frequently contain populations of antibodies with COOH-terminal specificity. For example, antisera directed toward the NH_2-terminus of G17 (1295) and the NH_2-terminus of G34 (L33) also had minor populations of COOH-terminally directed antibodies (Figure 2). These COOH-terminal-specific antibodies were of negligible importance in RIA but in immunocytochemical studies

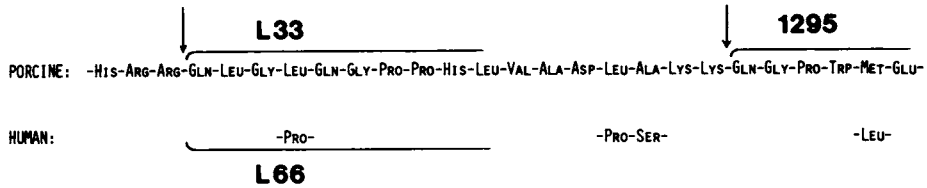

Figure 2. Representation of the antigenic determinants of antisera L33, L66, and 1295. For further details see refs. 8 and 11. Rabbit antiserum L66 was raised to synthetic (Tyr^7, Ser^9)1-15 human G34 coupled to bovine serum albumin by triazine (the conjugate was generously donated by the late Professor G. W. Kenner). In radioimmunoassays using 1–12 human G34 labeled with ^{125}I at His^9, antiserum L66 cross-reacts with human G34 with 50–100 times higher immunochemical potency compared with porcine G34. Conversely, in assays using 1–12 porcine G34 label, antiserum L33 shows about 50-fold higher immunochemical potency with porcine G34 compared with human G34. Evidently the Pro- for Leu-substitution at position 4 has a considerable influence on antigenicity with these antisera.

when the antisera were used at relatively low dilutions they cross-reacted with CCK cells in addition to gastrin cells (31). The contaminating antibody populations are relatively easily removed by adsorption to an appropriately immobilized ligand. Thus a COOH-terminal fragment of gastrin covalently linked to Sepharose beads was used to separate COOH-terminal antibodies from the NH_2-terminally directed populations in L33 and 1295 (31). However, it is frequently more convenient to obtain NH_2-terminal-specific antibodies without contaminating COOH-terminal-specific antibody populations by immunization with the appropriate peptide fragments. For example, antiserum L66, which is specific for the NH_2-terminal region of human G34, was raised by immunization with the synthetic 1-15 sequence of human G34 coupled to bovine serum albumin. However, advantages may be gained by immunization with the whole peptide, rather than with selected fragments, in that antibodies of unusual and unexpected specificity are occasionally obtained. One such gastrin antiserum (L6) is apparently specific for intact G17 and cross-reacts with neither COOH- nor NH_2-terminal G17 fragments (7). This antiserum is obviously very useful and yet could not have been obtained by immunization with gastrin fragments.

Origins and Interrelationships of Different Forms of Gastrin

Biosynthetic Relationships of G17 and G34

The availability of antisera directed toward the NH_2-terminus of G34 has made it possible to study directly the cellular and molecular relationships of G17 and G34 in greater detail than previously possible. In particular it has been possible to examine the suggestion (15) that G34 is a biosynthetic precursor that is cleaved at lysine residues in positions 16 and 17 by a trypsin-like enzyme to yield G17 and an NH_2-terminal tryptic peptide (NT G34) (Figure 3). Thus, in direct support of this idea, it was shown that in hog

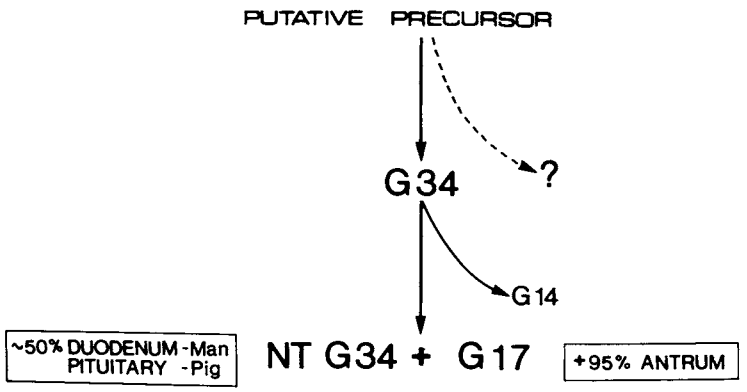

Figure 3. Schematic representation of the biosynthetic pathways in G cells by which tryptic cleavage of G34 might yield G17 and an NH_2-terminal tryptic peptide (NT G34) in equimolar amounts. In antral mucosa all but about 5% of G34 is presumed to be converted to G17, whereas in human duodenum and in pig pituitary only about 50% of G34 is converted to G17.

antral mucosal extracts there were almost equimolar amounts of peptides with the chromatographic and immunological properties of G17 and NT G34 (11). Moreover, in immunocytochemical studies, antisera L33, 1295, 1296, and L6 revealed a single population of antral G cells (31).

These results point to the fact that the antigenic determinants for G17 and NT G34 were localized in the same cell type, as would be expected if they were produced by cleavage of G34. These results also allow us to exclude the possibility that G17 and G34 are produced in separate populations of G cells. When used in other species antiserum L33 revealed only small amounts of immunoreactive material (in man, dog, cat) or no significant activity at all (in rat, mouse, guinea pig). It seems likely that there are marked species differences in the sequences of the NH_2-terminal regions of G34 that reduce or abolish immunoreactivity with L33. However, by using an antiserum (L66) that is specific for the NH_2-terminus of human G34, it has recently been possible to extend these studies to man. The results are in broad agreement with those obtained in pig. In extracts of human antral mucosa the total concentration of COOH-terminal immunoreactivity measured with L112 was $84.8 \pm 17.1\%$ (mean, $\pm$ S.E., $n = 5$) that measured with L66, i.e. there were virtually equimolar quantities of COOH- and NH_2-terminal G34 immunoreactivities. On gel filtration, over 95% of COOH-terminal immunoreactive gastrin eluted in the position of G17, and a similar proportion of NH_2-terminal G34 immunoreactivity eluted as NT G34. In immunocytochemical studies on serial sections antisera L66, L112, and L6 demonstrated identical cells, so that all cells that were revealed by L66 were also revealed by L112 and L6, and vice versa.

Thus, in man, as in pig, it seems likely that both G17 and G34 are produced in a single population of G cells. It is of interest that in human duodenum, unlike antrum, only about 50% of total gastrin immunoreactivity is attributable to G17 and the remainder is G34. It seems possible, then, that in duodenum there might be either a single population of cells with incomplete conversion, or two populations, one with normal conversion of G34 to G17, the other with little or no conversion of G34 to G17. The data available from morphological studies support the former idea in that there appears to be a single population of cells that are revealed by L66, L6, and L112, and therefore probably contain both G17 and G34 (Figure 4) (1); the reasons for the incomplete conversion in these cells are unknown.

The observation that antral and duodenal G cells apparently differ in their patterns of biosynthetic processing of the gastrins raises an interesting question when taken in conjunction with recent evidence that gastrin mRNA is 620 nucleotides long, a finding compatible with a gastrin precursor peptide of 111–140 residues (24). The precursor peptide is assumed to be converted to G34, and then G17, by a series of posttranslational processing events that include proteolytic cleavage and amidation of the COOH-terminus. Because COOH-terminal specific gastrin antisera require the COOH-terminal amide,

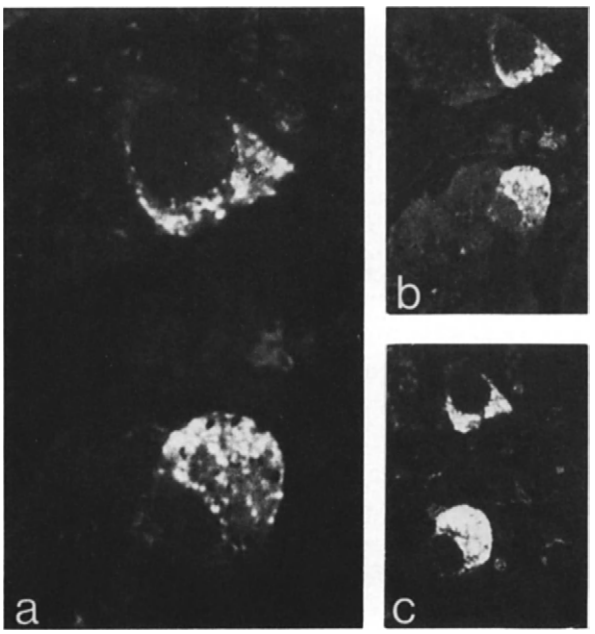

Figure 4. Two immunofluorescent gastrin cells in serial resin sections of human duodenal mucosa (formaldehyde fixation) demonstrated with three different antisera specific for (a) NT G34 (L66), (b) G17 (L6), and (c) COOH-terminus of gastrin (L112). (a) ×1800; (b) and (c) ×700.

they would not cross-react with either the initial product of translation or fragments generated from it if these were not amidated. It is conceivable, therefore, that the gastrin gene might be expressed in cells other than G cells but the translational product not recognized by COOH-terminal specific antisera. Such a situation could, for example, be associated with the production of a second active peptide from a different part of the precursor chain, since it is known that at least one other large precursor peptide contains the sequences of several different active peptides (ACTH, β-LPH, β-endorphin, β-MSH) that are produced in different cell types presumably by virtue of different pathways of posttranslational processing (21,23). The work of Noyes *et al.* (24) indicated that the sequence preceding G34 in its precursor was -His-Arg-Arg- so that treatment of a precursor with trypsin, which cleaves at Lys and Arg, would liberate the antigenic determinants for both L33 and 1295 (see Figure 2) irrespective of modifications at the COOH-terminus of the molecule.

With this in mind we have prepared trypsinized and control extracts from all regions of hog gastrointestinal tract for assays with antisera 1295, L33, and L112. The results are presented for L112 and L33 (Figure 5), but essentially similar results were obtained with 1295. The data clearly show that in hog, the antral mucosa is the only significant source of gastrin immunoreactivity, and all other parts of the gut contain less than 20 pmol/g. In antral mucosa the COOH-terminal-specific antiserum L112, revealed similar amounts of

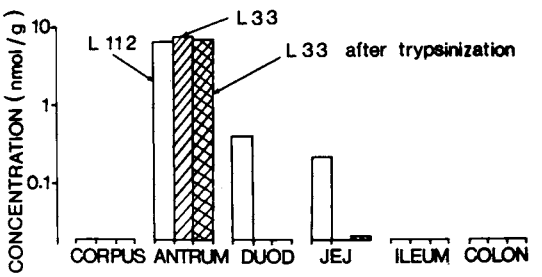

Figure 5. Immunoreactive material in extracts of hog gut estimated with L112 (open bars) and L33 either before (striped) or after (hatched) treatment of the extract with trypsin. Extracts were prepared by boiling tissues in water as previously described (5,11); similar results were obtained when the extracts were prepared by extraction in 0.5 *M* acetic acid and when acid or neutral extracts were assayed with antiserum 1295. Note that the ordinate is a log scale. The detection limit in this experiment was 20 pmol/g with both L112 and L33.

reactive gastrin to L33, but as expected L112 also demonstrated significant quantities of CCK immunoreactivity in the duodenum and jejunum. The results indicate that within the antrum there are not likely to be appreciable quantities of nascent gastrin precursor peptides, since trypsinization did not produce additional amounts of L33 immunoreactivity. The results also suggest that the gastrin gene is unlikely to be expressed in the nonantral regions of the gut. The present results therefore do not support the recent proposal that G34 is produced in the ileum and then converted directly to G4 in amounts up to 200 pmol/g (28). There remains a slight possibility that gastrin precursors might be produced in cells other than G cells and then rapidly processed in such a way as to destroy the antigenic determinants for the antisera we used, for example, by proteolytic cleavage or by extensive glycosylation or addition of other prosthetic groups.

GASTRIN TETRAPEPTIDE

Antisera like L112 that cross-react equally with G4 and larger forms of gastrin and CCK have been particularly valuable in the search for small COOH-terminal fragments of gastrin and CCK such as G4. The possible existence of G4 in tissues was originally suggested by the isolation from antral mucosa of small amounts of a peptide with the amino acid composition of 1-13 G17 (15). This fragment would be generated if G4 was produced by endopeptidase cleavage of G17. A similar fragment was later found in the blood and tumor tissue of patients with gastrinomas (8). Subsequently, there have been reports of high concentrations of G4 in brain, antrum, and throughout the gastrointestinal tract (18,27–29). These reports were based on data obtained by RIA using antisera that showed 180 times lower immunochemical potency with G4 compared with G17; thus a small peak of immunoreactivity eluting in a similar position to G4 on gel filtration was multiplied by 180 to obtain an estimate of true concentration. This method has the obvious disadvantage that small errors are also greatly magnified. In

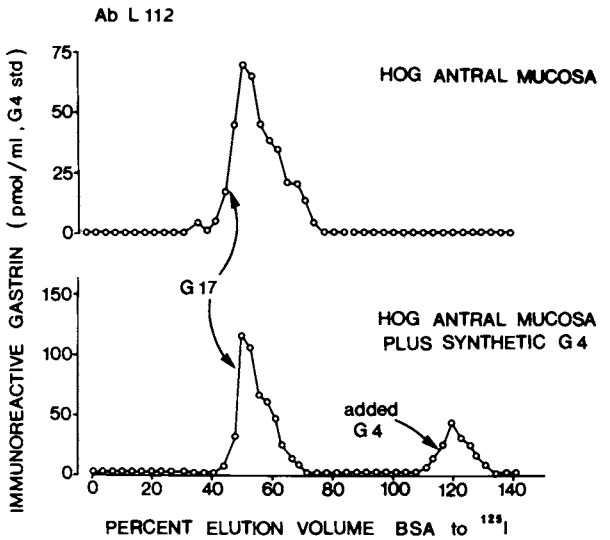

Figure 6. Separation on Sephadex G50 (1 × 100 cm in 0.02 *M* sodium barbitone) of extracts of hog antral mucosa assayed with antiserum L112. Antral mucosa was obtained within a few minutes of killing the pigs and immediately frozen on dry ice. The tissues were rapidly weighed and quickly plunged in boiling water (0.05 g/ml). Adjacent pieces of mucosa were extracted either with (lower panel) or without (upper panel) the addition of synthetic G4 immediately after adding the tissue to boiling water. Extracts were boiled for 1-2 min, tissues were homogenized and centrifuged, and supernatants were stored at −20°C prior to column chromatography. The overall recovery of G4 was about 60%.

contrast, if antisera are used that cross-react equally with G4 and larger forms of gastrin and CCK, this correction is not necessary. When applied to the RIA of immunoreactive gastrin in antral extracts of man (n = 5) and pig (n = 3), the latter antisera revealed a prominent peak of G17 immunoreactivity as expected, but no appreciable peak of activity in the region of G4. In contrast, exogenous synthetic G4, added early in the extraction of antral gastrin, was recovered after Sephadex G-50 gel filtration in a single clearly resolved peak in an overall yield of about 60% (Figure 6). The results indicate that had G4 been present in significant quantities in the original antral mucosa it would have been detected by these analytical methods. These data, together with the distribution results obtained with L112 (Figure 5), provide no support for the suggestion that there are large quantities of G4 throughout the gastrointestinal tract. The nature of the material identified by Larsson and Rehfeld (18) and localized to a specific cell type (TG cells) is therefore a mystery, and the need for the isolation and full chemical characterization of this material needs no emphasis.

Functional Aspects of Cellular Origins

Immunocytochemical studies with COOH-terminal-specific antisera reveal gastrin or CCK-like peptides to be present not only in mucosal endocrine

cells but also in gastrointestinal nerve fibers. Their presence in nerve fibers would be consistent with a neuroregulatory role for these substances. Their presence in endocrine cells is consistent with either a true endocrine role, i.e., peptides released into the circulation, or a paracrine role, i.e., peptides acting on neighboring cells in the mucosa, or both. In the context of paracrine regulation it is interesting that Larsson *et al.* (19) have recently noted that somatostatin cells in the antral mucosa have basal processes containing immunoreactive somatostatin that appear to project to adjacent mucosal cells. Presumably these basal processes are capable of directly delivering somatostatin to target cells in the gut mucosa. This idea is an extension of the earlier suggestions for paracrine regulation in which active substances were postulated to diffuse through the extracellular space in the mucosa (14). The basal projections, which are somewhat reminiscent of axonlike structures, provide a morphological basis for proposing regulatory mechanisms that are

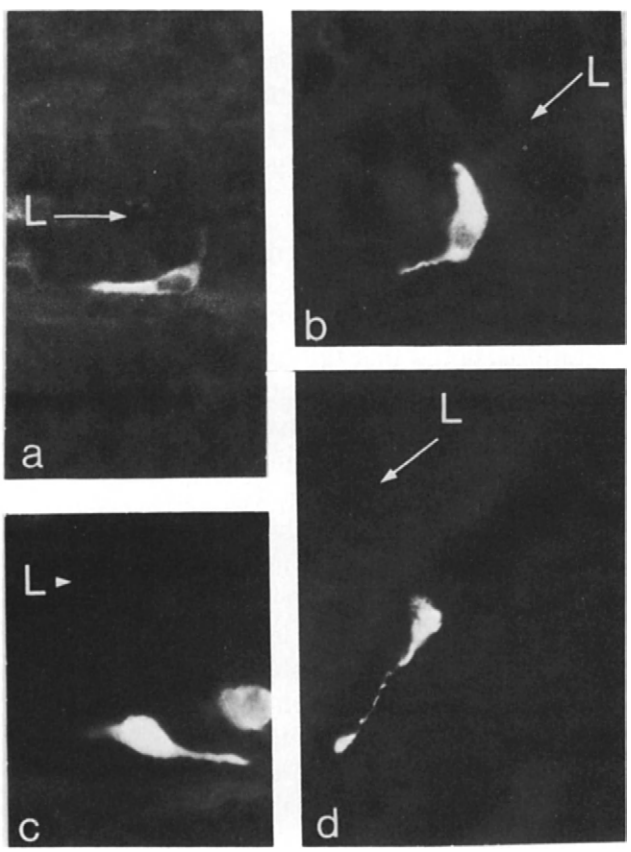

Figure 7. Immunofluorescent endocrine cells with basal processes demonstrated by antisera to (a) substance P (L83) in mouse ileum, (b) enteroglucagon (L56) in mouse colon, (c) gastrin (L48) in rat antrum, and (d) bombesin (L90) in chick gizzard. Frozen sections of tissues fixed in parabenzoquinone. L, lumen, ×450.

intermediate between true neuronal regulation, on the one hand, and endocrine regulation, on the other hand.

Basal projections of mucosal endocrine-like cells are not limited to somatostatin cells, but are fairly frequently encountered among a variety of different cell types, e.g., containing bombesin-, substance P-, and enteroglucagon-like immunoreactivity (Figure 7). In addition, antral G cells in the rat and mouse (although not in pig and man) also possess one or more basal projections containing immunoreactive material (Figure 7). Somatostatin inhibits the release of gastrin and many other peptides, including insulin, glucagon, growth hormone, and other pituitary hormones (20). The direct delivery of somatostatin to G cells therefore has a clear physiological significance, since it facilitates the specific regulation of gastrin release without influencing that of other hormonal peptides. In contrast, the physiological significance of basal processes on other cell types is less obvious. It is, of course, possible that rodent antral G cells are involved in paracrine control of adjacent cells. However, at the present time it also seems worth considering the alternative possibility that the basal projections of some or all endocrine-like cells in the gut are associated not with paracrine control but with some other function. The projections may, for example, provide a mechanism for increasing the surface area of the basal parts of the cell and thereby facilitate exocytosis of secretory granules in larger numbers, or more rapidly than would otherwise occur.

CCK and Gastrin in Gut Nerves

THE VAGUS

It is now generally accepted that the gastrinlike immunoreactivity in brain (32) is due to CCKs, mainly CCK8, that cross-react in some gastrin assays (3,5,10,22,26). However, Uvnäs-Wallensten *et al.* (30) have reported that true G17 immunoreactivity occurs in the vagus nerve, and in nerves of the gastrointestinal tract, and have suggested that whereas CCK8 is the predominant representative of this family of peptides in the CNS, G17 is the main representative in peripheral nerves. Recently we have examined the nature and transport of these peptides in the dog vagus and obtained substantially different results (9). We used both region-specific antisera and gel filtration to characterize and estimate the different forms of gastrin and CCK in the vagus. In five dogs in which a thoracic branch of the vagus had been ligated for 48 h we found a significant two- to three-fold increase in CCK8-like immunoreactivity on the cranial side of the ligature compared with a control branch that was not ligated. These vagal extracts did not contain detectable amounts of G17 (less than 300 fmol/g). In a second group of 10 dogs two ligatures were tied on one cervical vagus and loops were passed but not tightly tied on the other side. In seven of these dogs there was a significant increase of CCK8 immunoreactivity cranial to the first ligature (4.7 $\pm$ 0.7 compared with 0.6 $\pm$ 0.1 pmol/g, mean $\pm$ S.E.), and there was no

detectable change in concentrations of immunoreactive CCK8 between the ligatures. In these dogs there were again no detectable amounts of G17. However, in three of the 10 animals there were measurable amounts of G17 immunoreactivity that accumulated on the cranial side of the first ligature in concentrations comparable to those of CCK8 in the other animals. In this group of three dogs, there were also small amounts of G17, in addition to CCK8, in the control nerve, and between and distal to the ligatures. Again there was no change in concentrations of G17 or CCK8 between the ligatures compared with the control side. The animal-to-animal variation in the distribution of G17 in the vagus nerve is clearly unusual and merits further study. However, on the basis of the present results we can still draw several conclusions: (1) CCK8 is the predominant member of the gastrin-CCK family in the dog vagus, and G17 is of minor importance. (2) Both G17 and CCK8 are transported down the vagus toward the gut. (3) Over a period of 48 h there is neither significant synthesis nor degradation of peptides in the vagal fibers, since there was no change in concentration in the restricted segment between ligatures. On the basis of these results, therefore, we anticipate that the extrinsic nerves will account for some of the CCK8, and possibly a small amount of G17, in gut neurons. However, the present results by no means exclude the presence of CCK in neurons intrinsic to the gut as well.

ENTERIC NEURONS

Immunocytochemical studies indicate the existence of beaded nerve fibers with COOH-terminal immunoreactivity in the intestine of rat, mouse, and

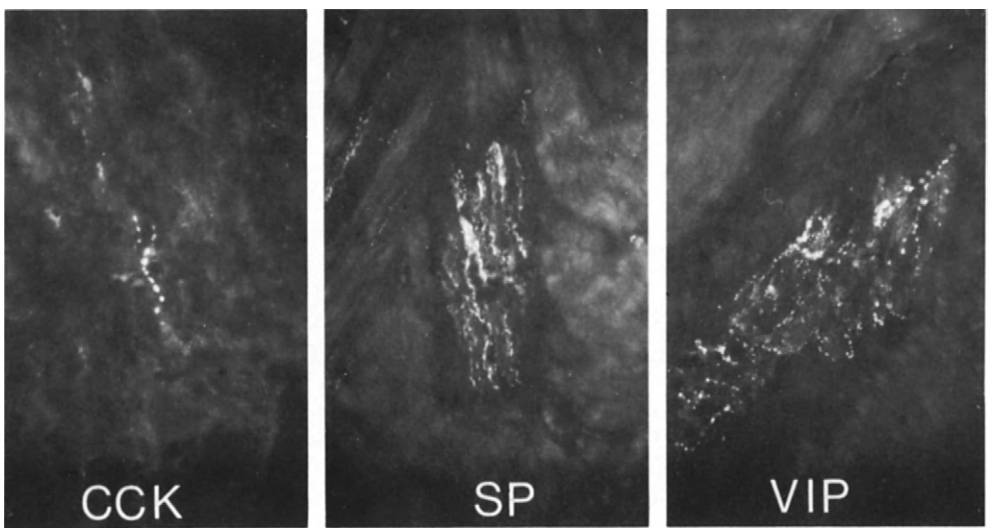

Figure 8. Immunofluorescent localization of peptidergic nerves in the myenteric plexus of pig ileum (frozen sections, parabenzoquinone fixation). Note the relative abundance of substance P-like (L83) and VIP-like (L85) immunoreactive nerve fibers compared with those demonstrated by antiserum L112, which probably contain CCK8 (×160).

pig. The fibers are distributed in the enteric plexuses and also in mucosa. However, nerve fibers with COOH-terminal immunoreactivity are generally less abundant than those containing other neuropeptides, e.g., VIP and substance P (Figure 8). There are obvious problems in attempting to quantify and characterize the CCK- and gastrinlike immunoreactivity in gut nerves,

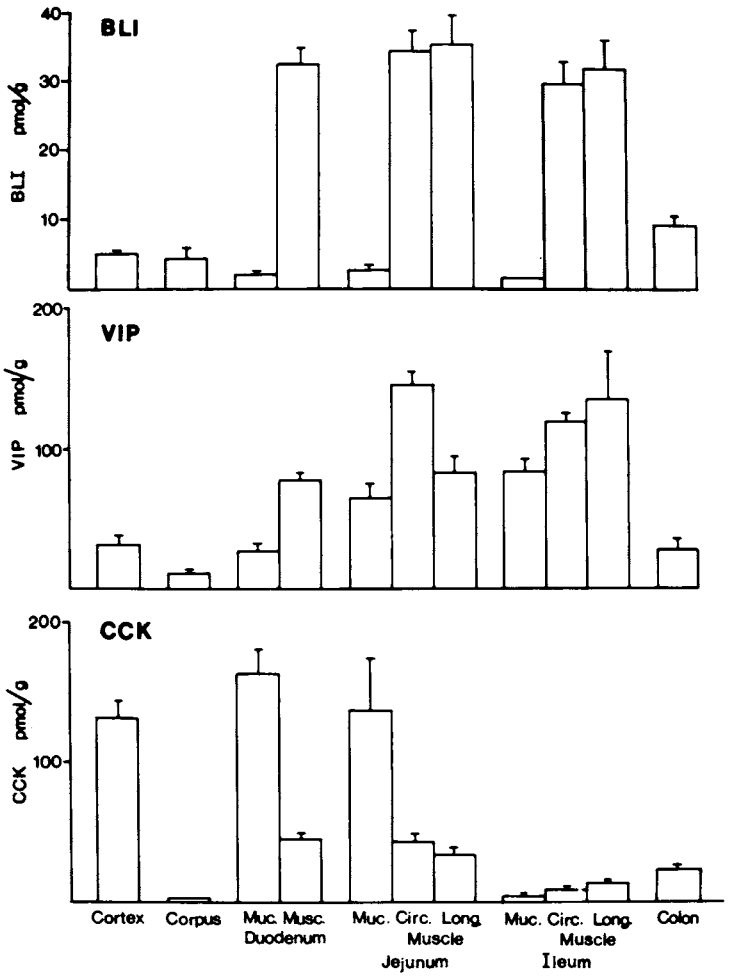

Figure 9. Distribution of immunoreactive CCK, VIP, and BLI in guinea pig gastrointestinal tract. For comparison, extracts of cerebral cortex are shown. Tissues were rapidly removed from guinea pigs killed by cervical dislocation and were frozen as soon as possible on dry ice prior to extraction in either boiling water (CCK) or boiling water followed by acidification to 0.5 *M* acetic acid (VIP and BLI). The whole thickness of the gastric corpus and the colon were taken. Longitudinal muscle layers of the jejunum (10 cm distal to the ligament of Trietz) and distal ileum (15–30 cm proximal to the ileocecal junction) were obtained with adherent plexuses as determined by visual inspection and methylene blue staining. Mucosa was removed by scraping, and circular muscle represents tissue remaining after removal of mucosa and myenteric plexus. Extracts were assayed with antiserum L48 (CCK), antiserum 1078 for BLI (12), and R501 (generous gift from Dr. N. Yanaihara) for VIP. Note that other VIP antisera, such as L25 (13) and 5603 (generous gift of Dr. J. Fahrenkrug), cross-react weakly with guinea pig VIP and reveal less than 10 pmol/g immunoreactivity in most parts of the gut.

since in tissue extracts the activity could arise from either endocrine cells or nerves. However, we have recently been able to study the amount and type of CCK-like immunoreactivity in the myenteric plexus of the guinea pig small intestine by taking advantage of the fact that in this species the longitudinal layer of the smooth muscle can be removed from the underlying circular smooth muscle layer with or without the adherent myenteric plexus (6). In addition, CCK was estimated in mucosal scrapings and in the remaining circular smooth muscle. The results are presented in Figure 9, which also shows comparable data obtained for VIP and bombesin-like immunoreactivity (BLI). In jejunum the concentration of CCK-like immunoreactivity in the longitudinal smooth muscle was about 25% that in the mucosa; there was a sharp gradient in mucosal CCK and concentrations in the terminal ileum were virtually negligible. A distal gradient also occurs in CCK immunoreactivity in the longitudinal muscle layer, but this was less marked than in mucosa. Thus in ileum longitudinal muscle, concentrations of CCK were about half those in jejunum longitudinal muscle, but were three times those in ileal mucosa. The CCK-like immunoreactivity of the longitudinal muscle was localized to the adherent myenteric plexus, since in strips of muscle without plexuses the concentrations were reduced by about 70% compared with immediately adjacent innervated strips (6).

An analysis of the molecular forms of CCK in extracts of guinea pig myenteric plexus indicated that over 80% of immunoreactivity was attributable to CCK8, the remainder being due to two minor components, one eluting in the void volume and the other just behind CCK8 on BioGel P10. Closely similar patterns of immunoreactive CCK were seen when extracts of guinea pig cerebral cortex were fractionated on the same columns. In contrast, in extracts of mucosa up to half of total CCK immunoreactivity was due to a factor that eluted in the position of CCK33. It seems reasonable

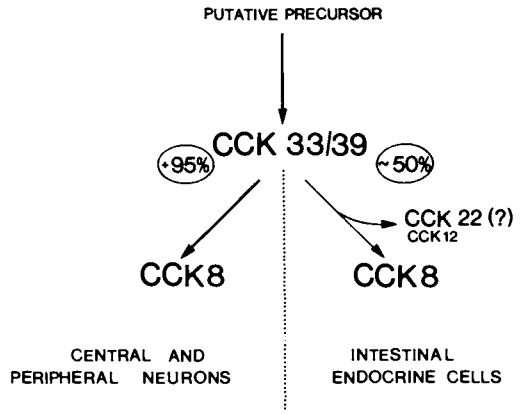

Figure 10. Schematic representation of the biosynthesis of CCK. It is proposed that CCK33 and CCK39 are generated from a large precursor peptide and in central or peripheral neurons converted more or less completely to CCK8, whereas in gut mucosal endocrine cells the conversion to smaller peptides is incomplete. (Modified from 13.)

to suppose that CCK33 and CCK39 are biosynthetic precursors of smaller molecular forms. The present evidence is therefore compatible with a mechanism for virtually complete conversion of larger molecular forms of CCK to CCK8 in central and peripheral neurons. In contrast, in mucosal endocrine cells there occurs incomplete conversion leading to the accumulation of significant amounts of CCK33-like immunoreactivity (Figure 10).

It is clear, from the data presented in Figure 9, that the relative distribution of CCK-like immunoreactivity in different regions of the gut differs markedly from that of VIP and BLI. Both VIP and BLI occur in significant amounts in guinea pig small intestinal myenteric plexus. However, unlike CCK, BLI is virtually absent from the mucosa, whereas VIP occurs in substantial amounts in both mucosa and muscle layers. Moreover, unlike CCK, neither BLI nor VIP show a tendency to predominate in the proximal small intestine. It is worth noting that a species difference exists in the distribution of BLI, in that in the rat BLI occurs in highest concentrations in the stomach (12), whereas in the guinea pig there are relatively low concentrations in the stomach (13).

PHYSIOLOGICAL ASPECTS

The distribution data presented here provide a framework for understanding the possible physiological functions of CCK and other neuropeptides in the gut, and for interpreting pharmacological data. For example, evidence shows that CCK stimulates contractions of the longitudinal muscle of guinea pig ileum by evoking the release of acetylcholine from the myenteric plexus (6,33). CCK8 might therefore play a neuroregulatory role in the myenteric plexus modulating acetylcholine release. In the case of bombesin there is evidence that this peptide releases gastrin, gastric inhibitory polypeptide, pancreatic polypeptide, and cholecystokinin after intravenous injection. Since there is little or no bombesin-like activity in the mucosa of the mammalian small intestine or pancreas, it is evident that neuronal BLI is unlikely to be directly involved in the release of GIP or PP. Bombesin-like peptides could, however, be involved in the control of gastrin release, since in the rat we have found BLI in nerve fibers of the antral mucosa (12); similar fibers also occur in the acid-secreting mucosa. The continued study of the origins and relationships between peptidergic nerves and endocrine cells in gut will help to clarify these and other functional interactions.

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Langherans Islets as the Neuro-Paraneuronal Control Center of the Exocrine Pancreas

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Pancreatic Islet Cells as Paraneurons

Since 1975 we have stressed that neurons (nerve cells) are not as specific a type of cell as was hitherto believed and that there is every gradation in cellular biological features between neurons and members of a large group of cells called paraneurons (5,6,8). Paraneurons are identical with or closely related to neurons in terms of possessing cytoplasmic granules or vesicles and producing secretions, including peptides, adenine nucleotides, and often amines, that may serve as bioactive messengers in the organism. Every paraneuron is a receptosecretory cell, recognizing adequate stimuli and responding to them by releasing its secretions. Endocrine cells are paraneurons whose secretory function is prominent, whereas a series of sensory cells represent paraneurons whose receptive features are predominant (6,8).

The pancreatic islet cells, together with their counterpart in the intestinal mucosa, the enteroendocrine cells, are fully qualified as paraneurons (7,11).

The previous doctrine advocated that neurons (nerves) supply endocrine glands to control the function of the endocrine cells. However, the latter cells, regarded as paraneurons, appear to us not necessarily under the regulation of neurons; they may at some time and in some places be equivalent or parallel in position with neurons. To support this possibility, more and more substances representing candidates for chemical messengers in the organism have been found in both neurons and endocrine cells.

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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Fujita, in 1979, while looking at light and electron micrographs of dog pancreatic islets, which had been used in previous reports (3,16), noted that neurons and islet cells were arranged laterally in parallel with each other along the sinusoidal capillaries, apparently to release their secretion concurrently into the bloodstream.

Insular Neurosecretion

In the previous view the nerves reaching the pancreatic islet were interpreted as regulating its endocrine function. Electron micrographs showing a synapse-like juxtaposition of a neuronal process and an endocrine cell were presented to stress the neuroendocrine connection in series (16,22,23, 25).

Only a few authors noted that numerous nerves appeared to terminate around the blood vessels in the islet as if to control the vascular wall (1,19). This interpretation has been disputable because the vascular wall in question is devoid of a muscle layer for control by the nerves, nor does the endothelium, so simply attenuated, appear to be the target of the nerve transmitters. No previous authors, as far as we are aware, have thought of the possibility that the nerves concentrated in the pericapillary space in the islets might release their secretions *into the blood.*

If we take an unprejudiced viewpoint, it seems unlikely that the secretions of the endocrine cells enter the bloodstream, whereas those of the nerves might be inhibited from doing so, because it is now known that the secretions of neurons ("transmitters") and of endocrine paraneurons ("hormones") are often identical or comparable in chemical and molecular nature.

We now believe that we have gathered sufficient morphological data supporting the occurrence of *neurosecretion* in the canine pancreatic islet (9). First, the prominent number of synaptic vesicle-containing axons extending to the pericapillary space may be accounted for only by the view that they are engaged in neurosecretion. In the pericapillary space the axons, usually covered by Schwann cell cytoplasm, are denuded of it toward the porous endothelium. The synaptic vesicles fill the swollen terminal and preterminal portions of axons and they tend to gather at the denuded side of the axon facing the capillary wall (Figure 1).

As detailed elsewhere (9), the pericapillary nerve terminals in the canine islet include, on the basis of synaptic vesicle morphology, adrenergic, cholinergic, and possibly dopaminergic and peptidergic types. The last type, containing endocrine-like granules and irregular profiles of small vesicles, may correspond to the VIP-like immunoreactive nerves, which are numerous in the dog pancreas (18).

Axons were found in close juxtaposition to the endocrine cells, but only a few of these were characterized by accumulation of synaptic vesicles, as a synapse or neuroendocrine connection in series. In most cases neuroendocrine connections appeared to be a simple juxtaposition or a connection in parallel.

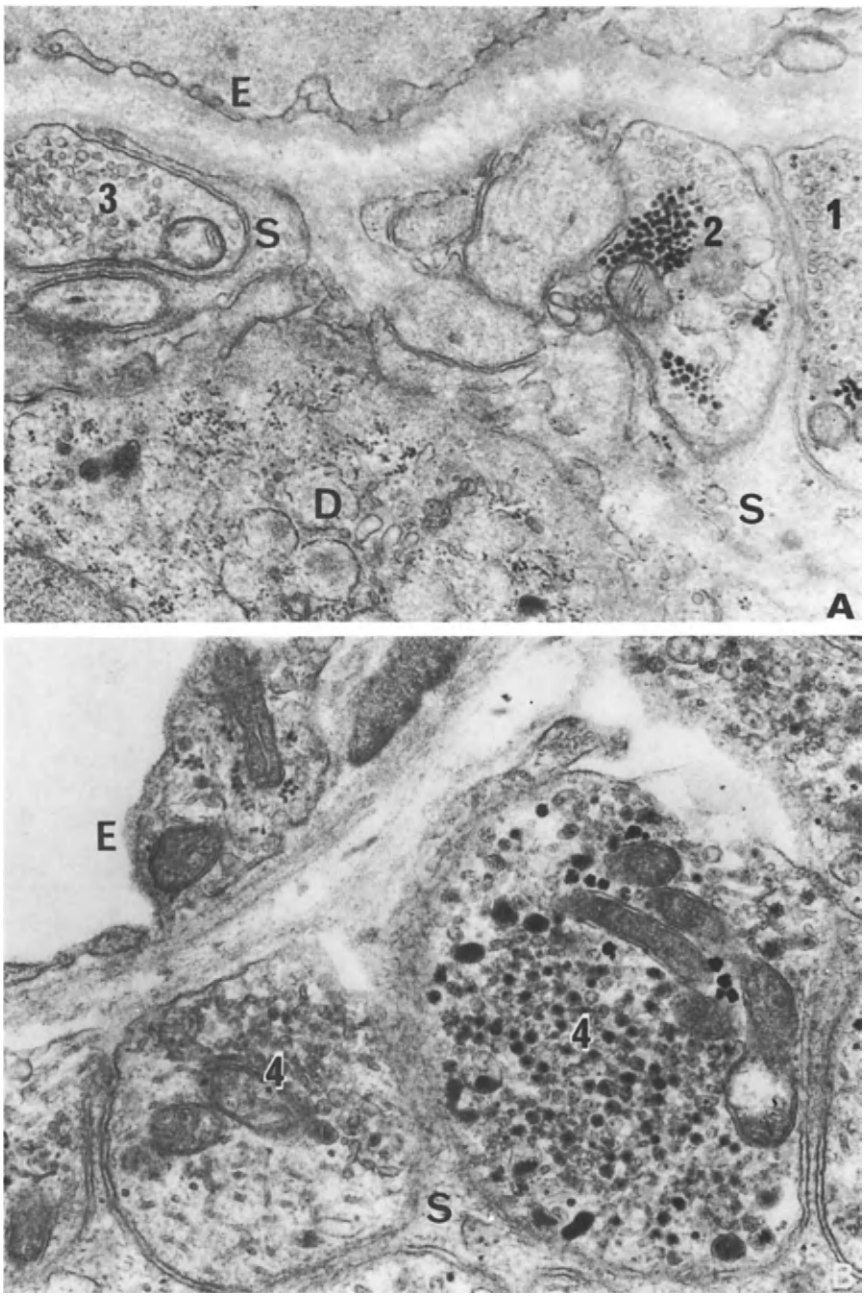

Figure 1A, B. Electron micrographs of portions of canine pancreatic islet. Nerves filled with different types of synaptic vesicles are apposed to the capillary endothelium (E). The nerves are enveloped in Schwann cell cytoplasm (S) but are denuded of it at the sites directly facing the endothelium. Nerves labeled 1, 2, and 3 in A are adrenergic, cholinergic, and unidentified in type, respectively. D is an endocrine D cell that is separated from the nerves by the Schwann cell. Nerves labeled 4 in B represent a special small-cored-vesicle type that may correspond to dopaminergic elements.

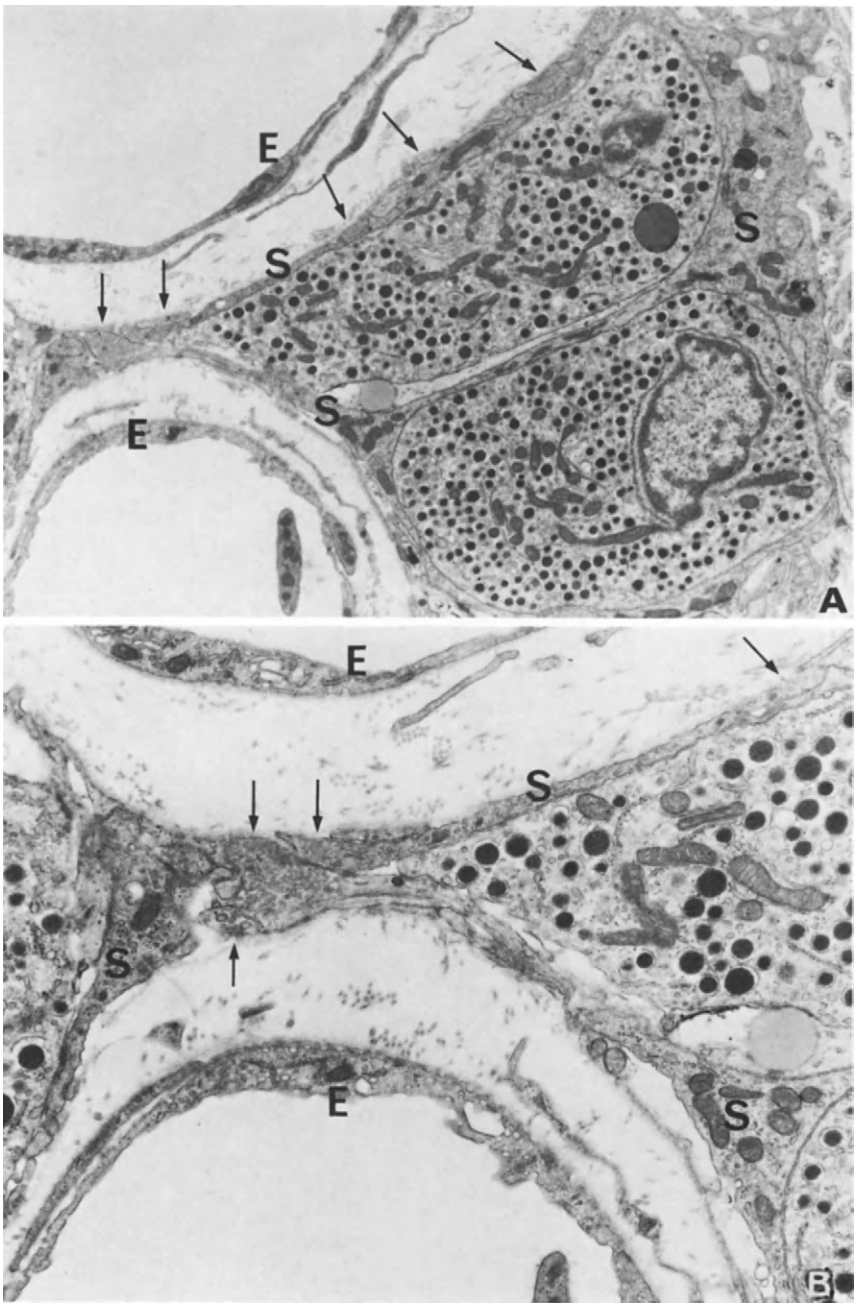

Figure 2. (A) Portion of a mouse pancreatic islet that, containing several profiles of nerve terminals and endocrine cells, represents a neuro-insular complex. A part of a Schwann cell on the right (S) extends its processes (S) through the endocrine cells. The Schwann cell processes reach the perivascular space. Nerve terminals containing numerous small vesicles (arrows) are often in direct connection with the tissue space (×5600). (B) Close view of a part of the picture shown in (A) (×14,000).

Neuroinsular Complex

After these observations in the dog, we confirmed the occurrence of vesicle-filled nerve terminals in the pericapillary space of islets in the mouse, although in this species insular axons suggesting neurosecretion were much fewer in number than in the dog. Figure 2 shows a case in which numerous nerve elements and endocrine cells are mixed in an islet (thus forming a neuroinsular complex) and are invested together by the attenuated cytoplasm of Schwann cells. The nerve processes apparently terminate in the pericapillary space with vesicle-filled swellings (arrows).

It seems reasonable to presume that in other species also the neurons in a neuroinsular complex may terminate on the blood capillaries within it, although the possibility that a part of the neuronal processes might extend outside of the complex is not excluded. The photograph and sketch made by one of us (T.F.) in his early light microscopic observation of the canine neuroinsular complex (Figure 2 in ref. 3), for instance, may reasonably be

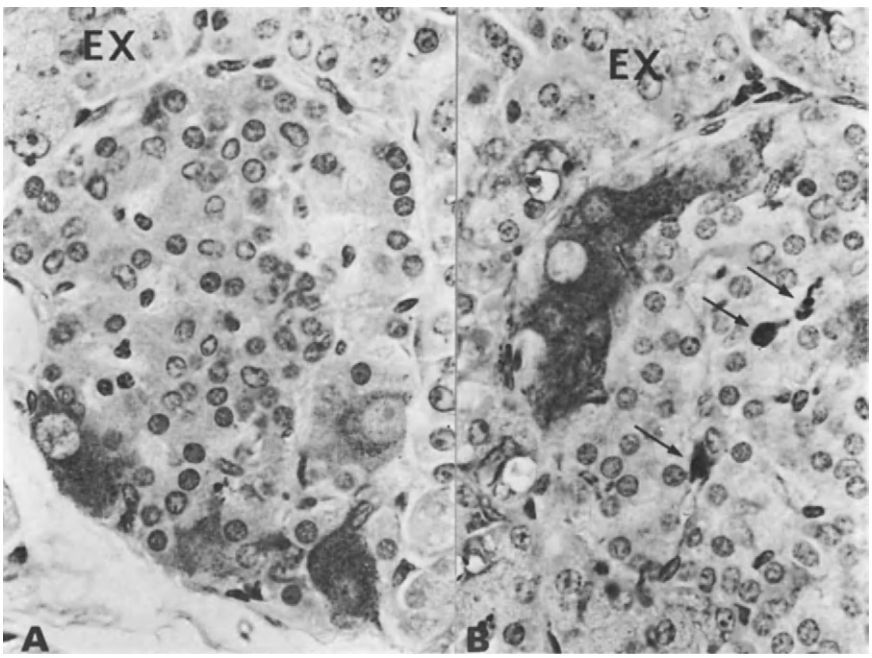

Figure 3. (A) Pancreatic islet of the mink. VIP-like immunoreactivity containing nerve cells are located in the peripheral portion of the islet. They possess fine granules in the cytoplasm that exhibit strong, VIP-like immunoreactivity. EX indicates a portion of the exocrine pancreas. Indirect immunoperoxidase method using rabbit anti-VIP serum (R-502). Counterstained with hematoxylin (×550). (B) VIP-like immunoreactivity containing structures in the pancreatic islet of the mink. Nerve cell bodies with VIP-like immunoreactivity are located in the periphery of the islet facing the exocrine pancreas (EX). Notice that the nerve terminal-like structures indicated by the arrows show strong VIP-like immunoreactivity. The method of specimen preparation is the same as that for (A) (×550).

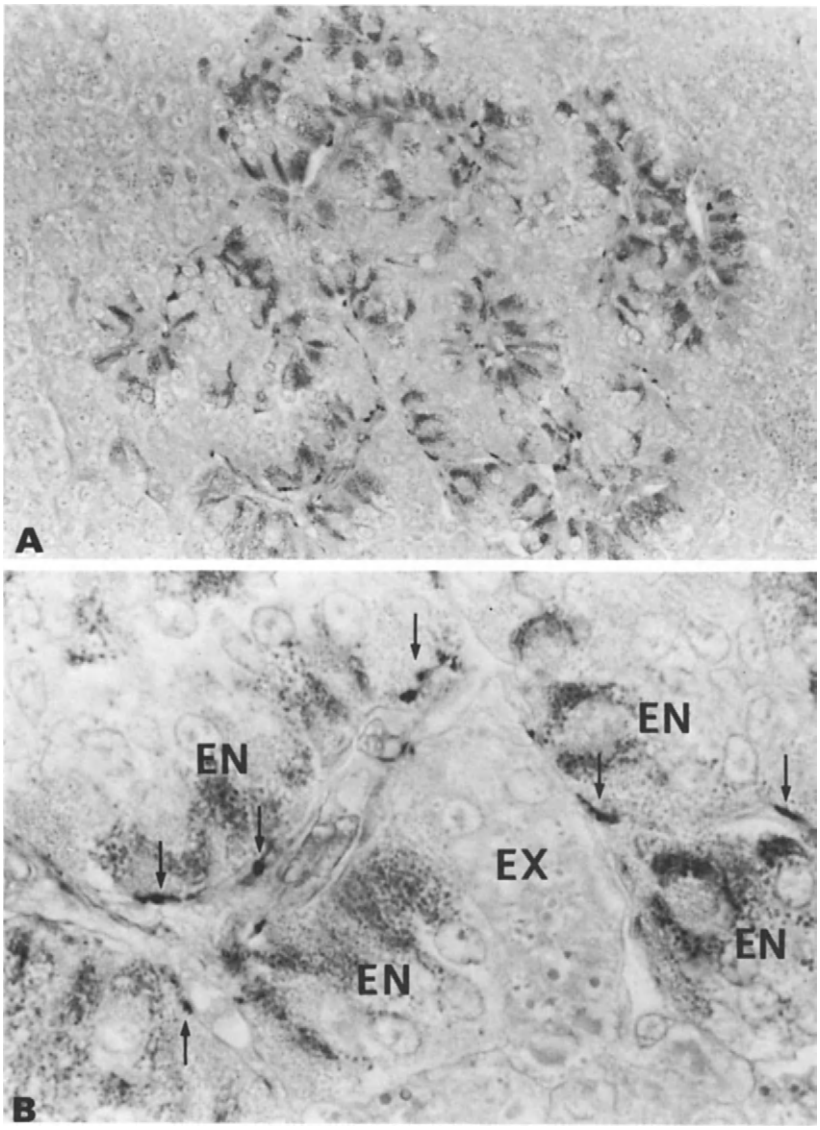

Figure 4. (A) Overview of the pancreatic islet of the Japanese rat racer snake, showing the distribution of VIP-immunoreactive nerve terminals along the surface of the islet cell cord. Many islet cells show weak, but distinct, VIP immunoreactivity. Indirect immunoperoxidase method using rabbit anti-VIP serum (R-502). No counterstaining (×500). (B) Closer view of a peripheral portion of the pancreatic islet of the Japanese rat racer snake. Both endocrine (EN) and exocrine (EX) pancreatic cells are included in this figure. VIP-immunoreactive nerve terminals (arrows) are located in the connective tissue space. Secretory granules of endocrine cells show VIP immunoreactivity. Method of specimen preparation is the same as that for (A) (×1500).

interpreted from the present viewpoint as indicating neurosecretion by a neuron within the complex.

The pancreatic islet of the mink, *Mustela vison*, probably provides the most interesting material for study in this context. In this animal many islets contain one or more nerve cells. One may say that the islets represent neuroinsular complexes. The neuronal somata are directly juxtaposed with islet cells. Our immunocytochemical examination using the antivasoactive intestinal polypeptide (anti-VIP) antiserum, R-502 (26) (kindly provided by Prof. N. Yanaihara at the Shizuoka College of Pharmacy) revealed that the majority (about 60%) of the neurons in mink islets contained VIP-like immunoreactivity both in the somata and processes. The neuronal processes run twisting through the intercellular spaces of the islets. The presumed terminal portions of the processes contain more condensed VIP-like substance and some of them are seen to terminate close to blood capillaries (Figure 3).

VIP-Immunoreactive Nerves in Snake Islets

During our study on the phylogeny of the endocrine pancreas we had an occasion to examine the islets of the Japanese rat racer snake, *Elaphe quadrivirgata* (Boie). Applying immunocytochemical examination with the antiserum mentioned earlier, we were surprised by the abundance of VIP-immunoreactive nerves in the islets. As single or small-bundled fibers of beaded profiles, they were found mostly in the pericapillary space, i.e., between the capillary endothelium and the islet cell cord (2) (Figure 4A, B).

Electron microscopic observation confirmed the occurrence of numerous nerve fibers with terminal and preterminal swellings filled with synaptic vesicles (Figure 5). The vesicles were mainly oval or elongated in shape and devoid of any visible core substance. In addition, the nerve swellings contained more or less numerous round granules with a large, round core of relatively high electron density. They measured about 120 nm in diameter. There seemed to be gradations from nerves containing a large number of the granules and a few vesicles to those containing only sparse granules and numerous vesicles. Because of the similarity to the immunocytochemical finding mentioned earlier, we suppose that most, if not all, of the nerves containing the granules correspond to the VIP-immunoreactive fibers. Possible inclusion of a cholinergic component, however, is not excluded. The possibility also remains that VIP-like substance and a known transmitter, like acetycholine, might be contained in one and the same axon (Figure 5).

At any rate the nerves are invested in Schwann cell cytoplasm except in the pericapillary space. Some of the naked portions contain numerous granules and vesicles facing the capillary endothelium, which is pierced by numerous, twisted channels corresponding to the pores in the more attenuated capillary endothelium in mammals (15). It seems reasonable to suggest that the neural

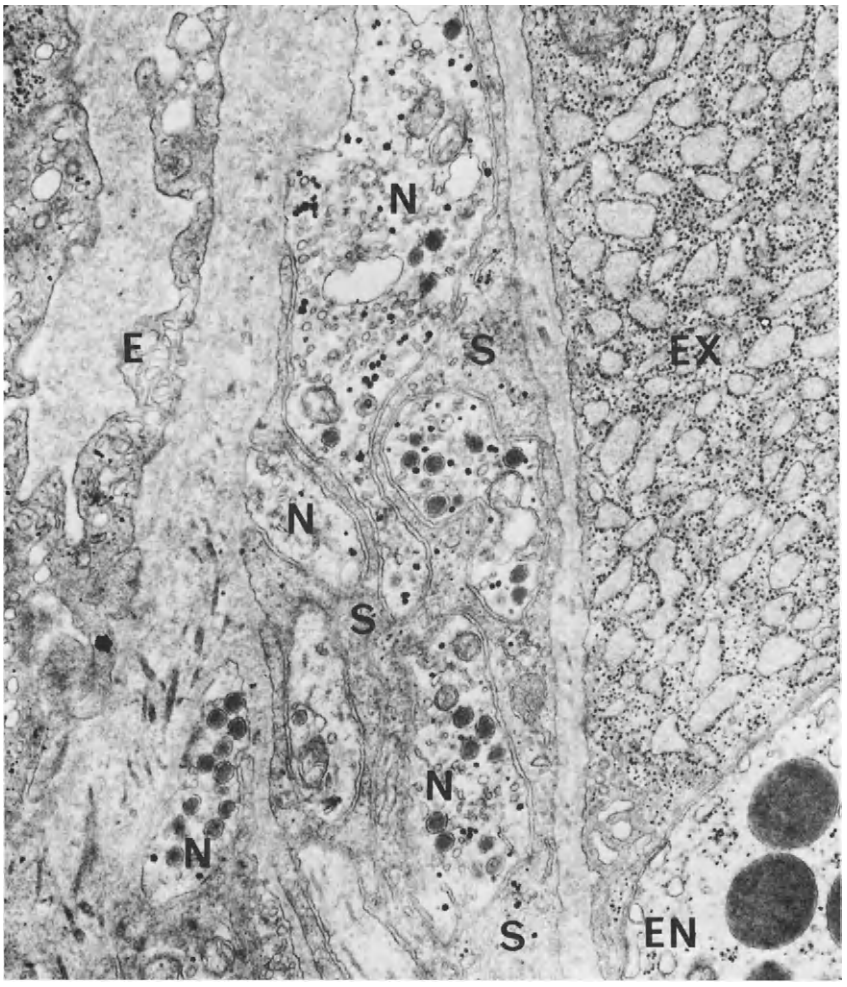

Figure 5. Electron micrograph of the pancreatic islet of the Japanese rat racer snake showing fine structure of nerve terminals (N) located in the pericapillary space between the capillary endothelium (E) and endocrine (EN)/exocrine (EX) pancreatic cells. Notice that the nerve terminals (N) are naked on the capillary side, but they are separated from the endocrine/exocrine cells by a thin layer of Schwann cells (S) (×27,000).

secretions, including the VIP-like substance, may largely enter the bloodstream here, besides acting on adjacent endocrine cells by diffusion.

In the Japanese rat racer snake, the VIP-immunoreactive nerves are concentrated in islets. The exocrine parenchyma of the pancreas contains much smaller numbers of nerves, which occur mainly perivascularly. No VIP-immunoreactive neuronal somata could be found in our preparations, and it is likely that the VIP-immunoreactive fibers in the snake pancreas are extrinsic (2).

Insuloacinar Portal System

The long-forgotten findings of early histologists (21,24) on the microcirculation through the pancreatic islet have recently been rediscovered and reevaluated. It is now established in mammals that an arteriolar vas afferens enters the islet to form its capillary bed, which in turn issues vasa efferentia to the capillary bed of the exocrine parenchyma (Figure 6). The vasa efferentia, although capillary in nature, represent more or less straight routes connecting the two capillary beds and therefore may be called insuloacinar portal vessels (4,10,12).

Because of this microcirculatory design, the islet hormones can be conveyed to the exocrine tissue in very high concentrations. Their possible secretagogous and trophic actions on the exocrine pancreas have been discussed (12). Special attention should be given to the experiment in perfused rat pancreas showing a marked effect of insulin on the acinar tissue in potentiating the action of pancreozymin (14). Glucagon, somatostatin, and

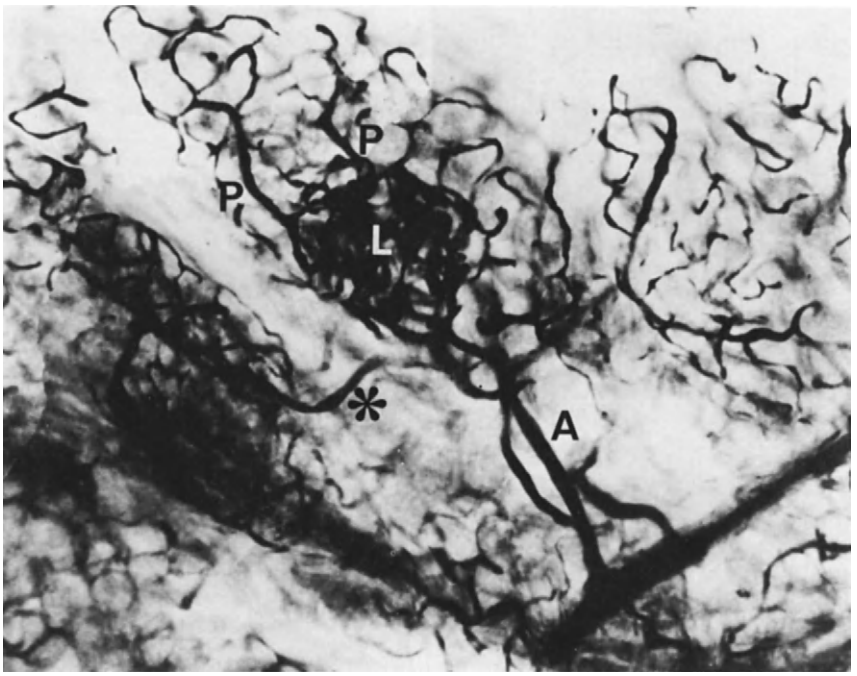

Figure 6. Photograph of the India-ink-injected pancreas of the rat showing the microcirculatory design of the islet. An arteriolar vas afferens (A) enters the islet (L) and forms a capillary network. On the other hand, venous vasa efferentia (P) come out from the islet capillary bed and then nourish the exocrine pancreas. Thus an insuloacinar portal system is formed. An arteriole marked by the asterisk branches into capillary network in the exocrine pancreas without passing the islet.

pancreatic polypeptide are known to exert depressive effects on the exocrine pancreas.

Significance of Insular Neurosecretion

In the preceding section we stated that neurons or their process terminals are quite numerous in the pancreatic islets in certain mammalian and reptilian species and we demonstrated some morphological data suggesting the release into the blood of the secretions of the neurons. The electron micrographs of the insular nerves in the dog are similar in appearance to neurohemal organs like the median eminence and the neurohypophysis.

Just as the neurosecretions in the median eminence are conveyed to the adenohypophysis via the hypophyseal portal vessels, the insular neurosecretions are conveyed to the exocrine pancreas via the insuloacinar portal vessels. They must exert their actions on the exocrine cells.

VIP is chemically related to secretin, and a secretin-like activity of low potency has been shown in some mammals, including the dog (17,20). In the pericapillary space of dog islets we identified, besides the peptidergic-type axons that may support the possible neurosecretion of VIP, different types of axons that were presumed to contain acetylcholine, noradrenaline, and dopamine. It is likely that all of these "neurotransmitters" exert their actions on the exocrine pancreas when they are conveyed there. This new view may help to resolve the existing controversies regarding the effects and mechanisms of vagal and sympathetic stimulation on the pancreatic secretion (20). The reason the exocrine pancreatic cells generally are so poorly innervated in spite of their reactivity to autonomic nerves (20) now seems elucidated. The possible occurrence of dopamine as an insular neurosecretion is especially worthy of attention as this monoamine is known to exert a very strong secretin-like effect in the dog (13).

The hypothesis of insular neurosecretion was a product of the concept of paraneurons. In the islet the endocrine cells, the paraneurons, seem to be not so much under the control of neurons as working in parallel with the neurons. The latter seem to use their secretions more as hormones than as transmitters in the islets of certain species.

The existence of the insuloacinar portal system suggests that the neurons and paraneurons may be gathered in the small islets in order to control the exocrine pancreas. Those that have command of the islet have control of the whole pancreas.

To make the story more complete and convincing, additional experiments and observations remain to be performed. In the dog there is the need to demonstrate increased amounts of VIP and other "neurotransmitters" in the pancreatic blood in response to nerve stimulation. In the mink the terminals of the intrainsular neurons must be made clear. The insuloacinar portal circulation established in mammals remains to be confirmed in the snake. Furthermore, the secretagogous effect of VIP on the pancreas should be examined in the mink and the snake.

SUMMARY

This chapter proposes that the nerves in the pancreatic islet release their secretions into the capillary blood and that these substances are transferred to the exocrine pancreas to regulate its secretory activity.

In the dog, nerve terminals are especially numerous in the pericapillary space of islets and typical images of a neurohemal organ are demonstrated under the electron microscope. Nerves showing a similar relation to blood capillaries are seen in snake islets. VIP-like immunoreactivity is demonstrated in these pericapillary nerves of the Japanese rat racer snake. VIP-like immunoreactivity is localized also in the neurons in the pancreas of the dog and mink, the latter animal possessing numerous VIP-positive neurons intermingled with ordinary islet cells. The possible secretin-like activity of VIP, which is evidenced in the dog, is stressed. At least in the dog, other "neurotransmitters" apparently are released as neurohormones and may have secretagogous effects on the exocrine pancreas.

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Classification of Gastroenteropancreatic Nerves

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The need for some classification of the nerves of the gastroenteropancreatic system is apparent now that an increasing number of functionally and chemically defined nerve types is being recognized (7,15). Table 1 lists those active substances that have been shown beyond reasonable doubt to be present in axons within the gastroenteropancreatic system. Except for norepinephrine, each of these substances is found in intramural nerve cell

TABLE 1
Active Substances Identified in GEP Nerves beyond Significant Doubt

Substance	Comments	Confirmed in man
Acetylcholine	Transmitter in several pathways	Yes
Norepinephrine (noradrenaline)	Except in rare cases, norepinephrine nerves of extrinsic origin[a]	Yes
5-Hydroxytryptamine	See Gershon (10)	No
Bombesin-like peptide	In enteric neurons	No
Cholecystokinin (CCK) terminal peptide	Disagreement whether dominant form is CCK8 or CCK4[b]	No
Enkephalins	Both Leu- and Met-enkephalin in enteric neurons	Yes
Somatostatin	In enteric neurons	No
Substance P	In enteric and extrinsic neurons	Yes
Vasoactive intestinal polypeptide (VIP)	In enteric neurons	Yes

[a]Furness and Costa (6).
[b]Larsson and Rehfeld (13); Dockray *et al.* (4).

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TABLE 2
Active Substances Suspected or Proposed in Intestinal Nerves

Substance	Reference and comments
Angiotensin	Fuxe *et al.* (9). Immunohistochemistry only
Gastrin	Uvnäs-Wallensten *et al.* (16), Dockray *et al.* (4)
Neurotensin	Schultzberg *et al.* (15). Immunohistochemistry only. No cell bodies located.
Pancreatic polypeptide	Loren *et al.* (14). Immunohistochemical demonstration in enteric neurons.
Adenosine 5′-triphosphate	Proposed as neurotransmitter by Burnstock (1,2).
γ-Aminobutyric acid	Jessen *et al.* (12).

bodies, that is, the neurons are part of the enteric nervous system. In some cases—for example, acetylcholine and substance P—a minority of axons are of extrinsic origin. Norepinephrine axons, with rare exceptions, arise from cell bodies in prevertebral (sympathetic) ganglia (6). Of the biologically active substances in gastroenteropancreatic nerves, only acetylcholine and norepinephrine have been shown to be neurotransmitters. The peptides included in Table 1 have been found in enteric nerves of a wide variety of mammals (8), and although they have not yet all been demonstrated in man, it seems likely that they occur in all mammalian species. 5-Hydroxytryptamine has been included because of the very strong evidence that suggests it to be present in enteric nerves (10) even though some doubts have been expressed about its presence and proposed role as a neurotransmitter (3,5).

Table 2 lists additional substances whose presence in nerves of the gastroenteropancreatic system is suspected but is either unconfirmed or open to doubt.

There are a number of fundamental, yet unanswered, questions about the different active substances in intestinal nerves. Foremost among these is whether all are neurotransmitters in the usually accepted sense of a substance that, when released by an action potential-dependent mechanism, has an acute effect on the excitability of an adjacent cell. With such basic questions still unanswered, it is not possible to nominate roles for the majority of nerve types. Furthermore, it is not yet possible to identify uniquely any of the nerves ultrastructurally in conventionally fixed material (11).

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Regulation of Metabolism by Gastroenteropancreatic Peptides

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Until recently, interest in the functional importance of biological activities demonstrable with peptide-containing extracts of gastrointestinal tissues was mainly concerned with their possible roles in regulation of the digestive functions of the gastrointestinal tract. These roles have been conceived as hormonal in nature, and experimental evidence has supported the concept of the gut as an endocrine organ with the capacity to integrate its exocrine secretory and absorptive functions in the processing of nutrients. At the outset, when nothing was known about the regulation of the metabolic functions of the pancreas, it was speculated that hormonal secretions of the small intestine might also regulate the endocrine functions of the pancreas by a mechanism analogous to that of secretin in the control of the water and bicarbonate output of the gland (20). However, the confusing results of experimental studies designed to examine this possibility, and the later recognition of direct effects of glucose in arterial blood on the output of insulin and glucagon from the gland that appeared to be capable of accounting for the physiological regulation of these endocrine secretions, discouraged interest in the regulatory functions of gastrointestinal hormones in metabolism.

More recently, the recognition of differences between the metabolic responses to nutrients delivered by the physiological gastrointestinal route and by the parenteral intravenous route has led to the conclusion that the gastrointestinal tissues exert regulatory effects on the fate of nutrients absorbed from the gut. The most obvious of these phenomena is the difference between the response of the concentration of plasma insulin to delivery of glucose by the enteral and parenteral routes, which suggests that the glycemic stimulus to insulin secretion is not adequate to account for the response of the endocrine pancreas to glucose absorbed from the

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gastrointestinal tract (16,13,7). This led to the definition of the enteroinsular axis (19) and to the suggestion that humoral secretions of the gut potentiate the response of the beta cells to a rise of arterial glucose concentration. It was also inferred that the relatively greater output of insulin in response to absorption of glucose from the gut accounts for the superior "tolerance" of the system to glucose entering by the physiological route. Thus there has been a reevaluation of the importance of hepatic-portal vascular perfusion of the liver as a factor in the determination of physiological glucose disposal, and experimental evidence has suggested that the liver does not serve effectively as a trap for glucose entering by this route simply by virtue of the vascular arrangement (15,18).

There followed a search among peptides extractable from the gastrointestinal tract for agents that might take part in this physiological mechanism. In the course of this work the pharmacological insulinotropic action of peptides of the secretin-glucagon family was documented, and evidence has accumulated suggesting that one of these peptides, gastric inhibitory polypeptide (GIP), is well qualified to take part in this system as a hormone (3). Thus in studies of the response to the nonphysiological stimulus of ingestion of glucose alone, evidence has been reported suggesting that neither gastrin nor secretin are secreted in quantities sufficient to result in insulinotropic effects such as can be elicited with pharmacological doses of these peptides, whereas the blood levels of immunoreactive GIP attained in response to this stimulus are similar to those produced by intravenous infusions of highly purified GIP in quantities sufficient to enhance the insulin response of infusion of glucose to a similar degree. However, in interpretation of these apparently exemplary studies of the "copying" type, some important reservations must be made. First, under physiological conditions the interactions among the several peptides with potential metabolic effects of this type remain to be evaluated, as do interactions of humoral secretions with nervous mechanisms. As a second reservation, these studies take no account of the possible importance of relatively greater concentrations of the gastrointestinal peptides in hepatic-portal blood under physiological conditions. Although this factor would not appear to be important with respect to humoral stimulation via the systemic circulation, there is evidence that complex interactions between the gut and the liver occur in this system, which may modify the net effects of the enterosystemic hormonal mechanisms. As a third reservation, the identities of the highly purified exogenous agents and the endogenous immunoreactive agents have not been established, and the demonstrable heterogeneity of the endogenous immunoreactive peptides cannot be taken into account adequately in the copying experiments.

The great potential complexity of regulatory functions of intestinal peptides in the metabolism of nutrients is illustrated by consideration of the several ways in which these peptides might affect metabolism, as illustrated in Figure 1. It transpires that even in the relatively simple case of the metabolism of

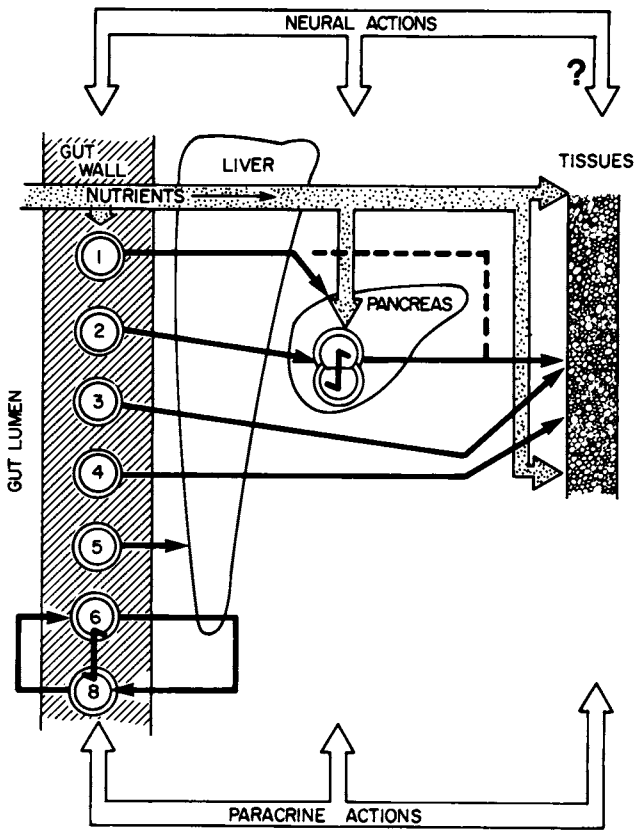

Figure 1. This scheme represents the several ways in which peptides elaborated in the gastrointestinal tract might take part in regulation of metabolism. In mechanism 1 the so-called enteroinsular action of gastrointestinal peptides is illustrated, together with the possible feedback effect of the pancreatic hormone on the intestinal hormone. In mechanism 2 a gut peptide acts directly on an endocrine cell in the pancreas, without interaction with a metabolite, and modifies the output of a pancreatic endocrine secretion, by direct or paracrine action in the islet. In mechanism 3 a gut hormone interacts with a pancreatic hormone at a target tissue in metabolism. In mechanism 4 a gut peptide acts directly on a target tissue. In mechanism 5 the action of the gut hormone is supposed to be confined to the liver, but this model does not exclude the possibility that the hepatic effect is associated with effects transmitted to other tissues by hormonal or other means. Mechanisms 6, 7, and 8 represent interactions between endocrine polypeptide cells in the gut, taking place via systemic, paracrine, or luminal routes, respectively, and it is clear that such effects might modulate the activities of endocrine cells involved in regulation of metabolism. The scheme also indicates the possibilities of interactions with aminergic or peptidergic neural mechanisms, and of paracrine actions at all levels.

glucose, models exist for each of the types of action illustrated in the scheme. The scheme illustrates the so-called incretin effect; that is, the potentiation of glucose-stimulated insulin secretion by humoral agents from the gastrointestinal tract, for which secretin serves as the original pharmacological model (9) and which seems to be a physiological action of GIP, as discussed earlier. It is also apparent that similar mechanisms might serve to affect

the functions of other endocrine cells of the pancreas, including the alpha cells, and pharmacological evidence has shown that GIP is a potential glucagonotropic agent (3). The physiological significance of this action of GIP is uncertain, but there is evidence suggesting that the glucagonotropic action of GIP may be manifest in pathophysiologic situations associated with hypersecretion of glucagon, particularly in uncontrolled diabetes mellitus (3) and in hepatic cirrhosis (8).

It is also apparent that endocrine secretions of the gut might affect metabolism by direct actions at the level of the tissues involved in the disposal of nutrients, rather than through the mediation of other endocrine secretions such as those of the pancreas. In the case of the metabolism of glucose, pharmacological models of such effects have been identified. An interaction between secretin and insulin in the metabolism of glucose in muscle in the human forearm has been described (5). It has also been shown that GIP can compete with glucagon for specific receptors on adipocytes and that GIP acts in this system as an antagonist of the action of glucagon (11). These examples represent interactions between gastrointestinal peptides and pancreatic peptides at the level of the target tissues, but pharmacological examples have also been described in which independent direct actions of gastrointestinal peptides appear to occur. Thus it has been shown that GIP can stimulate the production of glucose by the liver in dogs infused with somatostatin in doses that suppress the secretion of insulin and glucagon (17). Presumably this pharmacological effect represents further evidence of the action of GIP at glucagon receptors, but this has not been proved. There also exists less direct evidence of a possible effect of endocrine secretions of the gastrointestinal tract on metabolism, exerted through actions at the liver (15). These effects came to light in studies of the possible importance of portal perfusion of the liver, in which surgical porta-caval anastomoses in dogs had no effect on the metabolism of glucose given intravenously, but resulted in marked impairment of the disposal of glucose administered by way of the gastrointestinal tract. This could not be accounted for simply in terms of the diversion of portal blood to the systemic circulation, nor in terms of the changes in the insulin responses to administration of glucose by the physiological and parenteral routes, and led to the suggestion that an interaction between the gut and the liver is important in the disposal of glucose absorbed from the gastrointestinal tract under physiological conditions. It was inferred that this effect might depend on humoral secretions of the gut, acting on the liver, but it could not be determined whether the disposal of glucose favored by such mechanisms occurred in the liver or by means of an action transmitted to the so-called periphery.

Further evidence in support of an enterohepatic interaction in the metabolism of glucose absorbed from the gut has been obtained in studies of human volunteers infused with glucose together with insulin (6). In these studies it was shown that the substantial net retention of ingested glucose

within the splanchnic bed cannot be reproduced when glucose is delivered by intravenous infusion together with physiological or supraphysiological doses of insulin. It was again inferred that an enterohepatic interaction, probably humoral in nature, underlies the physiological response. The importance of this enterohepatic action and indeed its dependence on humoral mechanisms remain to be established, but these observations emphasize the current difficulty encountered in recognition of possible non-insulin-mediated metabolic effects initiated by signals originating in the gut. Thus in any situation in which the so-called enteroinsular axis affects the secretion of insulin, the associated metabolic effects may appear to be accounted for by the actions of insulin, and the occurrence of noninsulin-mediated events may be obscured. This problem calls for studies in which the effects of insulin can be controlled and identified in a quantitative fashion, and it appears that studies in experimental diabetes with controlled insulin replacement, and in diabetes in human volunteers under similar conditions (4), will be useful in unraveling the complexities of the system.

These examples in relation to the metabolism of carbohydrate serve to illustrate the problems already encountered. Analogous mechanisms probably operate in the regulation of metabolism of protein and fat, and these are beset with similar difficulties. In the case of the amino acids, there is good evidence of enteroinsulin potentiation of insulin secretion (10), and this also appears to be associated with enteroinsular stimulation of glucagon secretion (1). In this system the problem of first-pass hepatic uptake of the different classes of amino acids has yet to be addressed in a quantitative fashion, and even the recognition of enteroinsular effects of amino acids has not taken account of the prevailing concentrations of the individual amino acids, with their potential interactions in the pancreatic islets. In the case of fat metabolism, the problem of first-pass hepatic uptake is minimized by the fact that the major route of entry of triglyceride is by way of the thoracic duct to the systemic venous system. However, recognition of enteroinsular effects in the metabolism of this class of nutrients has been hindered by the lack of an appropriate vehicle for parenteral delivery of fat, since the available fat emulsions cannot be regarded as substitutes for clean preparations of chylomicra. These difficulties have been overcome in studies in animals, and in the case of the dog it is clear that enteroinsular mechanisms operate in the stimulation of secretion of glucagon in response to absorption of triglyceride from the gut (2). In relation to the metabolism of triglyceride, an experimental model also exists for a direct effect of a gut hormone on the fate of the nutrient, in the demonstration of an effect of GIP on the activity of lipoprotein lipase in the fibroblasts (12).

Speculation about the possible paracrine actions of peptides produced in the gastrointestinal tract further complicates ideas about the regulatory functions of peptides of gastroenteropancreatic origin in the regulation of metabolism. Clearly, paracrine regulation of any of the peptides active as

hormones in this system could serve to modify their effects on metabolism. Furthermore, pharmacological evidence suggests that some of the identified gastrointestinal peptides can act through humoral mechanisms to modify the release of potential paracrine agents. Thus GIP and perhaps other identified gastrointestinal peptides can affect the release of somatostatin from the pancreas (14), and this release may be associated with paracrine or endocrine effects of somatostatin. It appears likely that this represents only the first of a number of possible hormone-releasing or inhibiting factors secreted by peptidergic endocrine or nerve cells in the gut in response to various stimuli, metabolic or endocrine. The major problem in the investigation of paracrine mechanisms remains the difficulty of recognizing their effects, let alone quantitating them, by means of presently available techniques. These problems in the area of the regulation of metabolism by gastrointestinal-pancreatic peptide secretions are similar to those encountered in relation to other functions of this group of peptides. The endogenous immunoreactive agents are demonstrably heterogeneous and must be further characterized with respect to their physicochemical properties as well as their biological effects; interactions among these agents need to be examined and quantitated; interactions with nervous mechanisms must be evaluated; and these problems await methodological advances before they can be addressed effectively.

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Evidence for Neurohormonal Peptides in Ovarian Carcinoids: A Preliminary Report of Immunohistochemical Findings

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INTRODUCTION

Carcinoid tumors may arise from the ovary or testicle, usually in benign cystic teratomas ("dermoid cysts") (3,8,10,15). Their growth pattern is identical to that of gut carcinoids and may be of the following three types: insular carcinoids, trabecular carcinoids, or strumal carcinoids (18,19,20). The strumal carcinoid is composed of thyroid parenchyma together with a carcinoid component that usually is of the trabecular type (19).

One-third of the insular carcinoids are associated with clinical manifestations of the classical carcinoid syndrome (15,18), and one-tenth of the strumal carcinoids with signs of hyperthyroidism (20). Otherwise, carcinoids arising in the ovary are not known to be associated with any endocrine disorder that could be ascribed to the carcinoid (19).

Whereas there is one comprehensive report on the occurrence of endocrine cells in carcinoids in testicular teratomas (3), there are only a few immunocytochemical studies on ovarian carcinoids (15). Investigations on the occurrence of calcitonin revealed clusters of immunoreactive tumor cells in trabecular carcinoids and strumal carcinoids (6,11). The presence of triiodothyronine and thyroxine has also been described in the strumal carcinoids (12).

During the last few years evidence has accumulated that carcinoids produce neurohormonal peptides, regardless of the location of the tumor.

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Thus a majority of carcinoids of the rectum ("hindgut carcinoids") contain cells reacting with antisera to gastroenteropancreatic (GEP) neurohormonal peptides, such as pancreatic polypeptide (PP), somatostatin, glucagon, enkephalin, β-endorphin, substance P, and even insulin (1,2,7,24). These observations suggest that immunohistochemistry might assist in the classification of carcinoids. In the present study, a large number of clinically and histopathologically characterized ovarian carcinoids (8,18,19,20) have been reinvestigated by immunohistochemistry, using a wide range of antisera against neurohormonal peptides.

Material and Methods

CASES

The material comprised 73 formalin-fixed ovarian carcinoids, of which 38 were of the insular type, 22 of the strumal type, and 13 of the trabecular type. The material was collected from a number of hospitals throughout the world. The condition of the tissue material may have been affected by variables, such as ischemia at operation, differences in the choice of fixatives, inadequate penetration of the fixative to the carcinoid tumor, and age of the paraffin blocks. Paraffin-embedded material was available in 19 cases (5,6, and 8 in each of three groups). In 57 cases only three to six unstained slides were available; consequently, only a limited number of antisera could be tested (marked by an asterisk in Table 1). Salient features of the clinical history of each patient were obtained from the record sheets of the preceding studies (8,18, 19, 20).

ANTISERA

The antisera, as well as details on source and working dilutions, are listed in Table 1 [the gastrin antiserum cross-reacts with cholecystokinin (CCK)]. Fluorescein-isothiocyanate-labeled and unlabeled goat-anti-rabbit-IgG antisera were purchased from the State Bacteriological Laboratory, Stockholm, Sweden. The peroxidase–antiperoxidase (PAP) complex was obtained from Dakopatts, Inc.; Copenhagen, Denmark.

IMMUNOHISTOCHEMISTRY

Deparaffinized sections were hydrated and rinsed thoroughly overnight. The sections were then processed for immunohistochemical demonstration of the different neurohormonal peptides listed in Table 1, using the indirect immunofluorescence (5) or the immunoperoxidase (PAP) (23) method. The antisera were applied for 24 h (PAP technique) or for 3 h at room temperature (immunofluorescence). Controls were run as recommended by Sternberger (23) and included the application of antiserum pretreated with excess antigen.

TABLE 1
Specification of the Antisera Used and Their Source of Origin

Antisera raised against	Dilution[b] IF	Dilution[b] PAP	Code	Source
Pure human pancreatic polypeptide (HPP)(*)		1/1280	HPP	R. E. Chance, Eli Lilly Res. Lab., Indianapolis, Ind., USA
Pure bovine PP (BPP)(*)	1/320		7823	J. E. Thorell, Dept. Nucl. Med., Malmö Gen. Hospital, Malmö, Sweden
Synthetic ovine somatostatin (*)		1/1280	19578	M. P. Dubois, Station Physiol. Reprod., INRA, Nouzilly, France
Pure porcine glucagon (*)		1/640	7811	J. E. Thorell, Dept. Nucl. Med., Malmö Gen. Hospital, Malmö, Sweden
Highly purified porcine vasoactive intestinal polypeptide (VIP)		1/5120	5603	J. Fahrenkrug, Dept. Clin. Chem., Copenhagen County Hosp., Glostrup, DK
		1/5120	7852	J. E. Thorell, Dept. Nucl. Med., Malmö Gen. Hospital, Malmö, Sweden
Synthetic Leu- or Met-enkephalin (*)		1/240	Leu-enk	R. J. Miller & K. J. Chang, Burroughs
	1/240	1/640	Met-enk	Wellcome Res. Lab., Triangle Park, NC, USA
Synthetic human β-endorphin (β-lipotropin 61-91)		1/640	7763	J. E. Thorell, Dept. Nucl. Med., Malmö Gen. Hospital, Malmö, Sweden
Synthetic bovine substance P	1/80		K16	G. Nilsson, Dept. Pharmacol., Karolinska Inst., Stockholm, Sweden
	1/20		SP-8	P. Emson, Med. Res. Council, Cambridge, England
Synthetic bovine neurotensin		1/640	HC-8	R. E. Carraway, Lab. Hum. Reprod., Dept. Physiol., Harvard Med. Sch., Boston, Mass., USA
Synthetic human calcitonin		1/160	AS292/5	I. MacIntyre, Endocr, Unit, Royal Post-grad. Med. Sch., London, England
Synthetic human gastrin 2-17		1/5120	4562	J. F. Rehfeld, Dept, Med, Biochem., Univ. Aarhus, Aarhus, Denmark
Synthetic porcine motilin		1/640	MBR-1105	N. Yanaihara, Shizuoka Coll. Pharmacy, Shizuoka, Japan

[a]Antisera marked with an asterisk (*) were used when only a limited number of paraffin sections were available for immunohistochemical analysis.

[b]IF, immunofluorescence (5); PAP, Peroxidase-anti-peroxidase procedure (23).

TABLE 2
Summary of Immunohistochemical Observations[a]

Carcinoid type	Total no.	Immunoreactive peptides[b]							
		PP	SOM	GLUC	VIP	ENK	β-END	CALCIT	NT
Insular	38	1	1	0	0	1	0	0	0
Strumal	22	7	0	4	1	3	1	2	0
Trabecular	13	2	4	3	0	1	0	0	1
Total No.	73	10	5	7	1	5	1	2	1

[a]No immunoreactivity was observed with antisera raised against substance P, gastrin, or motilin.

[b]PP, HPP, and/or BPP (Table 1); SOM, somatostatin; GLUC, pancreatic glucagon; ENK, enkephalin; β-END, β-endorphin; CALCIT, calcitonin; NT, neurotensin. Antisera against neurohormonal peptides marked by an asterisk in Table I (i.e., PP, SOM, GLUC, and ENK) have been tested in 73 tumors, whereas all the other antisera have been tested only in 19.

Results

Seventeen of the 73 carcinoids (23%) contained tumor cells displaying immunoreactivity with antisera against various neurohormonal peptides (Table 2). The most prevalent ones were PP, glucagon, somatostatin, and enkephalin. Only rarely did tumor cells show immunoreactivity with antisera against vasoactive intestinal polypeptide (VIP), β-endorphin, calcitonin, and neurotensin (Table 2). No tumor cells displayed immunoreactivity with antisera against gastrin–CCK, motilin, or substance P.

Differences were observed with regard to the type and frequency of immunoreactive cells in the three types of carcinoids. Fifty percent of strumal carcinoids harbored immunoreactive cells, whereas they were present in 5 and 30% of the insular and trabecular types, respectively.

Characteristically, tumor cells storing immunoreactive PP, glucagon, enkephalin, or calcitonin had a patchy distribution (Figure 1), whereas cells reacting with antisera against somatostatin, VIP, β-endorphin, or neurotensin were more uniformly distributed (Figure 2). In either case, the immuno-

TABLE 3
Classification of the Ovarian Carcinoids According to Histopathological Type and Number of Identified Neurohormonal Peptides Occurring in One and the Same Tumor

Carcinoid type	Total No.	Number of identified peptides per tumor						
		None	1	2	3	4	5	6
Insular	38	36	1	1				
Strumal	22	11	5	3	2	1		
Trabecular	13	9		1	2		1	
Total	73	56	6	5	4	1	1	

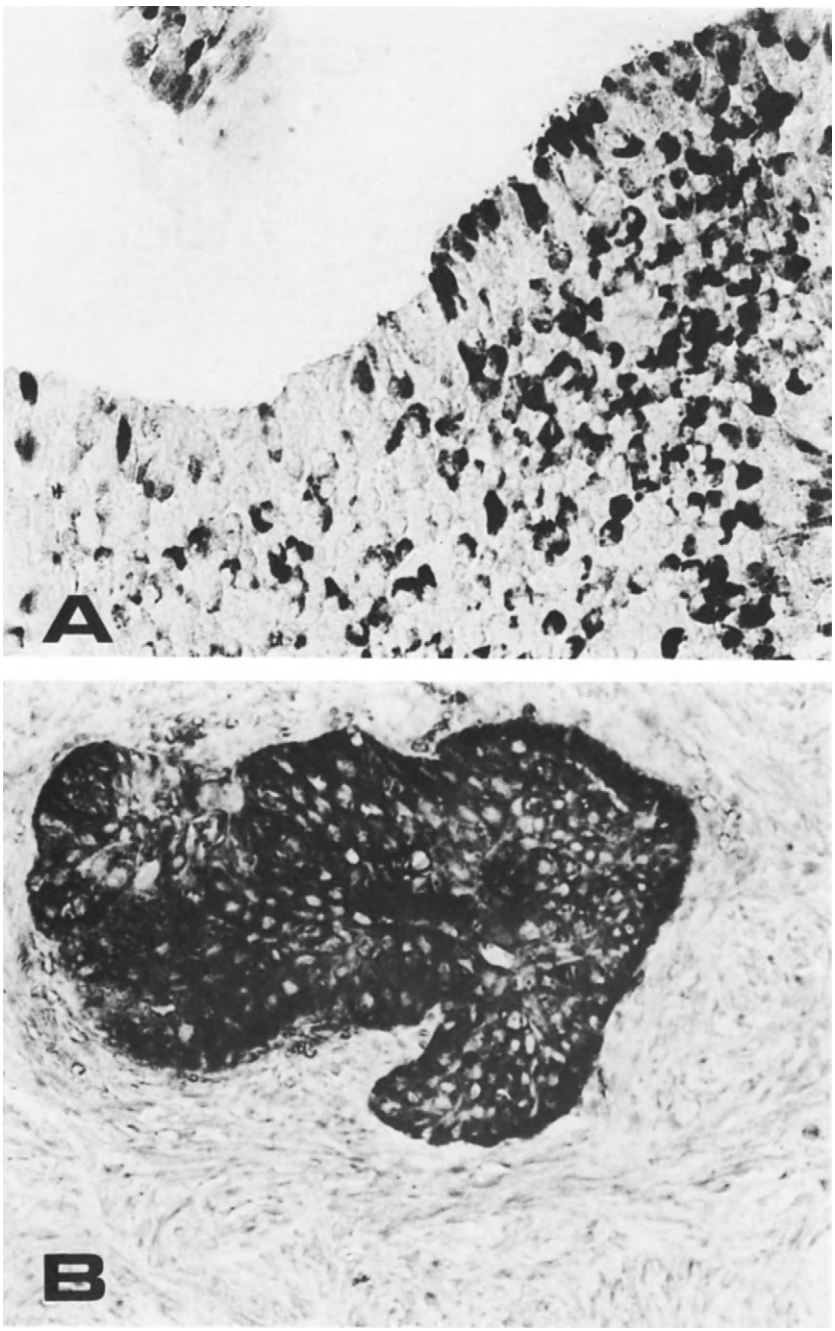

Figure 1. A strumal ovarian carcinoid, displaying intense immunoperoxidase (PAP) staining with antisera against (A) PP and (B) enkephalin, exemplifying the patchy distribution of the immunoreactive cells ×440(A); ×280(B).

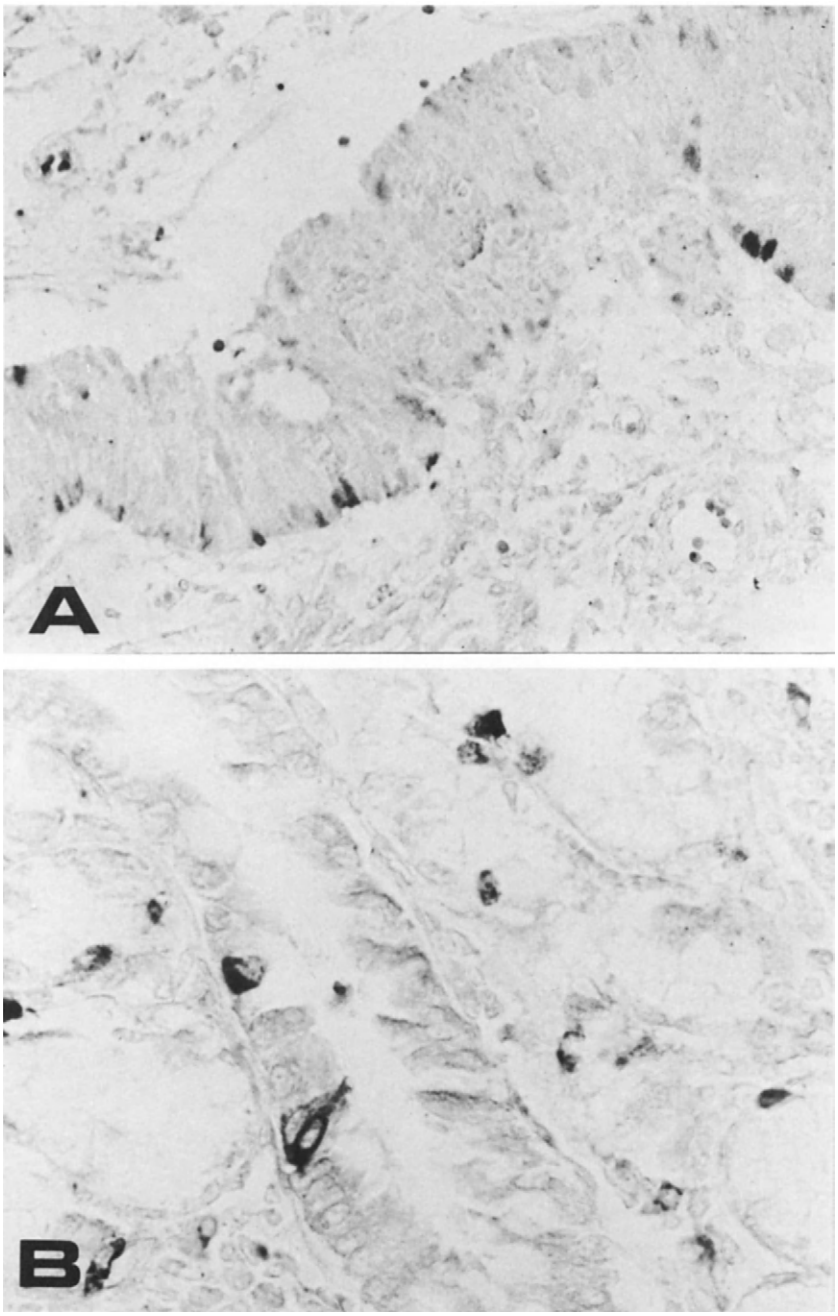

Figure 2. Two ovarian carcinoids, one (A) strumal; one (B) insular, showing widely disseminated immunoreactive tumor cells; in (A) with antisera against glucagon and in (B) against somatostatin. Most cells are of open type, i.e., equipped with apical processes reaching the lumen of the glandlike structures. PAP procedure; ×280(A); ×440(B).

reactive cells constituted only a minor fraction of the entire tumor cell mass. Eleven of the 17 carcinoids with GEP neurohormone immunoreactive cells were multihormonal (Table 3).

As previously reported, one-third of the patients with insular carcinoids showed the carcinoid syndrome. The presence of immunoreactive cells was not reflected by any specific symptom of endocrine disorder of any other kind.

Discussion

From earlier studies, the light microscopic appearance (growth pattern, tumor cell morphology, staining characteristics) pointed to a close relationship between ovarian carcinoids and endocrine tumors arising in the gut and pancreas. The present immunohistochemical findings make such a comparison even more justified; in fact, all the immunoreactive peptides detected in cells of ovarian carcinoids have previously been found to occur in intestinal and/or pancreatic endocrine tumors (1,2,7, 16,24).

Ovarian carcinoids resemble gut endocrine tumors in that several neurohormonal peptides may occur in one and the same tumor (16). The spectrum of neurohormonal peptides demonstrated in ovarian carcinoids is rather similar to that found in hindgut carcinoids (1,2,7,24). The present findings thus point to a similar origin for the ovarian carcinoids and for the endocrine tumors in the gastrointestinal tract. This conclusion draws strong support from an analogous study of 53 carcinoids in benign testicular teratomas, where 11 (i.e., 21%) contained tumor cells showing immunoreactivity with antisera against PP, somatostatin, or glucagon (3).

Apart from the cases with the classical carcinoid syndrome (18,19,20), the present study has not revealed any conspicuous constellation of clinical symptoms that could be ascribed to the presence of GEP neurohormonal peptides. An example of such a constellation of symptoms is the patient described by Cocco and Conway (4) with hypergastrinemia and Zollinger-Ellison syndrome who was cured by extirpation of an ovarian mucinous cystadenoma, another kind of tumor in which GEP endocrine cells have been found to occur (9,13,14, 21,22).

Whereas carcinoid tumors are rare elsewhere in the body (2,10), they are next to epidermoid carinomas in frequency in tumors arising in benign cystic teratomas of the ovary. The reason for the relatively high incidence of carcinoids in ovarian and testicular dermoid cysts is unknown. A putative connection between malformations and carcinoid tumors (8) suggests pathogenesis at the genome level (17).

Summary

Ovarian carcinoids as a rule arise in benign cystic teratomas ("dermoid cysts") of the ovary and are histopathologically subdivided into three main types. A limited number of sections and paraffin blocks were available for immunohistochemical analysis of 38 insular carcinoids, 22 strumal carcinoids,

and 13 trabecular carcinoids of the ovary, fixed in formalin. Tumor cells immunoreactive with antisera against PP, somatostatin, glucagon, VIP, enkephalin, β-endorphin, calcitonin, and neurotensin were found in 5% of the insular carcinoids, as well as 50 and 30% of the strumal and trabecular ones, respectively. In all, immunoreactive tumor cells were observed in 17 ovarian carcinoids (i.e., 23%). In 11 of these it was possible to demonstrate more than one neurohormonal peptide. The spectrum of immunoreactive peptides was similar to that observed in gut endocrine tumors, indicating that the ovarian carcinoids arise from gastroenteropancreatic derivates in the dermoid cysts.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Cancer Society (Project No 806-5XA), the Swedish Medical Research Council (Project No 12X-718), the Cancer Research Foundation at Malmö General Hospital, and the Faculty of Medicine at the University of Lund, Sweden.

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Distribution, Origin, and Pathology of the Gut Peptidergic Innervation

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INTRODUCTION

The gut is innervated by a neuronal network, both extrinsic and intrinsic, which is heterogeneous in morphology, histochemistry, and function (22). This network innervates every structure of the gut wall, including the two muscle layers, blood vessels, mucosa, and submucosa. Its main function, the coordination of gut motility, epithelial secretion, and blood flow, is carried out by means of a variety of neurotransmitters (22). The actions of some of these, such as acetylcholine and noradrenaline, are well established, whereas for others, such as the various neuropeptides, a neurotransmitter function has yet to be fully proved.

The existence of a large and complex peptidergic component of the autonomic nervous system has recently been recognized (43). The peptides found in the nerves of this system include vasoactive intestinal polypeptide (VIP), substance P, cholecystokinin (CCK) and its tetrapeptide amide, tetrin, bombesin, the enkephalins, thyrotropin-releasing hormone (TRH), and possibly somatostatin. Although they are distributed throughout the entire length and width of the gut, the nerves containing particular peptides often have a characteristic distribution within the gut wall and a well-defined set of actions compatible with their localization. We shall, therefore, discuss the main actions of most of the individual peptides and their distribution in the enteric nervous system as revealed by immunocytochemistry and radioimmunoassay.

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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Individual Peptides: Actions, Distribution

SUBSTANCE P

Substance P was discovered by von Euler and Gaddum (19), who noted that cholinergic stimulation was accompanied by the release of a substance capable of inducing a potent atropine-resistant gut muscle constriction and vasodilatation. Substance P was further characterised as an 11 amino acid peptide and found to be identical to Leeman's sialogenic peptide (38). Substance P is widely distributed in the central and peripheral nervous system and large quantities are found in the gut. Its principal localization is in the autonomic innervation (Figure 1). Numerous fine, varicose nerve fibers are seen in most structures of the gut wall, including the two ganglionated plexuses, the muscle coat, the mucosa, the submucosa, and the blood vessels. This anatomical distribution corresponds to its wide spectrum of pharmacological actions on the gut. Substance P powerfully contracts gut musculature (10,57), either by a direct action (52,57) or possibly by its release from excitatory noncholinergic interneurons of the myenteric plexus after high-frequency nerve stimulation (30). In addition, although this action is still poorly understood, substance P may be the putative gut sensory neurotransmitter involved in peristalsis (22).

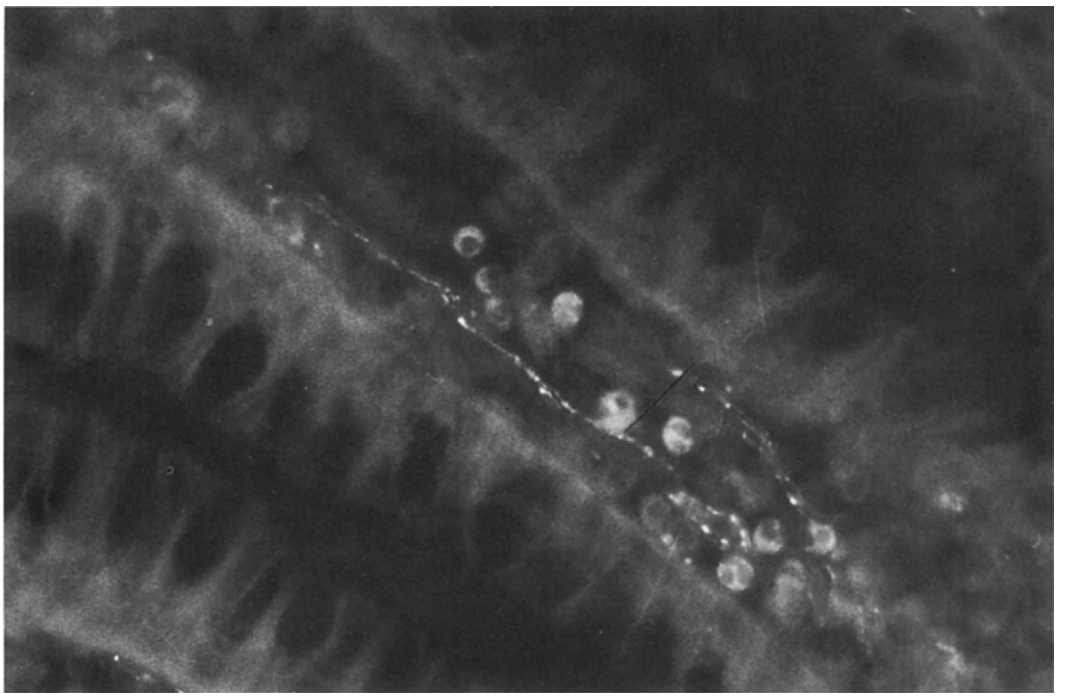

Figure 1. Human colonic mucosa stained with antibodies to Substance P. Numerous fine varicose nerve fibers are seen in close proximity to the intestinal epithelium (×625).

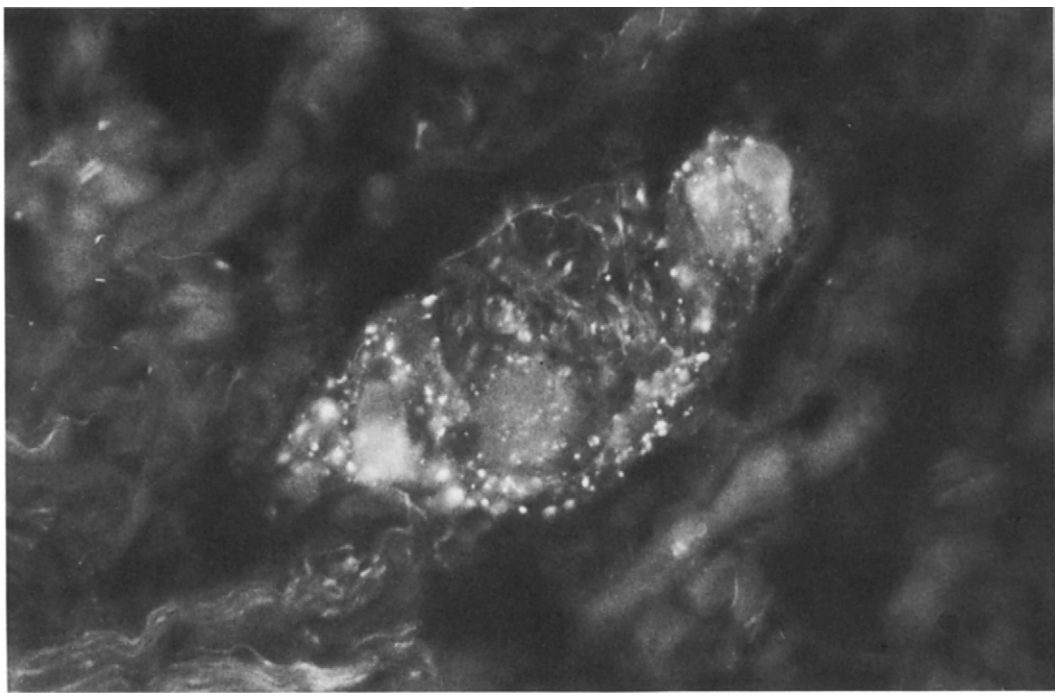

Figure 2. VIP-containing nerve fibers in Meissner's plexus of the human colon (×450).

VIP

Vasoactive intestinal polypeptide (VIP) was discovered after the finding of a potent vasodilatory action in certain fractions of extracts of porcine gut (50). It was later found to be a twenty-eight amino acid peptide with considerable amino acid sequence similarities with the members of the secretin family, glucagon, gastric inhibitory polypeptide (GIP), and secretin. VIP is found both in the brain and in peripheral nerves. The gut contains large quantities throughout its entire length. Numerous brightly stained VIP-containing fibers are always found in all layers of the gut wall (Figure 2), but principally in the mucosa and submucosa; this distribution supports the suggestion that the role of VIP is that of a powerful stimulator of water and electrolyte secretion by the gut mucosa (23). Numerous VIP receptors have recently been reported along most of the length of the gut mucosa (33). The two enteric plexuses are richly innervated by VIP; however, the VIP neuronal cell bodies are mainly found in the submucous plexus (Figure 2). This provides anatomical evidence for interconnections between the two main enteric plexuses of the gut wall and suggests yet another role for VIP in the gut: the coordination of functions of the two plexuses (28). Williams and North have recently reported potentiation of electrical activity of myenteric neurons after the addition of VIP at low concentrations (56). VIP-containing nerve fibers are

also found in the circular smooth muscle, and in the guinea pig, they are almost absent from the longitudinal muscle, thus explaining the recently reported lack of direct effect of VIP on the guinea pig longitudinal muscle layer (29,52). Like the actions of peptides on other organs, the potent relaxant effect of VIP on gut muscle is slow in both onset and recovery (27). A rich perivascular VIP innervation is also seen in all areas of the gut, in agreement with its powerful vasodilatory properties. Its postulated role as a neurotransmitter is validated by neurophysiological studies that indicate that the atropine-resistant gastric muscle relaxation and intestinal vasodilation may be mimicked by local microinjections of VIP (20). In addition, VIP is found in the synaptosomal fraction (24), and a large rise in tissue levels of VIP is found after local parasympathetic stimulation (5,20). The vagus nerve has recently been shown to contain VIP (20).

THE ENKEPHALINS

The enkephalins are a pair of pentapeptides that differ by only one amino acid (methionine or leucine) at the C-terminal end (25). Unlike VIP-containing nerves, enkephalin-containing nerves are mostly present in the muscle layer (especially longitudinal) (Figure 3) and in the myenteric (Auerbach's) plexus of the guinea pig, matching the distribution within the

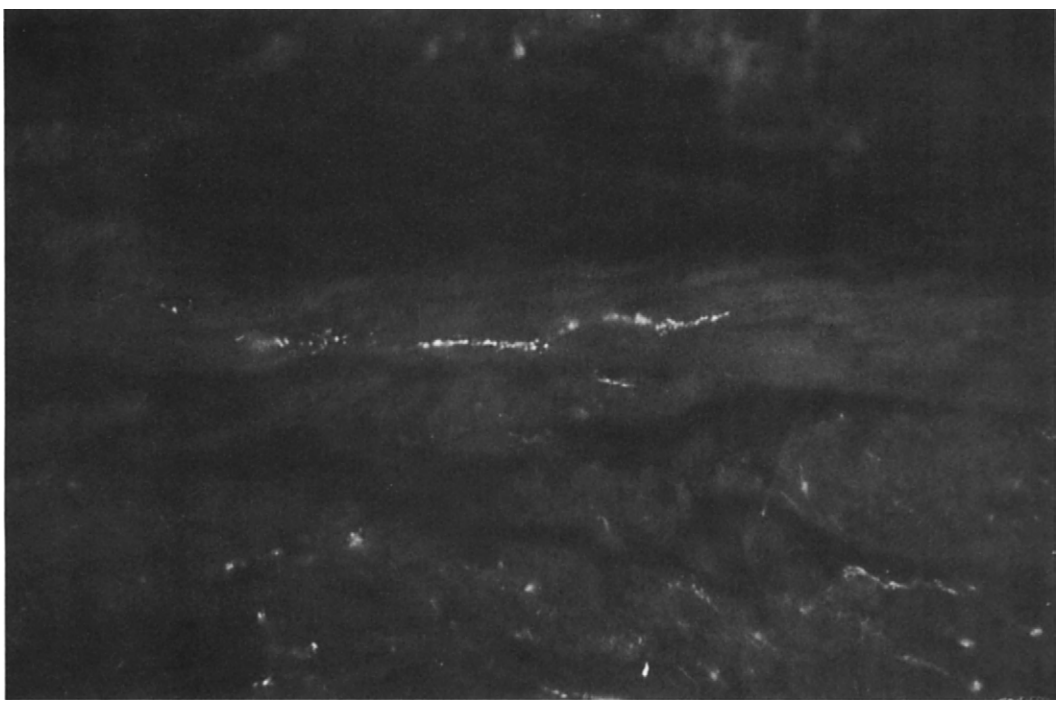

Figure 3. Human colon. Muscularis mucosae containing numerous Met-enkephalin reactive nerves (×450).

gut of opiate receptors that are mostly found in the myenteric plexus (42). The enkephalins may also be present in interneurons within this plexus. The enkephalins exert a wide range of actions in the gut. These include the reduction of intestinal motility and secretion, relaxation of gall bladder tone, and contraction of the sphincter of Oddi, as well as suppression of pancreatic bicarbonate or enzyme secretion after endogenous or exogenous stimulation (32). In addition, enkephalins inhibit both the release of acetylcholine and the firing of neurons of the myenteric plexus (13,41) and act on the cell processes rather than their perikarya of the cell (41). This finding fits well with the findings of naloxone-sensitive opiate receptors in the cholinergic nerve endings of the guinea pig inferior mesenteric ganglion (41,42). Activation of these receptors leads to a decrease of acetylcholine release (7,17,54). The enkephalins may thus play a part in gut motility. This concept is further supported by recently reported interference with the peristaltic reflex caused by enkephalin (27).

GASTRIN-CCK

Both gastrin and CCK, as well as their common C-terminal tetrapeptide amide, have been found in the central and peripheral nervous tissue (34,35,36). In the brain, CCK predominates in the cerebral cortex, whereas gastrin is found mostly in the hypothalamus and pituitary gland (48,49). In the gastrointestinal tract separate nerves containing gastrin and CCK have been reported, those of gastrin being found in the vagus, distal small intestine, and proximal colon, whereas CCK nerves are seen in both plexuses of the colon. In addition, the C-terminal tetrapeptide amide (tetrin) of both gastrin and CCK can be extracted in significant quantities from both the gut and pancreas, where immunocytochemistry localizes it in endocrine cells of the gut (35) and in the innervation, especially that of the endocrine pancreas (34). The postulated additional role for this "gut hormone" as a central and peripheral neurotransmitter is supported by the potassium chloride-induced calcium-dependent release of CCK from superfused brain slices and the finding of large concentrations of CCK and gastrin in the synaptosomal-vesicle fraction of brain cortex and hypothalamus (49).

SOMATOSTATIN

The 14 aminoacid peptide, somatostatin, originally extracted from the hypothalamus (8), has been found in large quantities in the gastrointestinal tract of man and numerous other mammals (2). It is principally localized in endocrine cells of the gut (47) and is thought to have a paracrine or locally controlling role. Somatostatin is the "inhibitory" hormone of the gut (4). It not only blocks the release of most gastrointestinal hormones but also inhibits many gut functions. Its localization in the gut innervation has been only sporadically reported (12,26). However, its neuronal localization would fit well with the reported inhibition of acetylcholine and adrenaline release by somatostatin in organ-bath experiments (11).

BOMBESIN

In contradistinction to somatostatin, bombesin appears to be the "releasing" hormone of the gut. Its potent gastrin-releasing properties were described first (40), and it was later found to be a significant releaser of most gastrointestinal hormones (6). Bombesin was first extracted from the skin of the discoglossid frog *Bombina bombina* (18) and was later found to be extractable from the mammalian gut, brain, and lung (46). In mammals, in the gut the autonomic innervation is its principal localization, whereas in the lung (55) and in the avian gut (53), bombesin is mostly present in endocrine cells.

THYROTROPIN-RELEASING HORMONE (TRH)

TRH was the first hypothalamic-releasing hormone to be characterized (9). It has recently been reported to be present in significant quantities in the gut (39) and pancreas (31), where by immunocytochemistry it appears to be localized in the autonomic innervation. TRH exerts a number of inhibitory effects on the gut such as the inhibition of pentagastrin-induced acid secretion (15), the prevention of glucose and xylose absorption (16), and the impairment of gastric motility induced by distension (14).

THE INTRINSIC NEURONAL ORIGIN OF THE GUT PEPTIDERGIC INNERVATION

The intrinsic neuronal origin of the peptide-containing nerves of the gut was suggested in 1976 (37) and 1978 (1) as a result of direct observations. It was further supported by neurophysiological experiments (21) as well as by the finding of peptide-containing nerves in organotypic tissue cultures of mouse embryonic gut devoid of extrinsic innervation (51). We have recently been able to validate fully and further expand these observations by immunostaining two types of preparations (28). These were, first, extrinsically denervated gut segments and, second, separately cultured myenteric and submucous plexuses of the gut wall that were necessarily devoid of both extrinsic and intrinsic nerve connections (Figure 4A and B).

DENERVATION EXPERIMENTS

Segments of guinea pig cecum including the taenia coli were denervated by crushing or freezing the perivascular nerves of the mesentery, 3 weeks before the animal was killed. The effectiveness of the denervation procedure was assessed by the disappearance of the adrenergic innervation, which is undoubtedly of extrinsic origin. No differences between the denervated and the control segments of the gut were observed in the immunostaining and the radioimmunoassayable peptide content of VIP, substance P, and enkephalin.

TISSUE CULTURE PREPARATIONS

After careful dissection, the submucous and myenteric plexus, free from connective tissue, were separately placed on glass cover slips in modified

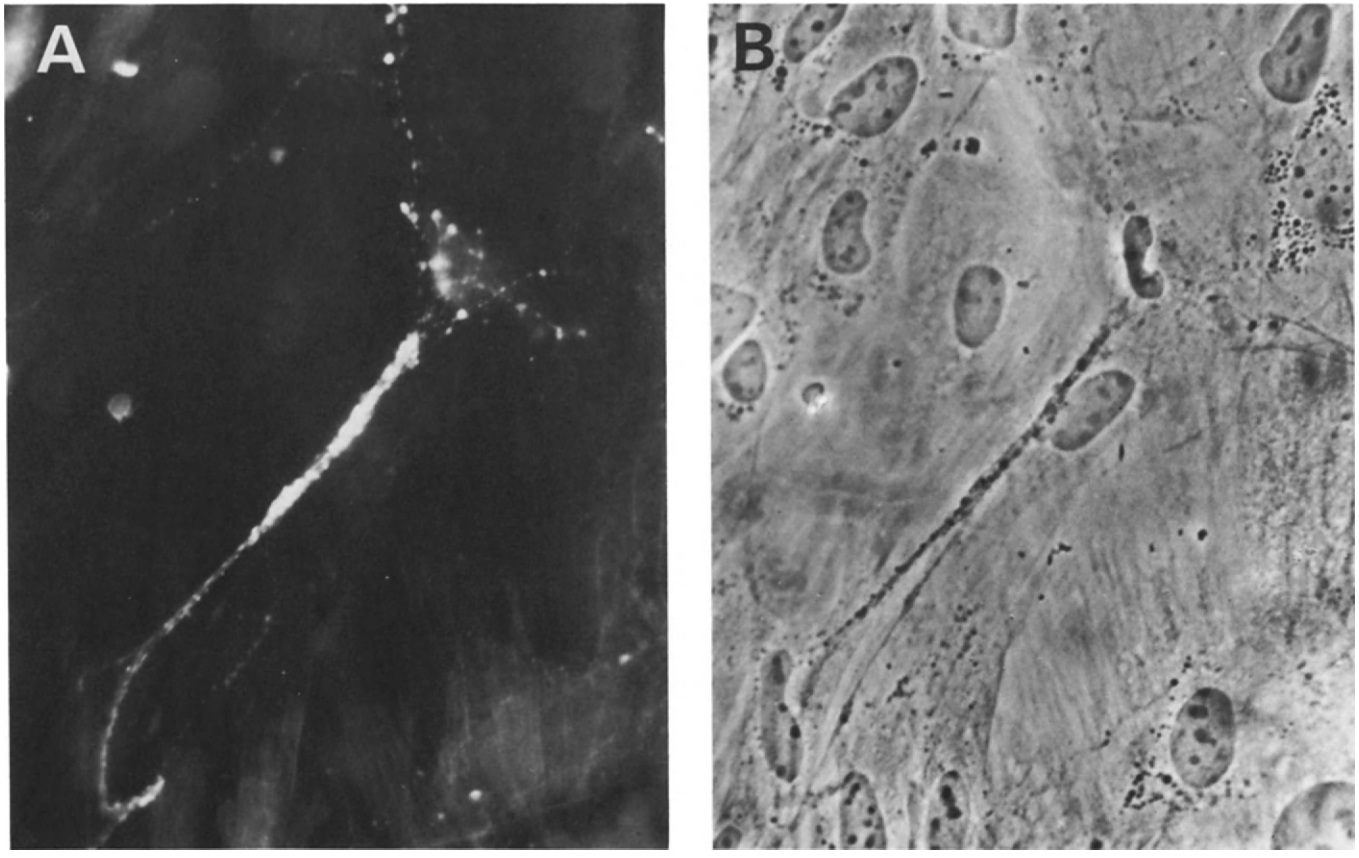

Figure 4. Culture of submucous plexus immunostained for VIP. (A) Immunofluorescence. (B) Phase contrast (×500).

Rose chambers and grown in tissue culture medium 199 at 37°C for 1-2 weeks when the preparations were immunostained. Numerous VIP, substance P, and enkephalin-containing nerves were seen to have developed into a dense network of fibers (28).

PATHOLOGY

The intramural ganglion cells of the gut autonomic innervation are known to be involved in a number of gastrointestinal diseases that show severe motility disturbances.

DEGENERATION OR ABSENCE OF AUTONOMIC GANGLION CELLS OF THE GUT

A number of gastrointestinal disorders associated with severe constipation show major degenerative changes or decrease in the number of ganglion cells. These include Chagas' disease or American trypanosomiasis and Hirschprung's disease.

Chagas' Disease.

Chronic gastrointestinal Chagas' disease is the result of an infection by the flagellate protozoon *Trypanosoma cruzi.* Periganglionitis and degeneration of the intrinsic cell bodies are the main histological features (Figure 5). This leads to severe denervation of the corresponding gut segment and subsequent megaesophagus, megacolon, and megaduodenum. We have recently been able to study the content and immunostaining of VIP, substance P, somatostatin, and enteroglucagon-containing nerves and endocrine cells of the gut from patients with Chagas' disease (45) (Figure 6). VIP and substance P nerves were markedly reduced in number and intensity of the immunostaining (Figure 7). Their peptide content was also significantly decreased to below half of that found in "normal" controls. In addition, we have recently observed similar changes in experimentally induced Chagas' disease. This was produced by infecting BALB/c isogenic mice with 2×10^5 *Trypanosoma cruzi* "Y" strain in the circulating form (45).

Hirschprung's Disease.

The bowel of children with severe hypoganglionosis and Hirschprung's disease also shows marked reduction (more than 50%) in the number, immunostaining, and content of the peptidergic nerves in the gut wall (Figure 8). The extent of these changes depends on the degree of the aganglionosis (3).

It is interesting to note that in patients with other autonomic nervous system disorders like the Shy Drager Syndrome or generalized (extrinsic) autonomic failure, the immunostaining and content of the gut wall peptidergic nerves do not significantly differ from those of normal control (Figure 6).

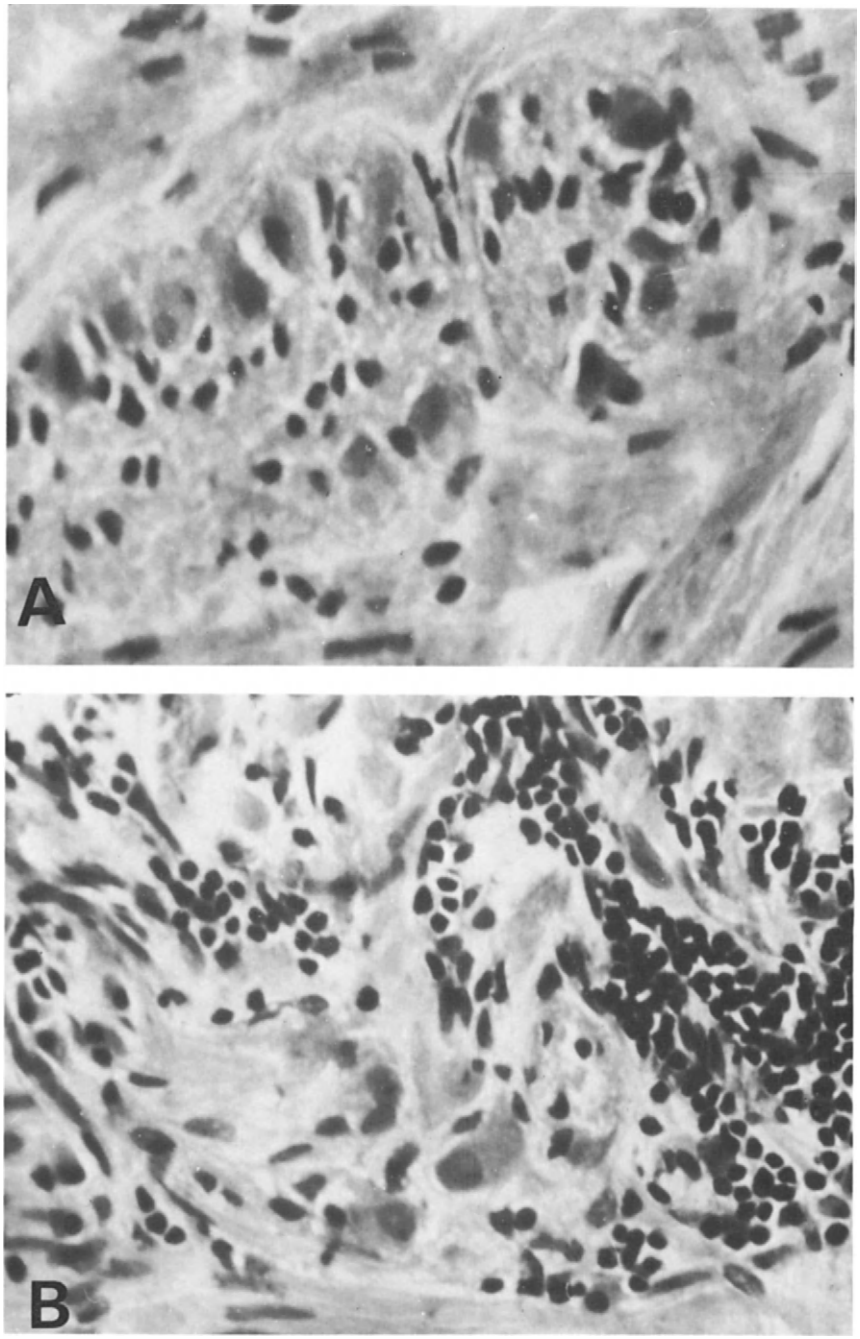

Figure 5. Human Chagas' disease. Colonic mucosa. Haematoxylin-eosin staining. (A) Normal appearance of Auerbach's plexus. (B) Perigangliontis and degeneration of ganglion cells in Chagas' disease (×400).

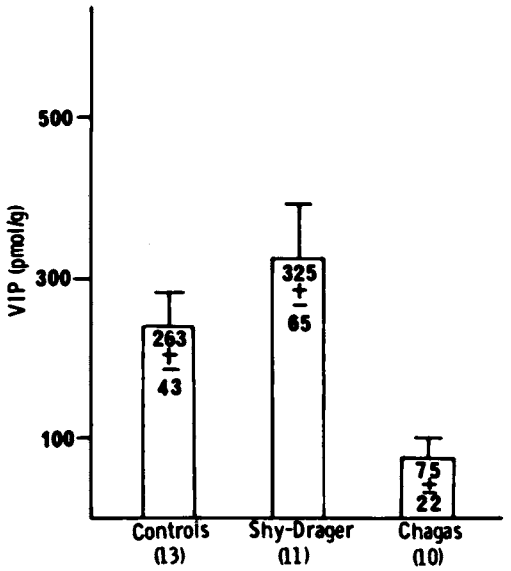

Figure 6. Gut VIP content in human Chagas' disease.

GASTROINTESTINAL GANGLION CELL HYPERPLASIA

A significant increase in the ganglion cell numbers and the presence of "neuromatous hyperplasia" has long been noted in the bowel from patients with Crohn's disease. Further insight into the nature and significance of these changes has followed the recent finding of a marked hyperplasia of VIP-containing nerve fibers and ganglion cells in this disease (44). VIP nerves appear brightly stained, thickened, and in disorganized meshes (Figure 9). Although these changes are seen in all areas of the gut wall, they are particularly noticeable in the mucosa and submucosa. Numerous intensely immunostained VIP nerve aggregates are also frequently seen. These abnormalities are present in both the "granulomatous" and the apparently "noninvolved" areas of the gut wall. Both plexuses appear enlarged and irregular and they seem to be even more easily distinguishable because of the exaggerated number of VIP nerve fibers in them, surrounding mostly bright immunopositive cell bodies, a feature rarely seen in samples of normal colon. These immunoreactive VIP-containing cell bodies are particularly noticeable in the submucous plexuses from which most of the VIP-containing nerves originate. The gut VIP content is also elevated to more than double that of "control bowel." Interestingly, the gut VIP content and immunostaining from patients with ulcerative colitis do not significantly differ from that of other "controls" ("normal" gut obtained from "carcinoma patients" or during bypass operation for obesity).

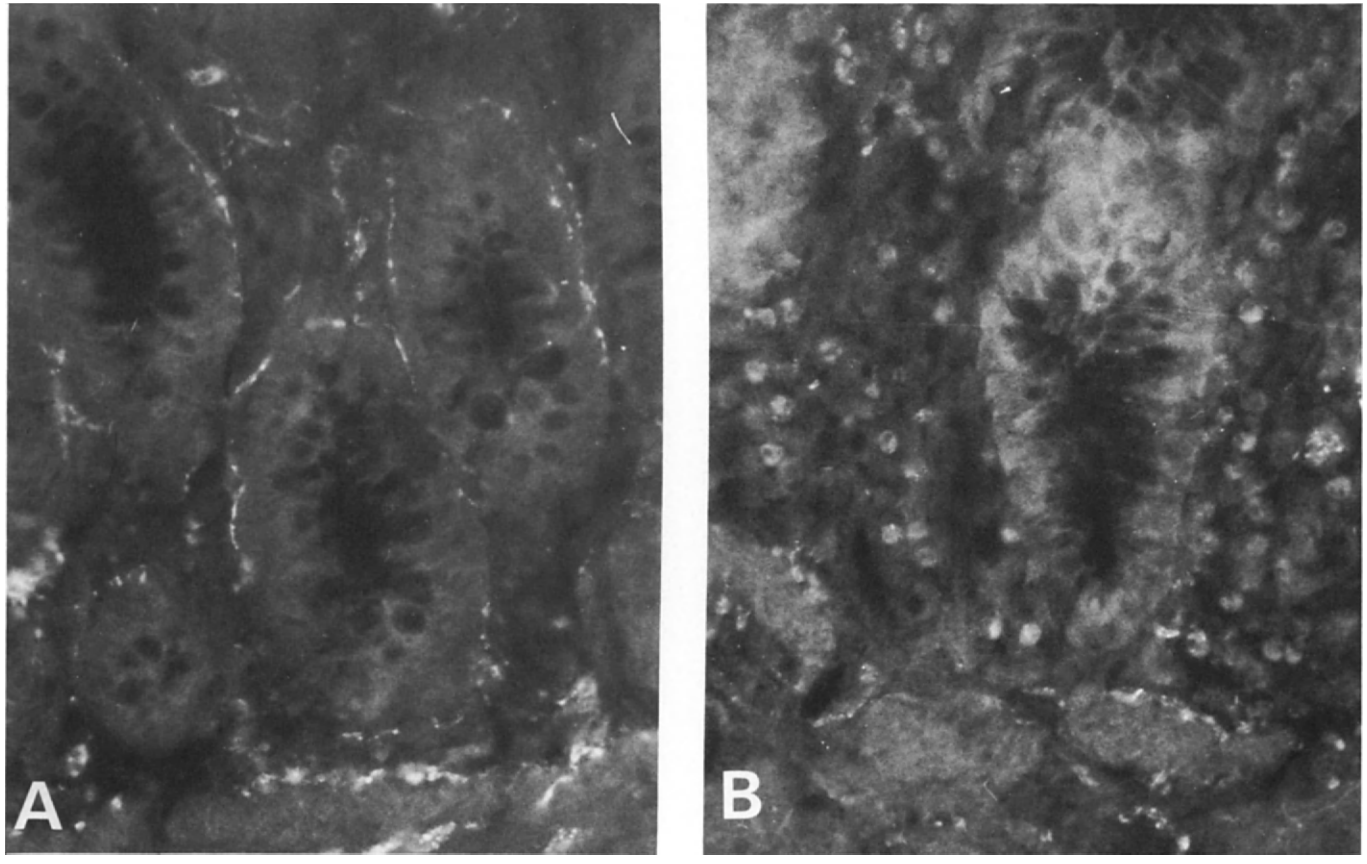

Figure 7. Human Chagas' disease. Colonic mucosa immunostained for VIP. (A) Normal control. (B) Chagas' disease (×300).

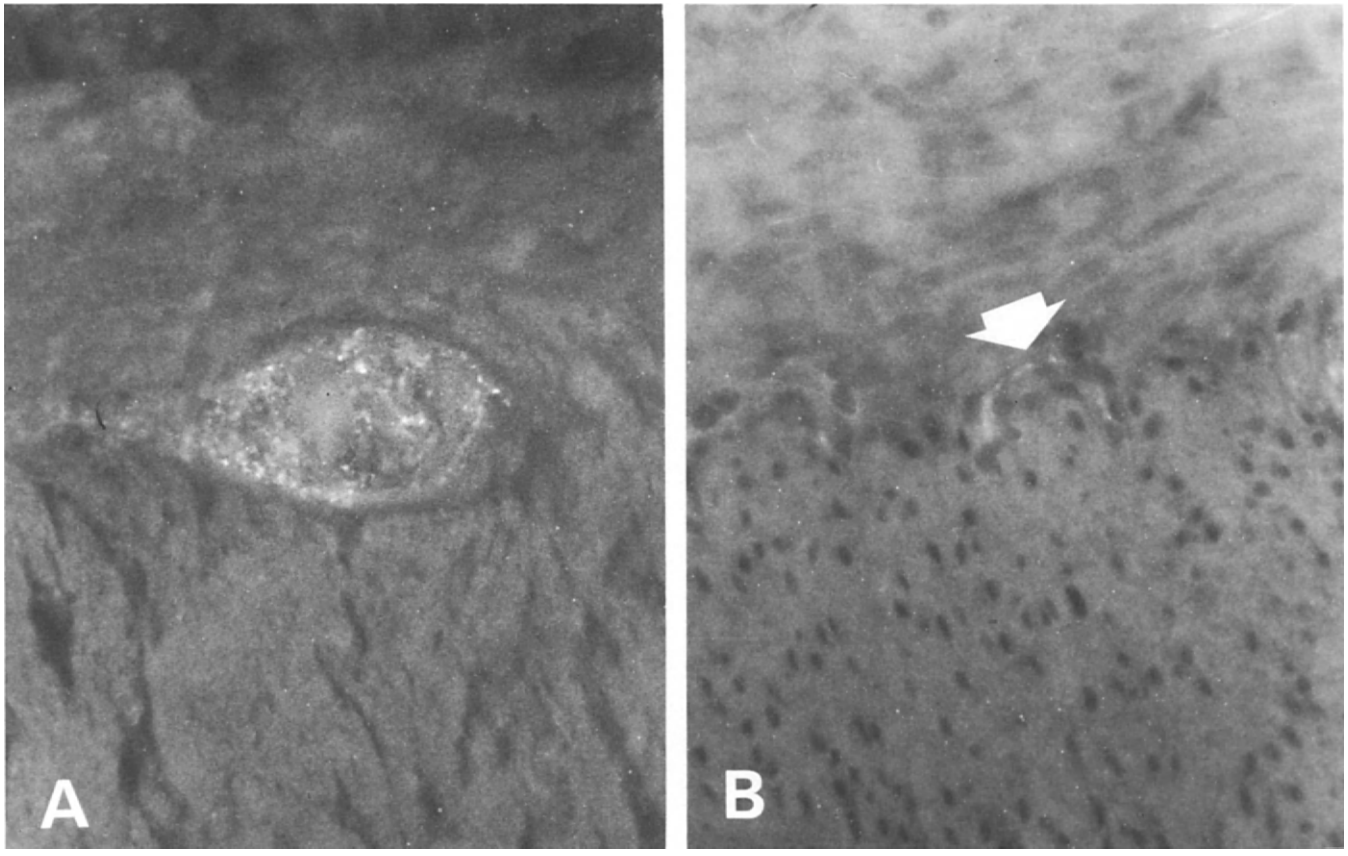

Figure 8. Hirschprung's disease. Colon. (A) Normal Auerbach's plexus immunostained for substance P. (B) Almost total absence (arrow) of Auerbach's plexus and substance P nerve fibers (×250).

Figure 9. Hyperplastic VIP nerves in Crohn's disease (×500).

Conclusions

The gut is richly innervated by a large and complex peptidergic system. Although generally these peptides are widely distributed throughout the length and width of the gut, marked differences are observed between them in respect to their localization within the various layers of the gut wall. These anatomical features support and broadly agree with the reported actions of these peptides on gut functions. It is now generally accepted that they control gut motility, secretion, and blood flow and that many of them act in a synergistic or antagonistic (bombesin-somatostatin) manner. The suggested intrinsic origin of the peptide-containing neurons as well as their possible anatomical and functional connections between the plexuses are now further validated by novel experimental procedures, which include the use of separate cultures of the two main ganglionated plexuses. These regulatory peptides are involved in a number of human diseases and experimental models, many of which are manifested by major motility disturbances. The continuous study of the pathological involvement of peptidergic nerves will undoubtedly lead the way to further understanding of their mode of action in health and disease.

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Storage and Release of Serotonin and Serotonin-Binding Protein by Serotonergic Neurons

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INTRODUCTION

Not long after the discovery of serotonin (5-HT) it became apparent that the amine might be involved in the regulation of some aspect of gastrointestinal function. In 1947–1948, 5-HT was found by Rapport *et al.* (48,49,50) to be the vasoconstrictive substance that had been known since 1911 (45) to be liberated from clotted blood and that also caused contraction of the intestine. After the identification of the structure of 5-HT (47,51) and its biosynthesis (37), it became clear that 5-HT was identical with material found in extracts of gastrointestinal mucosa that had been independently studied for some years by Erspamer (18), who had named this material enteramine. Before long, it was established that the major storage depot of 5-HT in the body is the gut, where the amine is stored in tremendous quantities in enteroendocrine cells of the mucosa (16,17,18, 65). These gastrointestinal cells were found to release 5-HT constantly into the circulation, causing portal venous blood to have a higher concentration of 5-HT than does peripheral arterial blood (64). The action of 5-HT on the gut was discovered to involve the neural tissue of the myenteric plexus (52,54). Since 5-HT could, moreover, be released from the mucosa on application of pressure (6), the first serious hypothesis concerning its enteric action envisioned a role for 5-HT in stimulating or initiating the peristaltic reflex (6,8). Enthusiasm for this hypothesis waned, however, when it was demonstrated that the mucosa could be depleted of 5-HT without blocking the peristaltic reflex (5). The role of 5-HT in the mucosa of the gut remains enigmatic today.

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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Another hypothesis concerning a role for 5-HT in the gut has emerged more recently. This hypothesis is that 5-HT is the neurotransmitter of a subset of intrinsic enteric neurons. Early pharmacological studies had revealed that myenteric neurons had receptors for 5-HT that were unique and different from nicotinic receptors for acetylcholine (ACh) (11,12,20,21). 5-HT, in fact, was found to be able to induce either a neurogenic intestinal contracture or, if muscarinic receptors for ACh were blocked, a tetrodotoxin-sensitive relaxation of the gut (7,11,21). 5-HT, therefore, can apparently activate intrinsic excitatory and inhibitory neurons. In addition, axons of the myenteric and submucosal plexuses were found to synthesize ^{3}H-5-HT from its precursor, 5-hydroxy(^{3}H)-D,L-tryptophan (^{3}H-5-HTP) and to become labeled and detectable by radioautography (25,26,31). Bulbring and Gershon (7) proposed in 1967, on the basis of these observations and from their pharmacological analysis of vagal relaxation of the stomach, the 5-HT participated in the vagal inhibitory pathway to the gut. Since this suggestion was made, a great deal of evidence has accumulated to support a role for 5-HT as a neurotransmitter. In fact, all of the five criteria generally required for moving a substance from the putative to the established category of neurotransmitters have now been satisfied for 5-HT in the enteric nervous system.

5-HT IN THE ENTERIC NERVOUS SYSTEM

The enteric nervous system contains 5-HT (29,53). The amounts of 5-HT in the myenteric layer of the gut are small in comparison to the mucosa (19); however, it appears likely that the turnover of enteric neural 5-HT is more rapid than that of the enteroendocrine cells (27). 5-HT, moreover, can be demonstrated in enteric neurons by histofluorescence (13), although not without difficulty (1,2,10,15). Enteric neurons, furthermore, synthesize ^{3}H-5-HT, not only from ^{3}H-5-HTP, but also from L[^{3}H]tryptophan (13). They also cross-react with, and are demonstrated immunocytochemically by, an antibody prepared against tryptophan hydroxylase obtained from central serotonergic neurons (32). The enteric neurons resemble central serotonergic neurons, additionally, in having a relatively high-affinity uptake mechanism (K_m = 50 μM) for the precursor amino acid L-tryptophan (23) and in having a specific high-affinity serotonin-binding protein (SBP) (40).

5-HT MIMICS ENTERIC NERVE STIMULATION

Studies with intracellular microelectrodes have now confirmed the ability of 5-HT to mimic the effects on myenteric ganglion cells of nerve stimulation (67). Two types of cell have been found in the gut using electrophysiological criteria (38,43,66). One of these has been called alternatively, the AH cell (38), the type II cell (43), or the tonic-type neuron (66). The activity of this neuron is marked by a period of prolonged after-hyperpolarization following discharge of an action potential (66). During this period the neuron is relatively inexcitable. When 5-HT is applied to the tonic-type neuron by

iontophoresis, however, the cell membrane shows a prolonged depolarization associated with hyperexcitability and often, a tonic discharge of spikes (67). This state is associated with an increased resistance of the perikaryal membrane. The effects of iontophoretic application of 5-HT are identical with the slow exhitatory postsynaptic potentials (EPSP) elicited in tonic-type neurons by repetitive stimulation of an interganglionic fiber tract leading to the ganglion being recorded from (66,67). The effects of 5-HT, moreover, can be antagonized by desensitization of the preparation to 5-HT itself or by adding methysergide (30 μM) to the incubating medium (67). These treatments block the effect of fiber tract stimulation as well as that of 5-HT. Substance P has an effect on tonic-type neurons that is similar to that of 5-HT; however, antagonism of the effects of substance P does not, like antagonism of the effects of 5-HT, block the slow EPSP in response to fiber tract stimulation (44).

INACTIVATION OF 5-HT

The enteric nervous system is well endowed with a mechanism to inactivate 5-HT. This mechanism is a specific reuptake of the amine. Axons of the enteric plexuses take up 5-HT (22,33,53). The uptake is saturable and has a low Km, 0.5 μM (22). It is Na^+-dependent and is antagonized by inhibitors of glycolysis. The uptake is quite specific in that it is not affected by norepinephrine (NE) up to 1000 times the 5-HT concentration (22,33,56); nor is the uptake of 5-HT altered by chemical sympathectomy with 6-hydroxydopamine (6-HD) (22, 33). The uptake of 5-HT, moreover, develops early in ontogeny and is present in the fetal rabbit gut a full week before uptake of NE can be detected (30,56). The enteric uptake of 5-HT, furthermore, is also dissimilar to the uptake of NE with respect to susceptibility to antagonism by drugs (33). In a manner that parallels the central amine uptake mechanisms, enteric uptake of 5-HT is maximally sensitive to inhibition by chlorimipramine (33) and fluoxetine (24), whereas uptake of NE is maximally sensitive to inhibition by desmethylimipramine (33). The molecular structural requirements for recognition by the enteric 5-HT uptake site seem to be exceedingly rigid (33). Adding a single methyl group to the aliphatic amine of 5-HT or removal of the ring hydroxyl group, for example, reduces the affinity of the compounds for uptake by a factor of 100. Analogues of 5-HT that are taken up by the 5-HT uptake mechanism include 6-hydroxytryptamine (6-HT) (33) and the indolic neurotoxins, 5,6-dihydroxytryptamine (5,6-DHT) and 5,7-dihydroxytryptamine (5,7-DHT) (34). These agents compete poorly with 5-HT itself (33), but they are extremely useful in studies of the enteric innervation. 6-HT has a high fluorescent yield (41), and, after chemical sympathectomy with 6-HD is very helpful in histochemically visualizing enteric serotonergic axons (33). The indolic neurotoxins, 5,6-DHT and 5,7-DHT, can be used (in combination with desmethylimipramine to prevent their uptake into adrenergic axons) to lesion selectively enteric serotonergic neurites (34).

Serotonin Neurons: Phylogenetically Old and Intrinsic to the Enteric Nervous System

The enteric serotonergic neurons in mammals seem to be intrinsic elements of the enteric nervous system. No stigmata of serotonergic neurites have been found in the vagus nerves (14). When the gut is grown for extended periods in organotypic tissue culture, moreover, the enteric serotonergic neurons persist (13,14). These neurons can be revealed in the cultures by histofluorescence (13), immunocytochemical demonstration of tryptophan hydroxylase (32), biosynthesis of ^{3}H-5-HT from L-[^{3}H]tryptophan (13), and the specific uptake of 5-HT (14). Histofluorescence of 5-HT, in particular, is especially vivid in cultured enteric neurons (13). Since extrinsic neurites degenerate in culture, the survival of serotonergic neurons in the cultures confirms that they are intrinsic elements of the nervous system of the gut. The enteric serotonergic neurons in mammals also appear to be a remnant of a phylogenetically old system. Serotonergic neurons have been detected in the gut in cyclostomes (2,36) and perhaps in amphioxus (59), although they do not exist in prevertebrates, such as tunicates and echinoderms (36). They may have evolved with cephalochordates, therefore, and they seem to have persisted throughout vertebrate evolution. The specific uptake mechanism of 5-HT has been found by radioautography in enteric neurons in teleosts, amphibia, birds, and six types of mammals (35), including humans (35,55).

Neural Release of 5-HT

One of the most important parameters of neurotransmission is the direct demonstration that the substance in question is actually released under physiological conditions by appropriate nerve stimulation. This is difficult to accomplish with respect to enteric 5-HT because the relatively small concentration of neural 5-HT is dwarfed by the immense concentration of 5-HT in enteroendocrine cells. In order to be meaningful, release of neuronal 5-HT has to be measured separately or in some way distinguished from release of 5-HT by the mucosa. Paton and Vane (46), for example, demonstrated that 5-HT is released from the stomach by vagal stimulation. They did not, however, identify the scource of the released amine, nor did they establish whether the release was a primary effect or was secondary to gastric contraction. Bulbring and Gershon (7) attempted to avoid these problems by destroying the mucosa through selective asphyxiation prior to stimulating the stomach and using scopolamine to prevent contraction of the stomach. They did find that electrical stimulation released 5-HT even after asphyxiation of the mucosa, and the release, moreover, was abolished by tetrodotoxin. They did not, however, show that release of 5-HT under their conditions was calcium dependent, as would be predicted for a synaptic release mechanism. In addition, they could not eliminate the possibility that 5-HT released from the mucosa during its asphyxiation was taken up by

enteric nerves and was the material subsequently released by electrical stimulation.

More recently, advantage has been taken of the difference in serotonergic mechanisms between enteroendocrine cells and enteric neurons. The enteroendocrine cells lack the specific 5-HT uptake mechanism (57) that characterizes enteric serotonergic neurites (22,53). The enteric serotonergic nerves then can preferentially be loaded with ^{3}H-5-HT by incubating the gut *in vitro* with the exogenous radioactive amine. In these experiments (39) the gut was everted (turned inside out), mounted in an organ bath, and perfused through the newly created serosal lumen. An electrode was inserted into the lumen, permitting the gut to be stimulated transmurally. This preparation has the ancillary benefit that the myenteric plexus is positioned very close to the serosal lumen, so that material released from the nerves rapidly appears in the perfusate. Since the interior volume of the serosal lumen, moreover, is small, there is relatively little dilution of released compounds. ^{3}H-5-HT (0.5 μM) was added to the material perfusing the serosal lumen. After 30 min of perfusion, ^{3}H-5-HT was discontinued, and the gut was washed for an additional 30 min. Light and electron microscopic radioautography revealed intense labeling of terminal axonal varicosities in both myenteric and submucosal plexuses; however, there was very little labeling of enteroendocrine cells (39). These observations indicate that there is no barrier between the serosa and the enteric nervous system that would impair recovery of released ^{3}H-5-HT in these experiments and that enteric neurons are the only source of ^{3}H-5-HT in the tissue.

Transmural stimulation of preparations loaded, as described, with ^{3}H-5-HT released the labeled amine (39). The amount of ^{3}H-5-HT released was dependent on the frequency of stimulation. More ^{3}H-5-HT was released as the frequency was increased, although the amount released per shock declined. The release of ^{3}H-5-HT, moreover, was abolished by removing external CA^{2+} (and including 0.1 mM EGTA in the medium), adding 30 mM Mg^{2+}, or adding 1 μM) tetrodotoxin to the medium. Stimulated enteric nerves therefore, release at least exogenous ^{3}H-5-HT in a manner that resembles a synaptic mechanism.

In these experiments release of SBP was also looked for (39). This protein is found in central serotonergic neurons (61,62,63), in neural crest derivatives that store 5-HT, such as thyroid parafollicular cells (3,40), and is also, as noted earlier, found in the gut (40). The function of SBP is unknown, but it may be a storage protein for 5-HT. In central serotonergic neurons SBP is rapidly transported proximodistally by fast axonal transport, as would be expected of a component of synaptic vesicles (60). Vesicles, moreover, derived from central synaptosomes have been found to be enriched fourfold in SBP over the synaptosomal supernatant. SBP may, therefore, be a component of serotonergic vesicles. 5-HT is probably stored in a very high concentration within the synaptic vesicles of serotonergic terminals; nevertheless the

vesicles do not swell. Intravesicular binding of serotonin by SBP could serve to reduce the intravesicular osmotic pressure below that which would exist if 5-HT were free in solution within the synaptic vesicles. If 5-HT does coexist in serotonergic synaptic vesicles with SBP in enteric neurons, and exocytosis is the mechanism of 5-HT release, then SBP would be expected to be released with 5-HT from the stimulated neurons. This expectation proved to be correct (39). SBP was detected in the fluid perfusing the gut. Stimulation increased the rate of SBP release. The stimulation-induced release of SBP, but not the spontaneous release of the material, was blocked in Ca^{2+}-free media. In contrast to SBP, no spontaneous or stimulation-induced release of the cytosol marker protein, lactate dehydrogenase, could be detected. The release of SBP on stimulation supports the view that electrical stimulation physiologically releases transmitter from enteric serotonergic axons. It also provides insight into the release and transmitter storage mechanisms. Release probably occurs by exocytosis, as this is the only physiological means of transporting a protein out of cells. Exocytosis, in turn, implies a vesicular form of storage.

In working with the everted-gut preparation, it was found that there is a barrier to the movement of 5-HT through the wall of the gut (28,29). Although ^{3}H-5-HT added to the serosal perfusion medium clearly reached, and was taken up by, elements of the enteric nervous system, very little of it was found to reach the fluid bathing the outside, mucosal surface of the everted intestine (28). In a like manner, when ^{3}H-5-HT (50,000 dpm/ml or 100,000 dpm/ml) was added to the mucosal side of the preparation almost no radioactivity (<150 dpm/ml) appeared in the serosal perfusate even after 15 min (28,29). It appears, therefore, that mucosal 5-HT does not reach the serosal perfusate. This phenomenon means that 5-HT in the serosal perfusate must be nonmucosal and thus of neuronal origin.

The release of endogenous 5-HT into the fluid perfusing the serosal lumen was therefore examined. The highly sensitive radioenzymatic method of Saavedra *et al.* (58) was used after initially desalting and concentrating the 5-HT in the perfusate by a modification of the method of Boireau *et al.* (4). 5-HT was spontaneously released to the perfusate (28,29); however, the rate of 5-HT release increased by over 250% ($p < 0.001$) when the preparation was electrically stimulated. Stimulation failed to increase the rate of endogenous 5-HT release when the gut was suspended in Ca^{2+}-free media. Endogenous 5-HT, like exogenous ^{3}H-5-HT, therefore, is also released by a Ca^{2+}-dependent mechanism. Further experiments with ^{3}H-5-HT revealed that electrical stimulation released ^{3}H-5-HT in preference to labeled 5-HT metabolites (28). The specific activity of ^{3}H-5-HT, in the perfusate, following stimulation, was also increased almost fivefold over that of the spontaneously released material and was about twice as high as that of the tissue. This indicates that newly taken up ^{3}H-5-HT is preferentially released by stimulation of the enteric nerves. These experiments indicate that 5-HT is

released in a manner expected for a neurotransmitter from stimulated enteric neurites. They also indicate that studies of the release of ^{3}H-5-HT provide a sensitive means of investigating the regulation of 5-HT release.

Adrenergic-Serotonergic Interaction

Wood and Mayer (68) found, using intracellular recording techniques, that exogenous NE antagonizes the slow EPSP in tonic-type neurons normally elicited by fiber tract stimulation (66). On the other hand, NE did not appear to affect the tonic-type neurons directly and did not block the effects of iontophoretically applied 5-HT. Since they believe 5-HT to be the mediator of the slow EPSP (66,67), they concluded that exogenous NE presynaptically antagonizes the release of 5-HT (68). They further proposed that such presynaptic inhibition of the release of a number of enteric neurotransmitters might be the general mode of action of sympathetic nerves in the gut. In support of this hypothesis, a reciprocally inhibitory adrenergic-cholinergic axo-axonic synapse has been found in the enteric nervous system (42). The effect of exogenous NE on the stimulated release of ^{3}H-5-HT was therefore examined in order to see if direct confirmation of the conclusions of Wood and Mayer (68) could be obtained. An attempt was also made to evaluate the effect of endogenous NE released from stimulated sympathetic nerves.

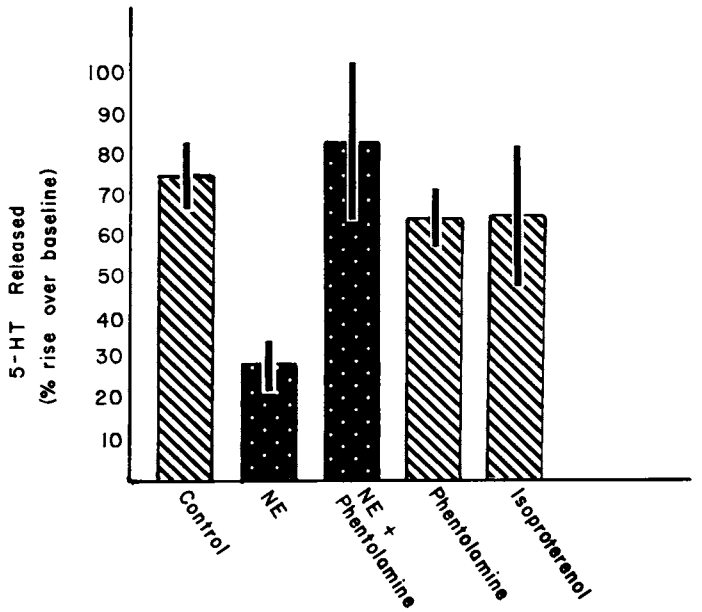

Figure 1. Release of ^{3}H-5-HT from segments of everted guinea pig ileum, preloaded by perfusion with ^{3}H-5-HT (0.5 μM) and nialamide (50 μM) and stimulated at 10 Hz. Release is expressed as the percentage rise above baseline efflux. Columns are the means of 6–10 determinations and brackets indicate the SE. The NE, phentolamine, and isoproterenol concentrations were 1.0 μM. Only stimulation in the presence of NE alone is significantly different from control ($p < 0.01$).

NE (1.0 μM) strongly inhibited the release of ^{3}H-5-HT from enteric nerves stimulated at 10 Hz (Figure 1). This effect of NE appears to be mediated via an alpha adrenoceptor, as it is blocked by phentolamine (1.0 μM) and is not mimicked by the beta agonist, isoproterenol (1.0 μM). The conclusions of Wood and Mayer (68) have, therefore, been confirmed. It is important to realize, however, in interpreting these experiments that transmural stimulation stimulates all of the nerves of the gut and that adrenergic nerves in the preparations are stimulated stimultaneously with serotonergic neurons. There is, therefore, at 10 Hz, a significant release of endogenous NE (42). The measured release of ^{3}H-5-HT, in consequence, is that released under whatever influence the endogenous adrenergic innervation may have on serotonergic terminals. If endogenously released NE is inhibitory to 5-HT release, as is the addition of the exogenous amine, then the alpha

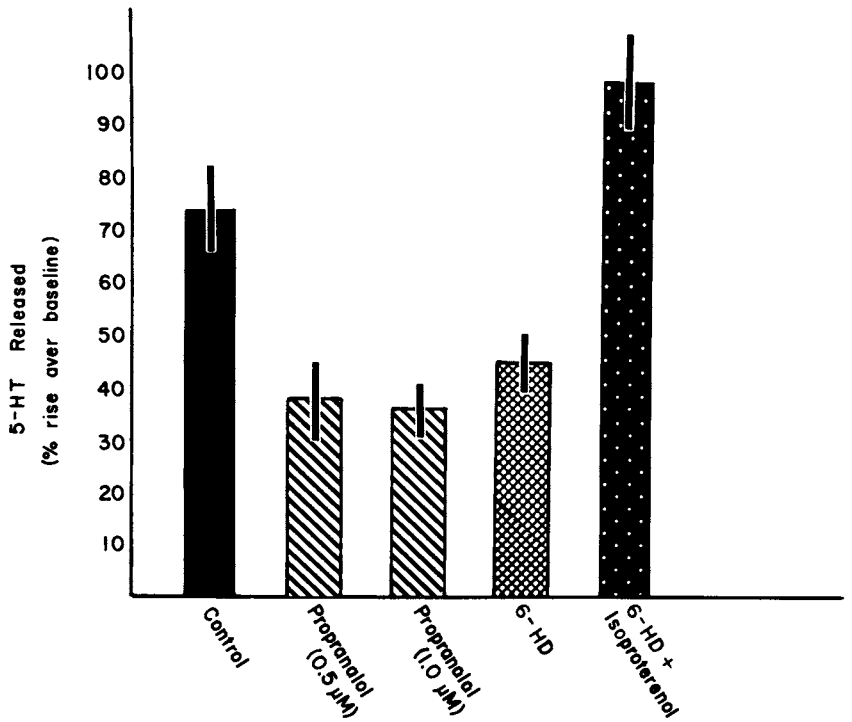

Figure 2. The effect of propranolol, 6-HD, and isoproterenol of the electrically induced release of ^{3}H-5-HT from everted segments of guinea pig ileum. Release is expressed as the percentage rise above baseline efflux. Columns are the means of 6–14 (6-HD) determinations and brackets indicate the SE. The gut was stimulated at 10 Hz. The concentration of propranolol was 0.5 μM or 1.0 uM, as indicated, and the concentration of isoproterenol was 1.0 μM. 6-HD (100 mg/kg, subcutaneously) was injected into animals 24,72, or 168 h prior to loading with ^{3}H-5-HT. Since no difference was found in release of ^{3}H-5-HT after 6-HD at any of the times indicated, the 6-HD data were pooled. The 6-HD + isoproterenol experiment was done 72 h after injection of 6-HD. Propranolol and 6-HD significantly reduced ^{3}H-5-HT release ($p < 0.01$). The inhibitory effect of 6-HD was reversed by isoproterenol ($p < 0.01$).

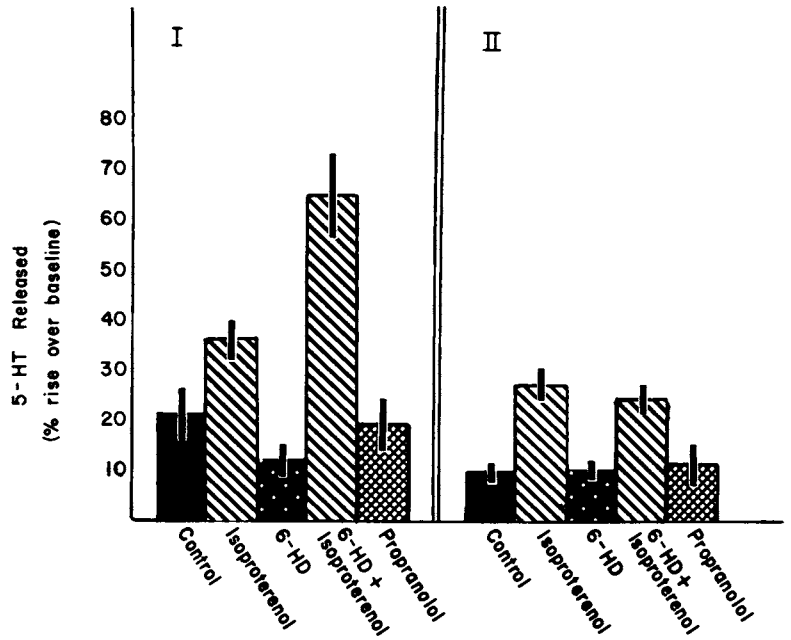

Figure 3. The effect of isoproterenol, 6-HD, and propranolol on the release of ^{3}H-5-HT from everted segments of guinea pig ileum stimulated electrically at a *low* frequency, 1.0 Hz. The preparations were subjected to two 3-min trains of stimuli, I and II, separated by a wash period of 15 min. Each column is the mean of 5–10 determinations and the brackets indicate the SE. 6-HD was given as described in the legend to Figure 2. The concentration of isoproterenol was 1.0 μM and that of propranolol was 0.5 μM. Isoproterenol facilitated release of ^{3}H-5-HT ($p < 0.05$, train I; $p < 0.02$, train II). Propranolol did not affect release of ^{3}H-5-HT.

adrenoceptor antagonist, phentolamine, would have been expected to potentiate the release of ^{3}H-5-HT. This was not the case (Figure 1).

In order to examine the effects of endogenously released NE more closely, preparations loaded with ^{3}H-5-HT were stimulated at 10 Hz in the presence of the beta adrenoceptor antagonist, propranolol, or after chemical sympathectomy with 6-HD (Figure 2). Both propranolol (0.5 μM and 1.0 μM) and 6-HD (100 mg/kg; 24, 72, or 168 h in advance) unexpectedly antagonized the release of ^{3}H-5-HT. The effects of 6-HD, moreover, were reversed by isoproterenol (1.0 μM). These observations suggest that enteric serotonergic terminals have beta adrenoceptors as well as alpha adrenoceptors and that the beta effect predominates for endogenously released NE.

In order to examine further the effect of beta adrenoceptors on release of ^{3}H-5-HT, the preparations were stimulated at a low frequency, 1.0 Hz, at which release of the sympathetic transmitter is not significant (42). The reasoning behind this experiment assumes that an effect of the beta agonist isoproterenol might not have been detectable at 10 Hz (Figure 1) because it was masked by the already maximal stimulation of beta adrenoceptors on serotonergic terminals by released endogenous NE. This is consistent with

the stimulation of ^{3}H-5-HT release by isoproterenol in preparations treated with 6-HD (in which release of endogenous NE has been eliminated). If this reasoning is correct, then isoproterenol should be able to facilitate ^{3}H-5-HT release when preparations are stimulated at low frequency. 6-HD, on the other hand, should have only a minimal effect or no effect on release of ^{3}H-5-HT under these conditions. These expectations were confirmed (Figure 3). Isoproterenol (1.0 μM) did facilitate ^{3}H-5-HT release at 1.0 Hz and neither 6-HD pretreatment nor propranolol (1.0 μM) antagonized the release of ^{3}H-5-HT at this low frequency of stimulation.

These experiments indicate that serotonergic axon terminals probably do have adrenoceptors that can influence transmitter release. Alpha adrenoceptors appear to inhibit 5-HT release, whereas beta adrenoceptors are facilitatory. It also appears that release of endogenous NE from stimulated adrenergic nerves can physiologically influence 5-HT release. The effect of endogenous NE, however, does not appear to be the same as that of the exogenously applied material. Perhaps anatomical restraints limit the diffusion of transmitter released from adrenergic nerves so that it does not reach the presynaptic alpha adrenoceptors. In any case, the beta effect appears to be dominant for the endogenous NE, whereas exogenous application of NE primarily has an alpha effect. This disparity points out how important it is not to draw conclusions about the role of neurotransmitters from observations of the properties of exogenously applied agents alone.

Conclusions

The image of the peripheral nervous system has changed during the past few years (9). For quite some time, most neurobiologists were happy to make do with ACh and NE as the neurotransmitters of the system. In recent years, however, the list of neurotransmitters and putative neurotransmitters has been expanding, and nowhere has the list been expanded more than in the gut. Among the many potential neurotransmitter candidates, besides ACh and NE, 5-HT has probably accumulated more evidence than any in its favor. It is very difficult, however, to establish beyond doubt that a given substance actually is a neurotransmitter. Because of the difficulty in demonstrating the 5-HT in the mammalian gut by histofluorescence (1,2,10, 15), some investigators have wondered whether the actual transmitter might be an indoleamine closely related to 5-HT, rather than 5-HT itself (10). The rigid structural requirements of the 5-HT uptake system (33), the many resemblances of enteric serotonergic neurons to their central counterparts, and especially the recent observations that 5-HT is released from stimulated enteric nerves, and that these nerves release unmetabolized 5-HT preferentially, however, would argue strongly that 5-HT itself, rather than a close relative, is the transmitter. The accessibility of the enteric nervous system would seem to make this system an advantageous one to use to study the cellular biology of its component neurons. With respect to the storage and release of 5-HT this has proved to be the case. It is, therefore, a shame that so little is known of

neuronal interrelationships in the gut. The enteric nervous system may be a simpler nervous system than the brain but less is known about its interneuronal connections. Studies, however, of the effect enteric neurons have on one another, such as the ability of adrenergic neurons to influence the release of 5-HT, promise to help rectify this shortcoming.

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Distribution and Release of Peptides from Peripheral Neurons

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Classically the peripheral nervous system has been divided into the autonomic nervous system and the somatic nervous system. Acetylcholine has been regarded as the transmitter in the preganglionic sympathetic motor fibers, in the pre- and postganglionic parasympathetic motor fibers, and in the peripheral somatic motor fibers. According to the rules of pharmacology, acetylcholine released from the postganglionic parasympathetic nerve endings activates cholinergic muscarinic receptors that can be blocked by atropine, acetylcholine released by preganglionic sympathetic and parasympathetic fibers activates cholinergic nicotinic receptors that can be blocked by hexamethonium, and acetylcholine released from the motor fibers activates cholinergic nicotinic receptors that can be blocked by curare.

Results that made the role of acetylcholine as the sole vagal transmitter questionable were described before the era of the peptidergic neurons. Martinsson showed in 1972 that gastric relaxation that can be induced by electrical vagal stimulation at certain frequencies could not be blocked by atropine (15). Later, it was found that release of gastrointestinal and pancreatic hormones caused by electrical vagal activation did not follow the pharmacological laws of cholinergic transmission. Thus gastrin release in response to electrical vagal stimulation in cats and dogs cannot be blocked by atropine (18,25). A similar atropine resistance has been demonstrated for vagally induced release of insulin, glucagon, and vasoactive intestinal polypeptide, VIP (5,22,23).

When, in our own experiments, we failed to block vagally induced gastrin release with atropine, we searched for gastrin in the vagal nerves, assuming that gastrin might be released directly from vagal nerve terminals. In order to

CELLULAR BASIS OF CHEMICAL MESSENGERS
IN THE DIGESTIVE SYSTEM

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test this idea, vagal biopsies were extracted for gastrin, i.e., they were boiled in water. Vagal water extracts from cat, dog, and humans were found to contain considerable amounts of gastrinlike immunoreactivity. The gastrinlike material from these extractions was further analyzed by gel filtration and was shown to contain gastrin-17 and in some instances also the precursor form gastrin-34 (24,28). Vagal extracts also contain somatostatin-like immunoreactivity (26). Several peptides have been shown to occur in the vagal nerves by immunohistochemical methods: gastrin cholecystokinin (gastrin-CCK), substance P, enkephalin, VIP, and somatostatin-like immunoreactivity can be demonstrated in the cat vagi (7,11,14), and substance P, enkephalin, and VIP-like immunoreactivity, in the human vagal nerves (13).

When the vagal nerves were ligated, the immunoreactive material accumulated close to the site of ligation, which means that the peptides were transported toward the peripheral parts of the nerves (11,14). The fact that the peptides are transported in the nerves indicates that they are produced within the nerves and not just taken up from the surrounding tissues.

The vagal nerve, like all other nerves, contains both sensory and motor fibers. The cell bodies of the motor fibers are located in the vagal motor area

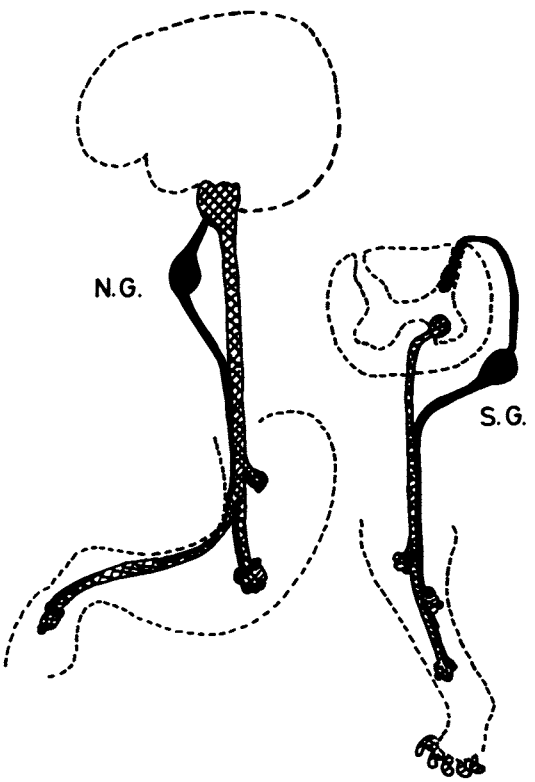

Figure 1. Schematic picture showing the motor (black) and sensory (hatched) fibers of the vagal nerve and the sciatic nerve. N.G., nodose ganglion; S.G., spinal ganglion.

in the medulla oblongata, and those of the sensory fibers in the nodose ganglion (Figure 1). When the nodose ganglion was analyzed for its peptide content, cell bodies reacting with gastrin-CCK, substance P, VIP, and somatostatin antibodies could be demonstrated (5,11,14). This means that these four peptides are present in vagal nerve fibers that, by definition, are sensory. Thus peptides in the vagi are transported distally regardless of whether they are located in nerves classified as motor fibers or in sensory fibers.

The full chemical identity of the vagal peptides is still to be elucidated. Some of the peptides have been partly characterized. As mentioned earlier the presence of gastrin-17 and -34 has been demonstrated in some vagal extracts (24). Dockray, Gregory, and Tracy have shown that CCK-8 often predominates over gastrin-17 in dog vagi. Strangely enough, some dog vagi contain a majority of gastrin-17 and some vagi contained both CCK-8 and gastrin-17 (2). The enkephalin-like immunoreactivity of cat vagi corresponds to met and leu enkephalin (ratio 4:1) when analyzed by high-power liquid chromatography (HPLC) and radioreceptor assay (Uvnäs-Wallensten, Stein and Lewis, unpublished). After gel filtration vagal substance P elutes in the same place as synthetic substance P, and as a larger molecule (7).

Our theory proposing that the gastrin released in response to vagal stimulation originated from vagal terminals was probably wrong. Today we have another "peptidergic" theory to explain the atropine resistance of the vagally controlled gastrin release mechanism. Several authors have shown that the peptide bombesin is a very potent stimulator of gastrin release (1), and of other gastrointestinal peptides. Furthermore short bombesin neurons have been shown to be present in the wall of the gastrointestinal tract of rats (3) and cats (Lundberg, Uvnäs-Wallensten, and Walsh, to be published). The cell bodies are located within the nerve plexuses, indicating that the bombesin cells are intrinsic to the gastrointestinal tract (3). These bombesin neurons might correspond to the postganglionic neurons of the vagal innervation of the endocrine cells in the stomach and the intestine. This hypothesis is supported by the results of experiments performed on cats in which gastrin and bombesin levels were measured in gastric venous blood after electrical vagal stimulation. A parallel increase in gastric vein gastrin and bombesin levels was caused by such stimulations, the ratio between the size of the release responses being 10:1 on a molar basis (Uvnäs-Wallensten and Walsh, to be published).

It should also be mentioned that the same peptides shown to occur in the vagi are also located in small neurons intrinsic to the gastrointestinal tract. Thus the peptide fibers found in the muscular and mucosal layers of the stomach and the intestine derive only to a small extent from vagal fibers (14).

Peptides seem to be almost universally distributed in the nervous system. They occur in the brain, in the vagi, and in the gastrointestinal tract. This widespread occurrence of peptides indicated to us that the peripheral somatic

nerves might also contain peptides. This question might be solved by studying the release of peptides following electrical stimulation of the sciatic nerve in an *in vitro* system. We therefore constructed a cat leg model, in which we could study the release of such possible neurogenic peptides from peripheral somatic nerves. This model consists of an extirpated cat leg that is hanging upside down. A catheter is introduced into the artery of the leg and the vascular system is then perfused with Tyrodes solution. The venous effluent is collected in tubes surrounded by ice. The perfusates can then be analyzed for their peptide content by radioimmunassay. Using this experimental design, we have stimulated the sciatic nerves and recorded the content of gastrin in the leg perfusates (21,27). As is shown in Figure 2, electrical stimulation of the sciatic nerves causes a release of gastrin, as illustrated by increased gastrin levels in the perfusates (27). This gastrin-like immunoreactivity has been identified as gastrin-17 by Dockray and Gregory (to be published). Gastrin can also be released from the cat leg preparation by adding pharmacological agents to the perfusion medium. One example is demonstrated in Figure 3, where the response to glibenclamid (belonging to

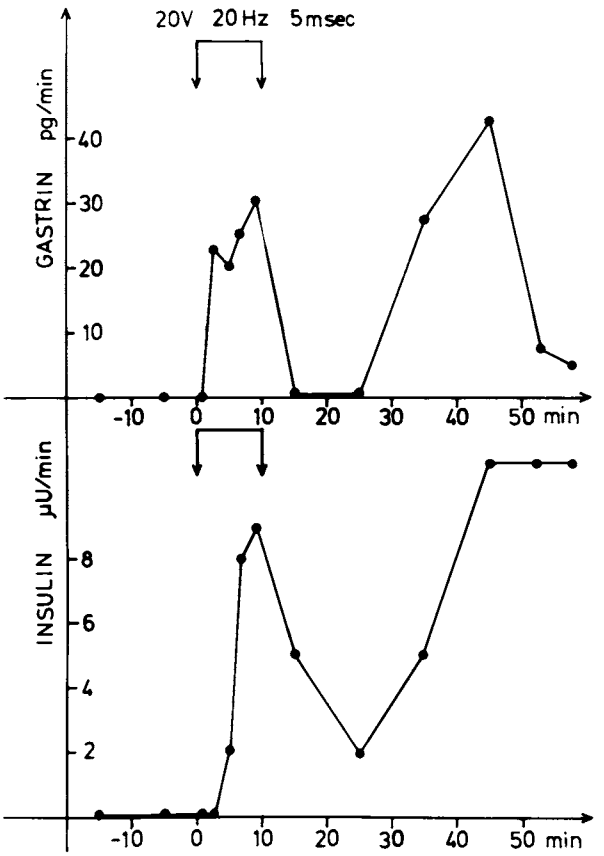

Figure 2. Release of gastrin- and insulin-like immunoreactivity following electrical stimulation of the sciatic nerve. Note the biphasic release response.

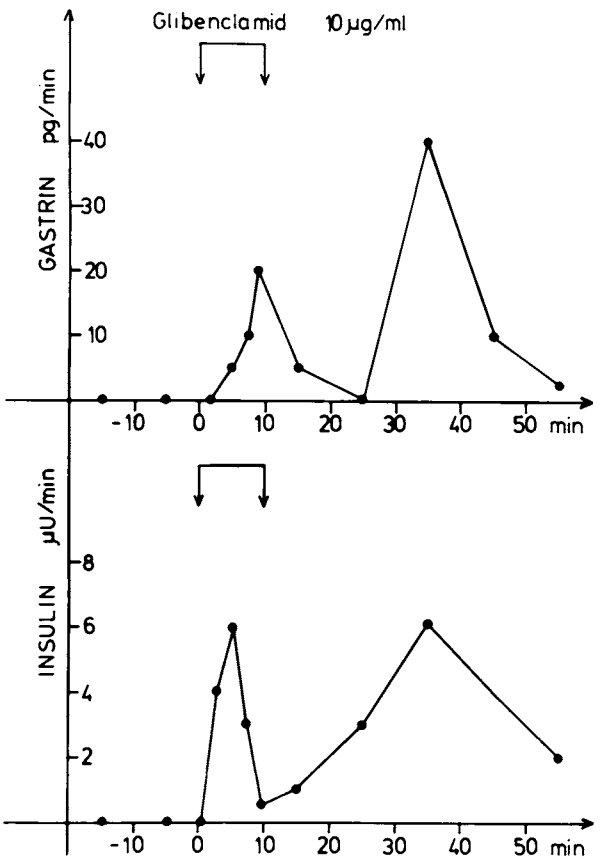

Figure 3. Release of gastrin- and insulin-like immunoreactivity following administration of glibenclamid to the isolated cat leg preparation. Note the biphasic response.

the hypoglycemic sulfonuric drugs) is shown (29). The release of gastrin in response to both electrical stimulation and sulfonuric drugs is for unknown reasons often biphasic, as is clearly shown in the present figures.

Another peptide was rather unexpectedly demonstrated in cat leg perfusates on electrical stimulation of the sciatic nerves, namely, insulin or an insulin-like peptide (21). Figures 2 and 3 illustrate that insulin-like immunoreactivity appears in the perfusates in a biphasic manner (in parallel with gastrin) both after electrical stimulation of the sciatic nerves and after addition of glibenclamid to the perfusion medium.

The Langendorff heart preparation is another experimental model that can be used to show release of peptides from peripheral stores. Following electrical stimulation of the vagosympathetic trunk or following administration of sulfonuric drugs, both gastrin- and insulin-like immunoreactivity appear in the perfusates (21,22) (Uvnäs and Uvnäs-Wallensten, to be published).

The morphologists have, of course, also looked for these peptides in the peripheral somatic nerves. Substance P, VIP, somatostatin, and

gastrin-CCK-like immunoreactivity have been demonstrated in the sciatic nerve using immunohistochemical techniques (14). As is shown in Figure 1, the somatic nerves are arranged in a similar fashion to the vagal nerves, i.e., they contain motor fibers and also sensory fibers. The spinal ganglia are the ganglia of the sensory fibers of the peripheral somatic nerves, thus corresponding to the nodose ganglion of vagal nerves. Substance P, somatostatin, gastrin-CCK, and VIP-containing cell bodies have been demonstrated in the ventral part of the spinal cord, and the enkephalin fibers should therefore be classified as motor fibers (14). Note the striking similarity between the distribution of peptide fibers in the vagus and in the peripheral somatic nerves (Figure 4).

When the sciatic nerve is ligated, the main flow of peptides seems to be efferent, independently of whether the peptides are in sensory or motor fibers (14), just as is the case in the vagal nerves.

For some reason the morphologists fail to find insulin in the sciatic nerves with immunohistochemical techniques. In contrast, insulin-like immunoreactivity can easily be demonstrated in acid ethanol extracts of the sciatic nerve. After ligation of the sciatic nerves a beautiful accumulation of insulin-like immunoreactivity occurs proximal to the site of ligation (Uvnäs-Wallensten and Uvnäs, to be published). Furthermore, the insulin extracted from cat pancreas and cat vagi appears to be identical in several chromatographic systems (Uvnäs and Uvnäs-Wallensten, to be published).

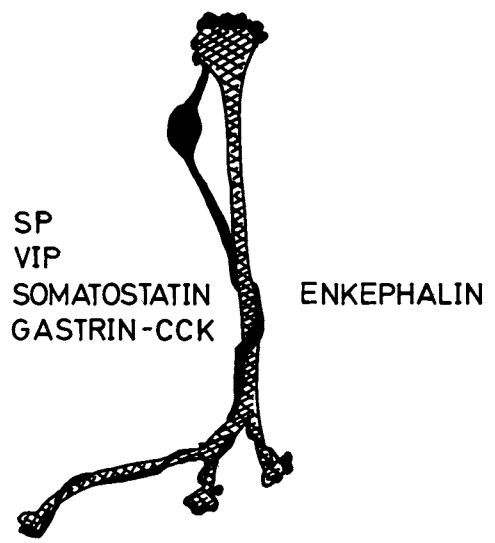

Figure 4. Schematic picture showing the distribution of *some* neurogenic peptides. Enkephalin has been demonstrated in motor fibers of the vagal and sciatic nerves, and VIP, substance P (SP), gastrin-CCK, and somatostatin in sensory fibers.

It should be stressed, however, that we still have to prove that the gastrin and insulin-like material occurring in our perfusates originates from the nerves. We think it is highly probable that it does, since a release of the peptides still occurs after pharmacological blockade of the nicotinic receptors on the striated muscles with curare (Uvnäs-Wallensten and Uvnäs, to be published). It has recently been shown that insulin occurs in almost every tissue (Roth *et al.*, in press), and therefore it cannot be excluded that some of the insulin released in our systems might be derived from a muscular depot, which might consist of pancreatic insulin bound to muscular receptors or of insulin produced in the muscles.

I want to close my trip around the nervous system of the body by returning to the vagi, in order to mention that these nerves seem to contain the highest amounts of insulin of all nerves hitherto investigated (Uvnäs-Wallensten and Uvnäs *et al.*, to be published).

We can only speculate about the functions of the neurogenic peptides. It is possible that some of them exert the same functions as they do as hormones, the difference being that effects are induced locally, i.e., they are restricted to the area corresponding to the extension of the nerves. Thus it is possible that insulin released from peripheral nerves increases the uptake of glucose in the muscles during physical exercise. The fact that insulin levels in the veins draining the muscles of the arm are higher than arterial insulin levels during physical exercise (10) supports this view.

As to the neurogenic gastrin or CCK, it might exert trophic actions (9). In fact, gastrin has been shown to cause hypertrophy of the pylorus when given to pregnant dogs or newborn puppies (4). Similar effects on striated muscles might also be induced by gastrin.

VIP has been proposed as a mediator of atropine-resistant, vagally induced gastric relaxation and of reflexly induced vasodilation in the stomach and the intestine (6). VIP in the sciatic nerve may regulate muscular blood flow. It is possible that the "cholinergic" vasodilator nerves demonstrated by Uvnäs 1966 (20) were in fact "VIPergic." As mentioned earlier, the bombesin-containing neurons might be a part of the vagal innervation of the endocrine cells of the gastrointestinal tract.

Recently, many peptides have been shown to coexist with the classical transmitters in neurons or endocrine cells. Thus noradrenaline and somatostatin seem to occur together in some neurons coming from the celiac ganglion in guinea pigs (8); some of the catecholamine-containing cells in the adrenal medulla also contain enkephalin (16,17); and, as recently shown by Lundberg *et al.*, some of the VIP-containing neurons innervating exocrine glands seem to contain acetylcholine as well as VIP (12). Thus it is possible that many physiological functions are regulated by the classical transmitters together with a peptide.

If so, this would be analogous to the cooperation between vagal cholinergic activity and gastrointestinal hormones described long ago. It is well known

that small amounts of gastrin or low-intensity vagal activation alone fail to produce gastric acid secretion, whereas when applied together a high rate of gastric acid secretion is produced (19). The difference between the two models is that the peptide that modulates the effect of the classical transmitters can be transported to the target organ either neurogenically or via the bloodstream.

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Experimentally Induced Endocrine Pancreatic Tumors in the Rat

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INTRODUCTION

Rakieten and colleagues (7) and Volk and colleagues (9) observed that the injection of streptozotocin together with nicotinamide produced insulinomas after 1 year in more than 50% of the injected rats. The tumors contained large amounts of insulin and, ultrastructurally, typical beta granules. Some of the animals had low blood glucose and elevated serum insulin levels. This model offered the possibility of reproducing experimentally some of the findings in human insulinomas, such as defective hormone storage, ductular origin, increased cellular activity, and multiple hormone production (2,3).

MATERIAL AND METHODS

Male Wistar rats weighing 250 g were injected with a single dose of 50 mg/kg streptozotocin intravenously. Ten min before this treatment and 180 min thereafter the rats were injected intraperitoneally with 350 mg/kg nicotinamide. Blood glucose was estimated 1 week after streptozotocin in the fed state and every 3 months after an overnight fast. At the end of the experiment (13–15 months) the animals were fasted overnight, anesthetized with pentobarbital (5 mg/100 g), and killed by bleeding. Blood glucose and serum insulin were estimated in all instances.

The pancreas was completely removed and carefully examined for nodules. These were enucleated and each was divided (if size permitted) into three portions. One portion was deep frozen at −20°C for hormone extraction, the second portion fixed in Bouin's solution for immunohistochemical investigation, and the third portion fixed in Karnovsky's fixative buffered with

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0.1 *M* sodium cacodylate with 0.03% calcium chloride for electron microscopic studies.

Results

None of the rats treated with streptozotocin and nicotinamide showed signs of spontaneous hypoglycemia even when fasted overnight. A slight decrease of the blood glucose levels developed in the course of the experiment. Two days before sacrifice, the difference in fasting blood glucose levels between rats with pancreatic tumors and those in which no tumors were found was significant. The tumor size was not correlated to the degree of hypoglycemia.

The serum immunoreactive insulin (IRI) levels at the end of the experiment tended to be higher in animals with pancreatic tumors compared to controls, i.e., animals without pancreatic tumors. However, this difference was not significant.

The IRI concentration in the pancreas of 20 rats treated with streptozotocin plus nicotinamide was 1.3 ± 0.1 mU/mg wet weight. There was no significant difference between rats with and without pancreatic tumors. The IRI concentration in the pancreatic tumors varied considerably. The mean value of 26 tumors was 50.8 ± 7.6 mU/mg, the lowest value being 11.0 and the highest 146 mU/mg.

Thirty-five pancreatic tumors were studied. The tumors were usually encapsulated and displayed a regular growth pattern. The majority showed a trabecular and some a medullary structure. In some cases, both growth patterns occurred in different areas of the same tumor. Some tumors contained large amounts of connective tissue. In these, proliferating ducts could also be seen. The tumor cells were often well granulated and larger than normal islet cells. However, in all tumors a varying number of tumor cells was poorly granulated or even completely degranulated.

Immunohistological investigation revealed insulin-containing cells in all tumors and a positive reaction in the majority of the cells. In addition, other hormone-producing cells were found in most tumors (Table 1). Somatostatin-producing cells were the second most frequently detected cells. They were evenly distributed among the tumors as single cells or clusters of

TABLE 1
Multiple Hormone Production in 35 Experimental Endocrine Pancreatic Tumors of the Rat

	n	Percentage	Reactive tumor cells (%)
Insulin	35	100	50-90
Somatostatin	21	60	3-50
Glucagon	13	37	<3
Pancreatic polypeptide	7	20	Single cells
Gastrin	0	0	—

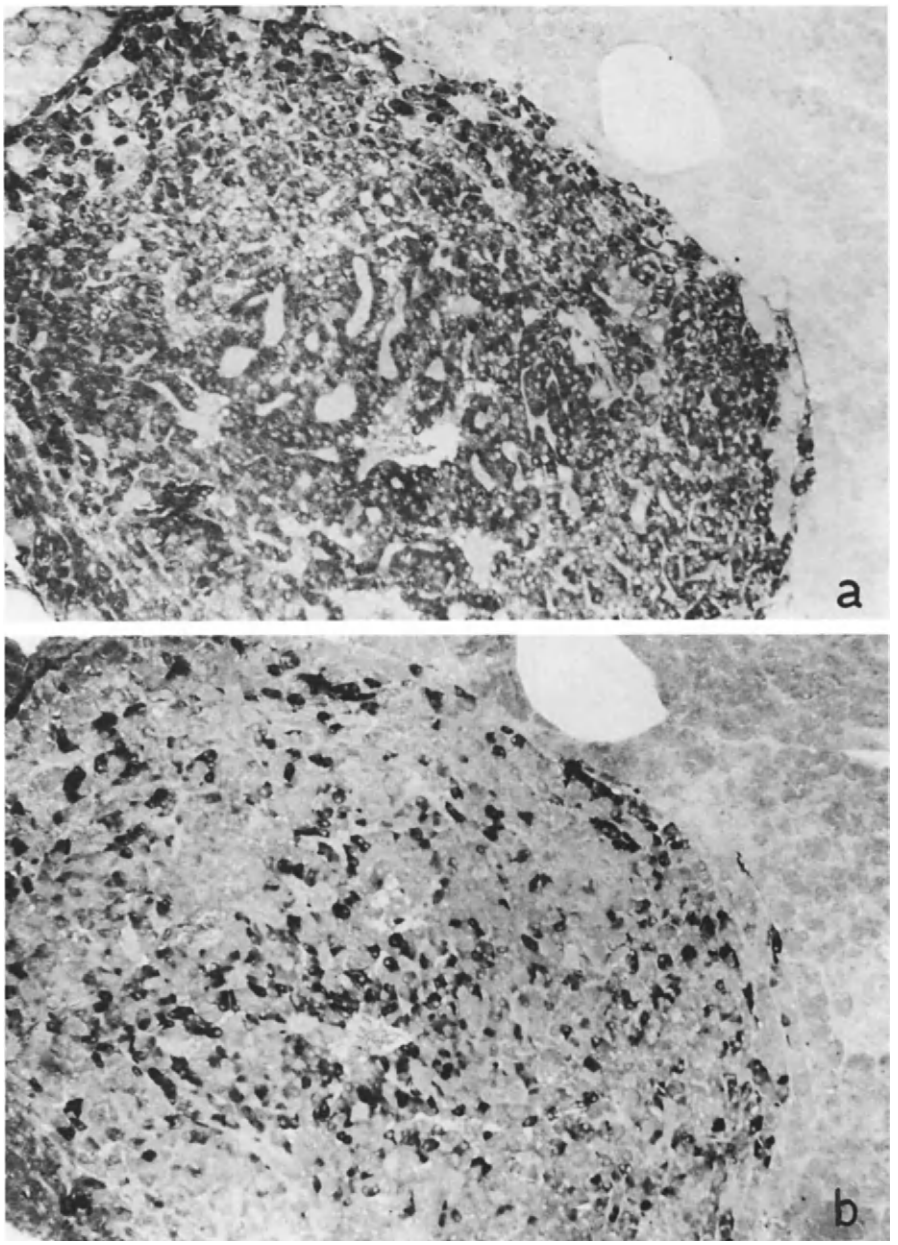

Figure 1. Consecutive sections of a pancreatic tumor of a rat treated with streptozotocin and nicotinamide. (a) Demonstration of insulin-containing cells. (b) Demonstration of somatostatin-containing cells. Bouin fixation. Paraffin, peroxidase-antiperoxidase method of Sternberger (8) using anti-insulin serum (a) and antisomatostatin serum (b). The hormone reactive cells appear dark brown or black (×160).

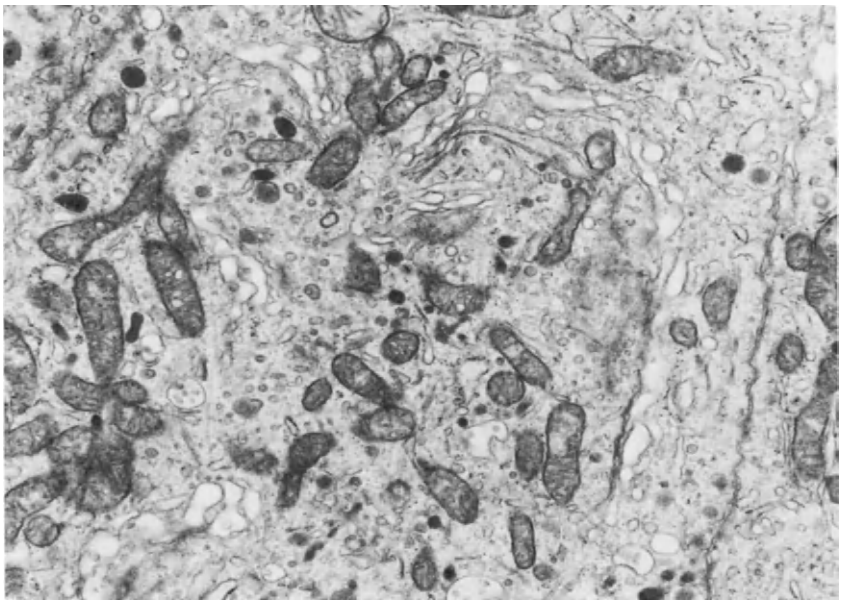

Figure 2. Portions of tumor cells containing only few typical beta granules and showing enhanced activity (prominent Golgi zone, numerous cytoplasmic vesicles) (×24,000).

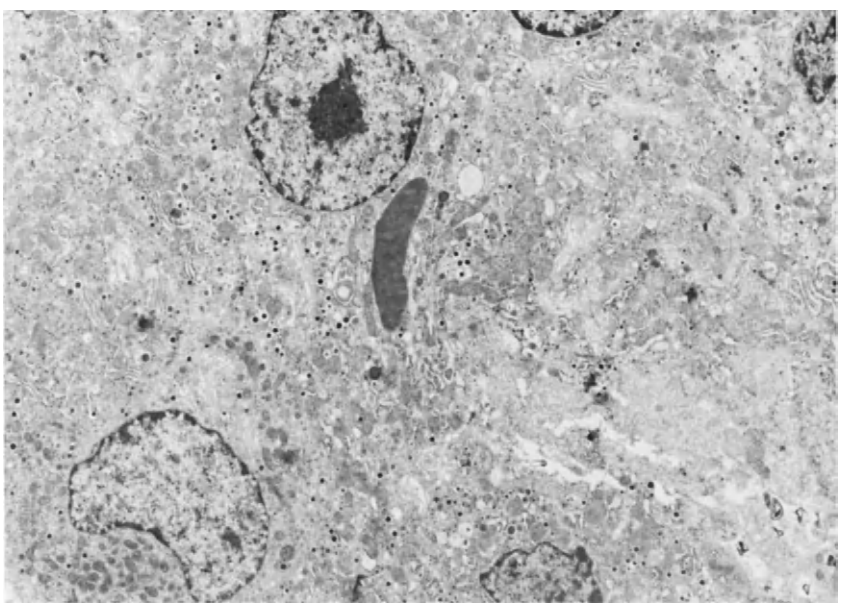

Figure 3. Area of a pancreatic tumor demonstrating virtually agranular cells next to cells with few beta granules. All cells show enhanced activity (prominent endoplasmatic reticulum and Golgi apparatus) (×6400).

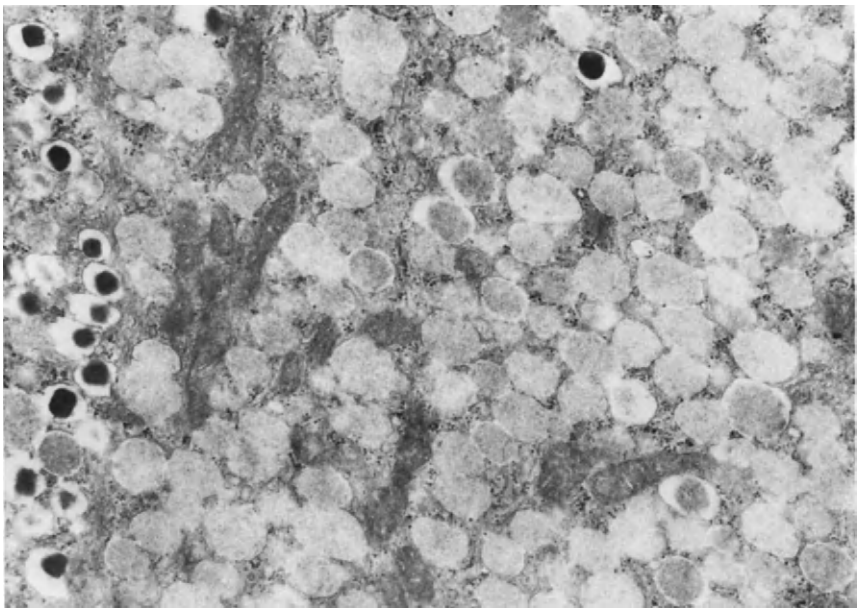

Figure 4. Portions of tumor cells from a pancreatic tumor of a rat treated with streptozotocin and nicotinamide. One cell is densely packed with pale granules resembling D cells of the normal islets but also a few secretory granules resembling beta granules. A portion of a tumor cell with numerous beta granules is shown at the left side (×24,000).

three to four cells (Figure 1). Tumors containing only somatostatin-producing cells were not found.

Ultrastructurally, all tumors contained cells with secretory granules. These varied in number, size, and shape. Beta granules were found in all tumors. Many tumor cells containing beta granules showed signs of high functional activity. They had a large dilated Golgi zone, empty vacuoles in the cytoplasm, and a well-developed rough endoplasmic reticulum (Figure 2). The number of secretory granules was markedly reduced in a varying number of tumor cells. Some cells were virtually agranular (Figure 3).

Tumor cells that resembled islet D cells occurred next in frequency. They were usually densely packed with pale granules that lacked any visible space between granular core and limiting membrane (Figure 4). However, sometimes the limiting membrane was less closely applied to the granular core. When a single or only a few electron-dense secretory granules (indistinguishable from beta granules) were found in an otherwise typical D cell, the designation became difficult.

Cells with typical alpha granules or PP granules were not encountered. This is not surprising if one thinks of the small number of cells identified by immunohistological techniques (Table 1).

Occasionally cells with electron-dense secretory granules and a tightly fitting limiting membrane were found. These cells correspond to the type IV (D_I) or "atypical" cells (Figure 5).

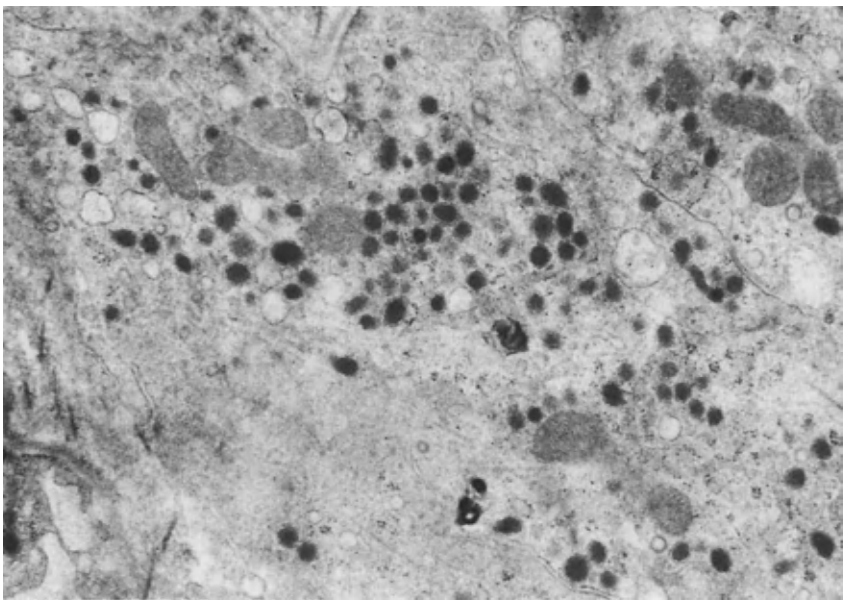

Figure 5. Portion of a rat pancreatic tumor containing only cells with electron-dense "atypical" (type IV) secretory granules (×24,000).

Discussion

Our study confirms the findings of others (5,7,8) that the simultaneous application of streptozotocin and nicotinamide is a reliable method to induce endocrine tumors of the pancreas. This may prove to be an experimental model for the study of the origin of these tumors and the mechanism of their hormone release.

The insulin concentration in the pancreatic tumors seldom amounts to the concentration found in normal islets (assuming an islet volume of 1% of the pancreas). This is in excellent agreement with the finding in human insulinomas (3). The morphological analysis confirmed the suggestion of defective storage capacity. A variable number of tumor cells were partially or completely degranulated as demonstrated by specific staining in the light microscope or by ultrastructural investigation. The latter revealed in addition signs of enhanced functional activity of the tumor cells.

The demonstration of multiple hormone production in the experimental pancreatic tumors was surprising. This has not been described by previous authors. However, it was not recognized until 1975 (1,4,6) that multiple hormone production by endocrine pancreatic tumors is a frequent event. Since all tumors of this series investigated by immunohistological methods contained insulin, and in all cases the majority of tumor cells reacted with anti-insulin serum, the designation "insulinoma" is correct (2).

A comparison of the occurrence of different hormone-producing cells in human insulinomas and these rat insulinomas reveals a remarkable

prevalence of D cells and a paucity of pancreatic polypeptide in the rat tumors. The reason for this is not known. Also remarkable is the absence of gastrin cells.

The finding of multiple hormone production in the streptozotocin-induced pancreatic tumors would be best explained by the assumption that these tumors originate from pluripotential stem cells, as has been suggested also for human pancreatic endocrine tumors (2). The finding of ductular proliferation in and around the tumors would support this view.

The frequent finding of pale granules in the rat tumors and their differentiation from D-cell granules awaits further studies. Only by applying immunohistology at an ultrastructural level or by investigating serial semithin-thin sections both with immunohistological and ultrastructural methods can the question of the stem cell and the identification of the somatostatin-producing cell be solved.

SUMMARY

Hormone-producing pancreatic tumors have been induced in rats by injection of streptozotocin and nicotinamide. After 1 year, fasting blood glucose levels were lower and the serum insulin levels higher in animals with pancreatic tumors. The insulin concentration in the tumors varied and rarely was as high as the insulin concentration of normal pancreatic islets. The majority of the tumor cells were insulin-producing B cells. A varying number of tumor cells were poorly granulated or agranular by light and electron microscopic investigation. The majority of the insulinomas contained other hormone-producing cells. Up to 50% somatostatin-producing cells and up to 3% glucagon-producing cells were found. Only scattered single pancreatic polypeptide-producing cells and no gastrin-producing cells were encountered. Ultrastructurally, cells containing mature, and a varying number of immature (pale), beta granules were seen. D cells and type IV (D_1) cells were scarce.

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In Vitro Studies of Canine Pseudo-Islets

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The endocrine cells of the canine pancreas are present in small, nonencapsulated islets or are scattered singly or in small groups in the parenchyma and ducts. This diverse histologic localization plus the larger, firm nature of the canine pancreas differs from that of the murine pancreas, in which the endocrine cells are in discrete islets and the organ is much less compact. Methods used for isolation of viable islets from the murine pancreas (5), as mechanical disruption by ductal distension followed by collagenase digestion of the minced tissue, have not been adapted to mass isolation of islets from the pancreas of large mammals, as the dog or man. In order to obtain a concentrated preparation of endocrine cells from the canine pancreas for cytological, physiological, and biochemical studies, another method has been developed (7,8). In brief, either the right (duodenal) lobe or, separately, the left (splenic) lobe was finely minced and subjected to trypsin digestion. The resulting suspension of single cells was placed on a Ficoll discontinuous gradient and centrifuged. Selected layers were suspended in media 199 with supplement, and aliquots were placed in flasks in a gyrotational culture bath at 110 rpm and 37°C. The technique is an adaptation of the rotational culture techniques described by Moscona (6). After 4 days the cultures consisted of numerous individual aggregates that were firm, solid, round to oblong, and from approximately 0.05 to 0.2 mm in diameter. Although the aggregates contained some acinar cells, they were primarily composed of endocrine cells, and thus they have been named pseudo-islets.

The pseudo-islets can be selectively removed and an exact number used for the type of functional-morphological studies that have been accomplished with isolated rat islets (4), or the pseudo-islets can be harvested in large numbers for biochemical studies and may perhaps be useful in transplantation studies.

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Because regional concentrations of pancreatic polypeptide (PP), insulin and glucagon exist in the canine pancreas, this method has allowed us to obtain pseudo-islets that differ in endocrine cell composition. In a previous study of the canine pancreas (1), we reported that PP concentration and F cells, which contain PP (2), were eight times greater in the distal portion of the right lobe than in the left lobe. In contrast, glucagon was 12 times more concentrated in the distal portion of the left lobe. The concentration of insulin in the left lobe was not quite twice as much as that in the right lobe. Our present studies of the pseudo-islets suggest that the number of A, B, and F cells prepared from each lobe reflect this difference in concentration of hormonal content.

For light microscopic study a pellet composed of 30–50 pseudo-islets was fixed overnight in Bouin's fixative, dehydrated, and embedded in paraffin. Sections 5μm thick were stained with hematoxylin-eosin or aldehyde-fuchsin-trichrome as tinctorial stains and with antibodies to insulin, glucagon, pancreatic polypeptide, and somatostatin by the unlabeled-antibody-enzyme method of Sternberger (9). For electron microscopic study the pellets were fixed at room temperature for 1/2 h in Karnovsky's fixative (3) (2% *p*formaldehyde and 4% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2) that was diluted in a ratio of 1 part fixative with 2 parts 0.1 *M* cacodylate buffer. The addition of zinc chloride (9 parts of the diluted fixative to 1 part 5% zinc chloride) greatly enhanced the preservation of the secretory granules of the F cells of the pseudo-islets. The addition of

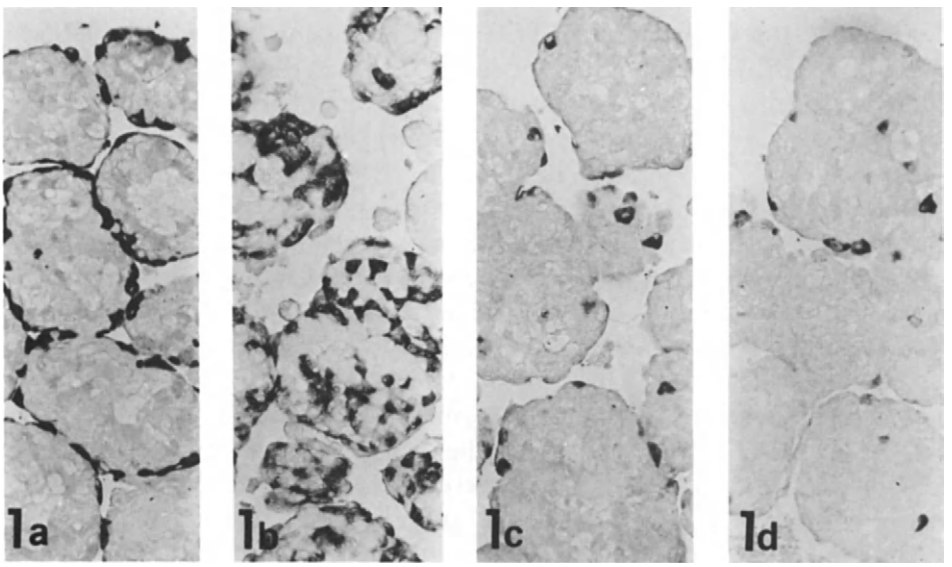

Figure 1. Sections of Bouin's-fixed pseudo-islets stained by the unlabeled-antibody-peroxidase-antiperoxidase method. The positive cells have a black cytoplasm (×400). (a) Stained with antibodies to insulin; (b) stained with antibodies to pancreatic polypeptide; (c) stained with antibodies to glucagon; (d) stained with antibodies to somatostatin.

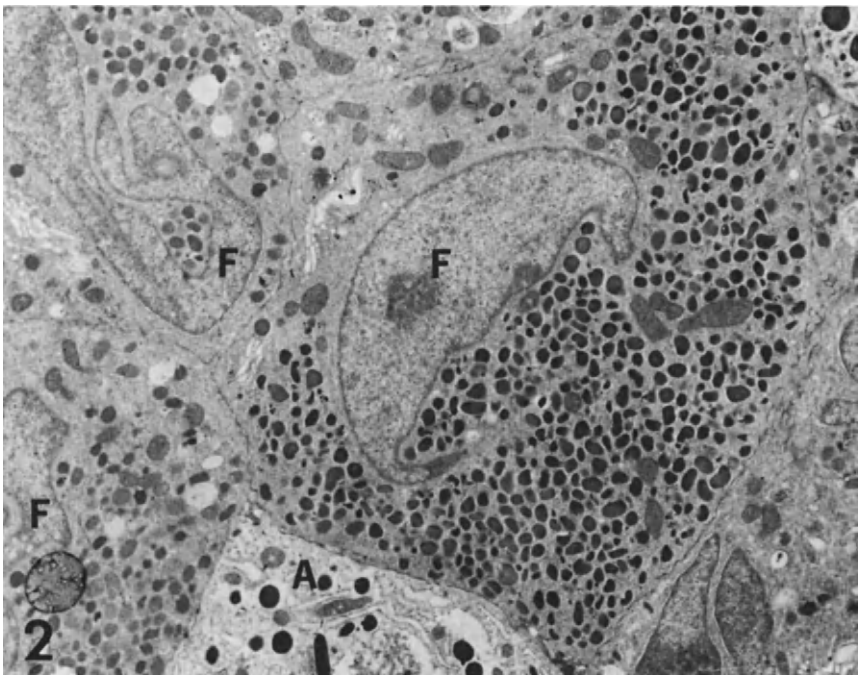

Figure 2. F cells of the pseudo-islets following fixation in a glutaraldehyde-paraformaldehyde solution containing 0.5% zinc chloride (A) acinar cell (×7000).

mercuric chloride to the fixative prepared in 0.1 *M* phosphate buffer gave similar results. The pellets were postfixed in 1% osmium tetroxide solution in 0.1 *M* cacodylate buffer, dehydrated, and embedded in an epoxy resin.

The pseudo-islets were composed of acinar cells and four endocrine cell types: A, B, D, and F cells (Figure 1). Pseudo-islets prepared from the right lobe contained more F cells than pseudo-islets from the left lobe. In pseudo-islets from both lobes, the A and B cells were located toward the edges, whereas F, D, and acinar cells were located throughout the tissue. As the pseudo-islets aged, vacuoles containing dead cells and cellular debris developed within the central area, and the endocrine and acinar cells contained autophagic vacuoles, swollen endoplasmic reticulum, and other signs of cellular injury. The endocrine cells contained numerous secretory granules and resembled those of the intact pancreas. F cells in pseudo-islets fixed in a solution with zinc chloride or mercuric chloride contained highly electron-dense secretory granules (Figure 2), whereas F cells not subjected to a fixative containing one of these metals had granules of very low electron density.

The viability of pseudo-islets in culture was examined by *in vitro* studies, measuring the insulin and PP release during stimulation. Glucose-stimulated insulin release was studied by incubation for two 30-min periods in low glucose media (50 mg%), followed by a 60-min incubation in media containing

a high glucose level (300 mg%), a level known to stimulate insulin secretion from isolated rat islets. The final media contained a low glucose level, and insulin present in all media was measured by radioimmunoassay. The results shown in Figure 3 indicate that insulin secretion increased significantly in the presence of high glucose (78.8 ± 23.9 to 309 ± 27.6 μU insulin/ml/h) and returned toward basal levels when the pseudo-islets were again incubated in media of low glucose level (134.7 ± 41.3 μU insulin/ml/h).

The release of PP from pseudo-islets during 4 weeks on culture was studied in an effort to evaluate the effects of culture time on PP secretion from the preparations. On days 7, 14, 21, and 28, pseudo-islets were transferred to separate vials for measurement of PP release during 90 min of incubation (37°C) in the absence (basal) or presence of carbachol (0.1 m*M*), a known PP secretagogue. The results from six separate isolations are shown in Table 1. Basal PP release remained stable during the first 3 weeks but began to rise by

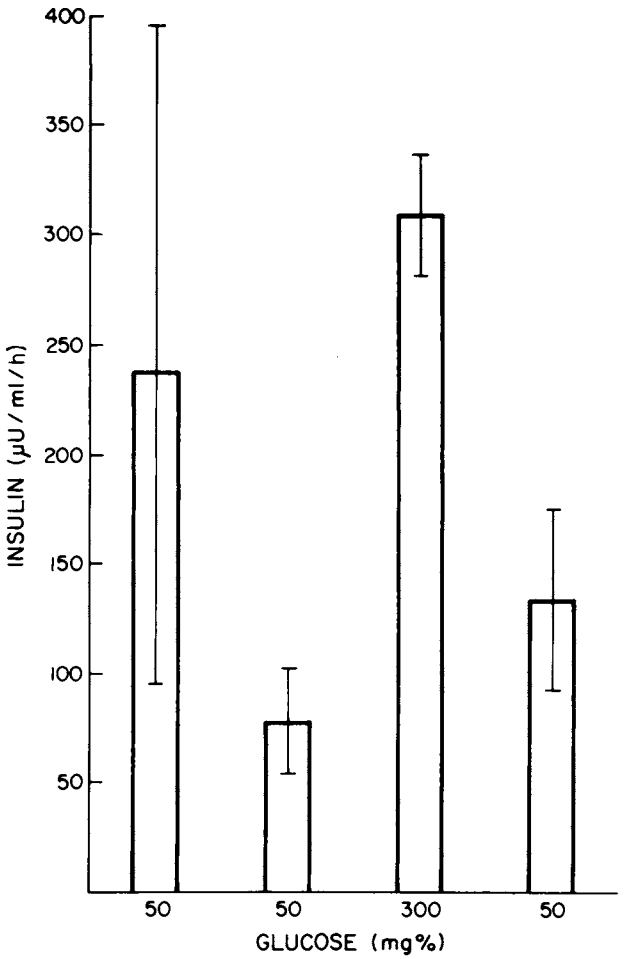

Figure 3. Insulin release from 2-week-old cultures of pseudo-islets by *in vitro* glucose challenge. Insulin is expressed as μU ml/h, ± S.E.M. (N = 3).

TABLE 1
Pancreatic Polypeptide Release from Isolated Canine Pseudo-islets in Culture ($N = 6$)

	Weeks on culture (pg PP/PI/90 min ± S.E.M.)[a]			
	1	2	3	4
Basal	49.2 ± 7.8	59.4 ± 10.7	53.8 ± 10.9	88.0 ± 26.8
Carbachol	235.4 ± 34.8	209.3 ± 48.4	174.4 ± 40.2	114.4 ± 21.6

[a]PP, pancreatic polypeptide; PI, pseudo-islet.

the fourth week. Carbachol stimulation resulted in an approximately fourfold elevation in PP values in the first week. This response dropped slightly during the second and third week, but markedly decreased by the fourth week of culture. This stability of PP secretory response over 14–21 days of culture has also been demonstrated for glucose-stimulated insulin secretion in these preparations (7).

These cytological and secretory studies suggest that cultures of isolated pseudo-islets provide an excellent *in vitro* model with which to study many parameters of PP and insulin secretion from dog endocrine tissue.

ACKNOWLEDGMENTS

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In Vitro Gastrin Release by Rat Antrum: Effects of Exogenous Prostaglandins

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An *in vitro* approach to the study of gastrin release eliminates some of the variables inherent in *in vivo* systems, such as influences due to vascular and neural stimuli. In an *in vitro* system the direct response of gastrin cells to agents to be studied can be measured under controlled and reproducible conditions, and these agents can be characterized as to their stimulatory, inhibitory, or modulating effects on gastrin release.

Gastrin cells are most concentrated in the distal portion of the mammalian antrum. Thus, unlike most gastrointestinal endocrine cells, a large number of one cell type can be obtained for culture from a small tissue sample. Such preparations have been used by us (2,3) in *in vitro* culture systems for the study of gastrin release.

The methodology of our culture preparation, briefly, is as follows: 3-mm strips of antral mucosa from weanling Wistar rats are subjected to pronase digestion at 37°C; the resulting free cells and fragmented glands are plated with culture medium into Falcon chambers and cultured at 37°C in an atmosphere of 95% air and 5% CO_2. By 3 days a state of equilibrium is reached, and well-preserved gastrin cells are shown to be present in the fragmented glands by immunocytochemical staining with antibodies to gastrin (2). Although gastrin cells were detected through 7 days of culture, the 3- to 4-day cultures contained the largest number of well-preserved cells. Cultures of this age were studied by electron microscopy (Figures 1,2) and used routinely for secretion studies.

Secretion Studies of 3- and 4-Day Cultures

Gastrin levels in the culture media, as determined by radioimmunoassay (12), were monitored at hourly intervals for 6- to 8-h periods. Unmodified

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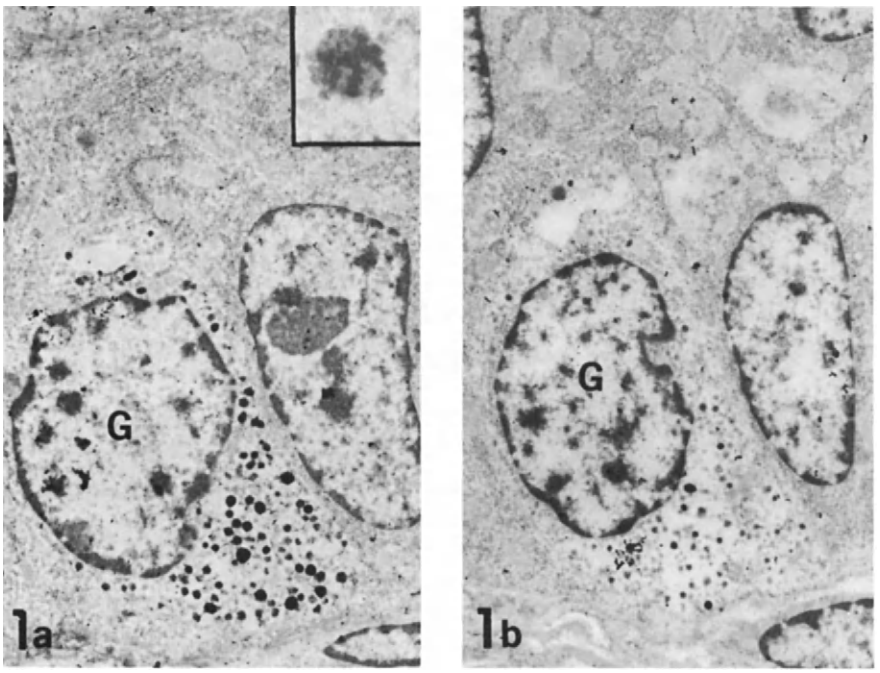

Figure 1. (a) Gastrin cell (G) of the rat antrum that has been stained with rabbit antiserum to gastrin (1:20,000) by the unlabeled antibody-peroxidase-antiperoxidase technique (11). The tissues were fixed in paraformaldehyde-glutaraldehyde, dehydrated, and embedded in an Epon 812-based resin. The highly electron-dense appearance of the storage granules is due to the immunostaining reaction product (insert). Staining was done on ultrathin sections mounted on nickel grids (×6000). (b) A near-adjacent section was treated with a 1:20,000 dilution of rabbit antiserum to gastrin that had previously been incubated with excess gastrin. The storage granules have no reaction product (×6000).

media bathed the cultures during hours 1, 3, 5, and 7, whereas media containing the substance or substances to be tested were present during hours 2, 4, 6, and 8. Such a design allowed the same cell population to act as its own control in relation to gastrin levels. The cultures maintained reproducible basal gastrin release and responded to the addition of a combination of dcAMP-theophylline by an increase in media gastrin levels (2).

In recent publications (3,4) we described the effects of agents modifying the microtubule-microfilament (MT-MF) system on basal and dcAMP-stimulated gastrin release and the effects of exogenous neurotransmitters on the cultured gastrin cells. Now, using the same experimental system, we would like to report on the effects of exogenous prostaglandins (PGs) on gastrin release.

As seen in Table 1, when a low concentration of PGE_2 is applied to the cultures, significant stimulation of gastrin release is observed when compared to basal levels. However, the progressively increasing concentrations of PGE_2 resulted in proportionately increasing inhibition of media gastrin

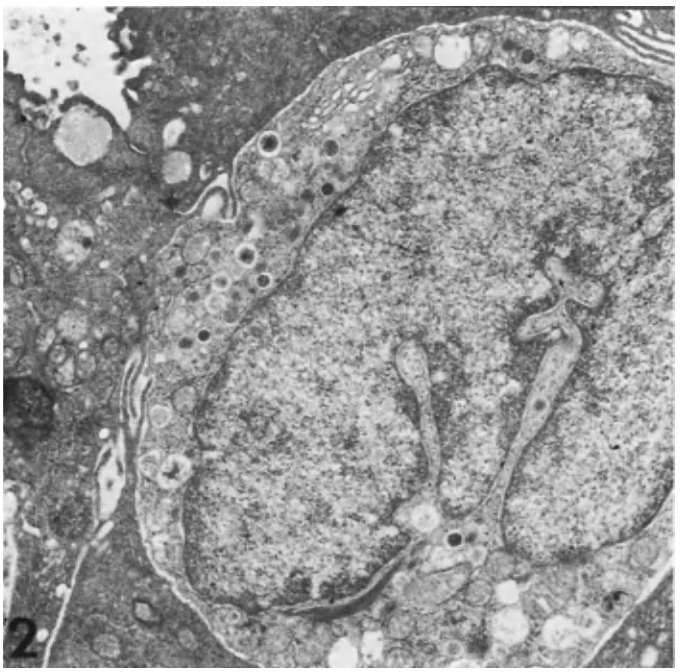

Figure 2. Gastrin cell (G) fixed in *p*-formaldehyde-glutaraldehyde solution postfixed in osmium tetroxide (×12,000).

release. In another series of experiments (not shown in tables), when cultures were preincubated in $1 \times 10^{-7}M$ norepinephrine for 1 h, the initial level of stimulation achieved by the low dose of PGE_2 was considerably higher (Δ:12.2 ± 4.5 = +73% change) than if no preincubation was done, although ultimately, inhibition of gastrin release did occur with 1 μg/ml PGE_2 (Δ:−0.9 ± 0.7 = −21%). Similar patterns of gastrin release were obtained when PGE_1 was applied to the cultures (Table 2). Although both PGE_2 and PGE_1 were

TABLE 1
Effect of Increasing Concentrations of PGE_2 on Gastrin Release

PGE_2	No. of Expts.	Difference in IRG[a] between paired basal and experimental hours (pg Culture^{-1} h^{-1} ± S.E.M.)	Change from basal (%)
10 ng/ml	4	1.83 ± 1.1	+13[b]
50 ng/ml		0.30 ± 1.5	+ 2
100 ng/ml		−0.69 ± 0.9	− 5
500 ng/ml		−2.20 ± 1.0	−15[b]
1 μg/ml		−4.70 ± 2.9	−33[b]

[a]IRG, immunoreactive gastrin.
[b]p is equal to or better than 0.05.

TABLE 2
Effect of Increasing Concentrations of PGE_1 on Gastrin Release

PGE_1	No. of Expts.	Difference in IRG[a] between paired basal and experimental hours (pg Culture^{-1} h^{-1} ± S.E.M.)	Change from basal (%)
50 ng/ml	4	0.37 ± 1.4	+ 4
100 ng/ml		1.35 ± 0.9	+13[b]
500 ng/ml		−3.28 ± 1.5	−32[b]
1 μg/ml		−4.70 ± 2.5	−46[b]

[a]IRG, immunoreactive gastrin.
[b]p is equal to or better than 0.05.

stimulators of gastrin release when tested at the low concentrations, PGI_2 proved to be considerably more potent in this regard (Table 3).

Prostaglandins E_2, E_1, and I_2 are known to cause accumulation of intracellular cyclic AMP (9). Since exogenous dcAMP has been shown to increase gastrin release *in vitro* (2,10), it was not surprising that prostaglandins E_2, E_1, and I_2 proved to be stimulatory in our system at the low concentrations tested. At the higher concentrations, however, the observed inhibition of gastrin release was thought to correspond to "desensitization" of membrane receptors for prostaglandins, as has been reported in other experimental systems such as frog erythrocytes (7). Norepinephrine (NE) pretreatment of cells resulting in a higher stimulation by subsequent PGE_2 application may be related to NE-stimulating or facilitating prostaglandin synthesis (8).

In a different series of experiments, preincubation of cultures was done with the various prostaglandins, at high concentrations for 1 h. The effects on gastrin release of subsequent application of dcAMP-theophylline, carbachol or norepinephrine were examined (Table 4). Although each of the substances applied individually resulted in significant gastrin release, preincubation with

TABLE 3
Effects of Increasing Concentrations of PGI_2 on Gastrin Release

PGI_2	No. of Expts.	Difference in IRG[a] between paired basal and experimental hours (pg culture^{-1} h^{-1} ± S.E.M.)	Change from basal (%)
10 ng/ml	4	16.2 ± 2.6	+73[b]
30 ng/ml		1.9 ± 1.3	+ 9
100 ng/ml		1.7 ± 1.8	+ 8
300 ng/ml		3.6 ± 2.5	+16[b]

IRG, immunoreactive gastrin.
[b]p is equal to or better than 0.05.

TABLE 4
Modulating Effects of PGE_2 on Gastrin Release[a]

	Agent used alone	Agent used subsequent to 500 ng/ml PGE_2 incubation
dcAMP-theophylline	22.3 ± 3.6	21.4 ± 3.2
Norepinephrine	30.8 ± 3.9	29.0 ± 2.4
Carbachol	7.0 ± 1.6	− 0.9 ± 1.5

[a]Expressed as the difference in immunoreactive gastrin between paired basal and experimental hours: pg culture^{-1} h^{-1} ± S.E.M.

PGE_2 yielded an inhibition of only the carbachol-induced gastrin release. Similar data were obtained after preincubation with PGE_1 or PGI_2 (not shown in tables). This pattern of response indicates that stimulatory mechanisms believed to involve the dcAMP system seem to behave differently with regard to prostaglandin effects those involving other mediators. Inhibition of carbachol-mediated actions by PGE_2 has also been reported using other experimental systems (9).

The physiological relevance of our data remains unknown. Oral PGE_2 has been reported to act as a potent inhibitor of meal-induced gastrin release in man (6). These findings may assume new significance in light of recent reports indicating release of prostaglandins by the stomach in response to hypertonic mucosal solutions (1,5). Prostaglandins may prove to be one of many regulating factors that contribute to determining the amplitude of gastrin cell response to intraluminal digestive products.

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In Vitro Studies of the Mechanism of Gastrin Release: Comparison of the Results between Isolated G-Cell and Organ Culture Preparations

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In order to understand the molecular aspects of hormone release it is important to be able to investigate hormone secretion under carefully controlled conditions. This is best accomplished *in vitro* by incubating the tissue of choice in chemically defined medium and measuring hormone secretion in response to the addition or deletion of one of a variety of test agents.

Over the past several years a number of laboratories, including our own, have utilized a variety of *in vitro* techniques to gain insight into the cellular mechanisms regulating gastrin release. In these studies gastrin secretion was studied from either intact antral mucosal tissue, maintained viable *in vitro* over short periods of time by a number of techniques, or more recently from isolated endocrine cells either maintained or grown in culture medium (5,6,8–14, 16).

The organ culture technique has been used with some success in our, as well as other, laboratories in the measurement of gastrin release *in vitro* (8–10,14). This system has several advantages over others presently in use: (1) the tissue appears morphologically intact and viable and is maintained in a differentiated, highly responsive state over short incubation periods, (2) the results are highly reproducible from one experiment to another, and (3) the technique is extremely simple and inexpensive to set up. The major

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disadvantage of the organ culture technique is that it does not allow an investigator to determine whether a putative secretory stimulant or inhibitor influences gastrin release by directly acting on the G cell or indirectly by affecting the release of another antral factor, which then in turn interacts with the gastrin-containing cell. Although this may cause a problem in data interpretation, this characteristic of the organ culture technique may be utilized to gain a new perspective in investigating the paracrine interactions of antral peptides and how they may influence gastrin release.

In earlier studies performed in collaboration with Trier (14), we noted that peptone as well as other *in vivo* stimulants of gastrin release induced an anomalous gastrin secretory response when added to medium bathing antral mucosal (organ culture) explants. It was found that medium gastrin levels were not enhanced above control values when the peptone-containing medium was in direct contact with the tissue, but were significantly increased during the subsequent period when peptone-containing medium was exchanged for unmodified medium. This and other supporting data led us to speculate that peptone, in addition to initiating certain cellular events necessary for gastrin release, induced the release of another antral peptide that inhibited the secretion of gastrin into the culture medium. This would account for the failure of peptone to stimulate gastrin release during the treatment periods when the concentration of the putative stimulant would be high, as well as the increase in gastrin release during the posttreatment periods, when the concentration of this substance would be diminished.

An alternative experimental approach that would circumvent the problems of data interpretation inherent in a tissue containing a heterogeneous population of endocrine cells, which is the case in the antral organ culture system, would be to isolate and subsequently purify the G cells free of extraneous cell types. In collaboration with Forssmann and Ito (6) we have developed a technique to achieve this end with some success. This was accomplished by a combined technique of selective pronase digestion followed by cell separation by unit gravity velocity sedimentation. It was determined that the G cells were purified approximately 50–100-fold by this technique in comparison with their original concentration in the intact mucosae, resulting in a final G-cell purity of 15–25%. The G cells remained viable throughout the purification procedure as determined by both morphological examination at the light and electron microscopic level and by functional responsiveness (11). It was in turn demonstrated that the *in vitro* gastrin secretory properties of G cells in response to a challenge with proteinaceous agents varied markedly between the isolated enriched G-cell preparation and the organ culture system. In the following studies we investigated the possible role of the antral peptides, somatostatin, and glucagon as paracrine regulators of gastrin release that may contribute to these discrepant findings.

METHODS

ORGAN CULTURE

Rodent antral mucosae were dissected free from muscle layers, cut into small portions (1–2 mm^3), and set down and incubated in organ culture dishes by a technique described in detail previously (14). After an initial equilibration period in unmodified Trowells T-8 medium, in an atmosphere of 95% O_2/5% CO_2 at 37°C, secretory testing was initiated. This was accomplished by dividing the cultures into control and treatment groups, incubating all cultures in unmodified medium during the first 15-min basal period, and completely exchanging the solution with the appropriate incubation medium at 15-min intervals thereafter. The cultures designated as "controls" received only unmodified medium throughout the study period, whereas the media containing various test agents were added to the culture dishes during the designated periods. The collected media were then stored at −20°C for subsequent gastrin and somatostatin radioimmunoassay.

ENRICHED G-CELL PREPARATION

An enriched fraction of rodent G cells was prepared by the combined techniques of selective pronase digestion, followed by cell separation by unit gravity velocity sedimentation, as described previously (6). Briefly, this method involves incubating the antra of 30–40 rats in pronase (0.15%)-containing KRB buffer (pH 7.4) for 90 min. This results in the proteolytic removal of cells from the mucosal surface to the neck region of the antral glands, leaving the mid- to basal glandular region intact, where the G cells are localized. These cells were then removed by exposing the tissue to high shearing forces. Cell aggregates were then disrupted by incubating the exfoliated basal glandular cells in DNase (20 μg/ml)-containing buffer followed by filtration (75μm). The G cells of this crude preparation were then further purified by unit gravity velocity sedimentation by applying the cells to the top of a cell sedimentation chamber containing a KRB buffer in which an albumin gradient (2–4%) had been established. The cells sedimented in accordance with their volume and were collected in a fraction collector. It had previously been determined, both by morphology and immunoreactivity, that the G cells with a cell diameter between 9 and 12 μm, elute in a discrete fraction in a region corresponding to the last 75–90% of the chamber volume and constitute between 15 and 25% of the cell population. It was in turn calculated that this represents an approximate 50–100-fold purification in comparison with the intact tissue. Cells from this fraction (50–200 × 10^3 cell/tube) were then added to siliconized tubes containing KRB buffer (1 ml) with and without the test agents for hormone secretory testing. The cell mixture was incubated in a shaking water bath at 37°C in an atmosphere of 95% air/5% CO_2 for the desired period of time. The incubation period was

terminated by centrifuging the tubes (500 *g*) at 4°C, separating the cells from the medium, and heat-extracting both at 96°C for 10 min. The samples were then stored at −20°C for subsequent radioimmunoassay.

GASTRIN RADIOIMMUNOASSAY

Gastrin was measured by a modification of the technique of Yalow and Berson as described by Walsh (13,19,20). Antibody 1296, kindly donated by John H. Walsh (CURE), was used in all assays. Human G-171, donated by Morton I. Grossman (CURE), was used as both standard and label. Gastrin was iodinated by the chloramine-T technique and purified by the method of Stadil and Rehfeld (18).

SOMATOSTATIN RADIOIMMUNOASSAY

Somatostatin was measured by a modification of the technique of Patel *et al.*. as previously described (12,15). Antibody 56, kindly donated by Jean Chayvialle, Lyon, France, was used in all assays (4) as well as in an organ culture experiment to be described below. Iodinated by the chloramine-T technique, 1-Tyr-somatostatin (Beckman) was purified by Sephadex G-25 chromatography (15). Cyclic synthetic somatostatin was used as standard. Appropriate concentrations of these substances were initially incubated at 4°C in 0.05 *M* phosphate buffer (pH 7.4) containing 0.25 *M* EDTA and 0.1% bovine serum albumin and after 48 h antibody-bound label was separated from free ^{125}I labeled somatostatin by second antibody precipitation. The assay had sufficient sensitivity to detect somatostatin concentrations as low as 4 pg/ml (ED_{50} = 15 pg/ml).

Results and Discussion

ORGAN CULTURE

As noted earlier, previous experiments have indicated that peptone induces a prominent increase in gastrin release from antral organ culture explants only during the posttreatment periods after peptone-containing medium is exchanged for unmodified medium (14). This "off response" to peptone and other *in vivo* secretory stimulants could be mediated by the release of an inhibitory antral peptide during the treatment periods. Of all the established antral peptides (1), somatostatin appeared to be the most promising candidate to mediate this response, since it has previously been reported to inhibit gastrin release in both *in vivo* and *in vitro* systems (3,8,10), and recent evidence suggests that instillation of a protein extract into the canine gastric lumen markedly stimulates somatostatin release into the antral vein (17). Indeed, preliminary experiments from our laboratory indicated that peptone and other proteinaceous agents directly stimulated somatostatin release from antral mucosal explants in organ culture (12). For this reason we decided to investigate further the relationship between medium gastrin and somatostatin concentrations in organ culture in order to

determine whether or not somatostatin may have a paracrine influence over gastrin release in this *in vitro* system.

In the initial experiments, therefore, we monitored medium somatostatin levels under various *in vitro* conditions that might alter the concentration of the hormone in the bathing solution. In addition to peptone, a second group of organ culture explants was incubated in glucagon-containing medium, since it had previously been reported to stimulate somatostatin release in other *in vitro* systems (2). Also a saturating concentration of somatostatin antiserum was added to the medium in another group of organ cultures to find all the available hormone. The results are shown in Table 1.

It can be appreciated that medium somatostatin levels remained fairly constant in the control cultures throughout the study. In contrast, incubation of antral mucosal organ culture explants in medium-containing 5% peptone directly induced a highly significant 15–20-fold increase in medium somatostatin levels that returned to control values during the posttreatment periods. The addition of a saturating titer of somatostatin antiserum (1:50) to

TABLE 1
Alterations in Somatostatin Release from Antral Mucosal Organ Culture Explants

		Periodic changes in medium somatostatin concentration[a]					
Group	n	1	2	3	4	5	6
Control	5	100	98 ±20	59 ±10	57 ±3	79 ±23	72 ±15
5% Peptone (2,3)[c]	5	100	1820[b] ±253	1890[b] ±281	184[b] ±50	99 ±20	95 ±17
5% Peptone (2,3)[c] + 1×10^{-5} M glucagon (4,5)[c]	5	100	1595[b] ±127	1723[b] ±94	209[b] ±8	97 ±10	3957[b] ±150
5% Peptone (2,3)[c] + somatostatin antisera (2,3)[c]	5	100	<20 U·D[d]	<20 U·D	<20 U·D	<20 U·D	322 ±122

[a]Somatostatin concentration expressed as a percentage of the medium hormone concentration measured during the basal period, period 1. The mean basal medium somatostatin concentration is 32 pg/ml.

[b]Represents a significant difference in comparison to the hormone concentration of control cultures measured during the same period.

[c]Number in parentheses represents the period(s) that the test agent was added to the medium.

[d]U·D below the detection limits of the radioimmunoassay.

the medium in the presence of peptone effectively abolished this increase, as hormone levels decreased below the detection limits of the radioimmunoassay. One surprising finding was that incubation of antral explants during periods 4 and 5 in medium supplemented with glucagon failed directly to stimulate somatostatin release into the medium as reported in other systems (2). However, somatostatin release was markedly increased 40–50-fold during the following period when glucagon-containing medium was exchanged for unmodified medium.

The gastrin secretory responses of the same antral organ culture explants to the preceding treatments are shown in Table 2. As reported earlier, gastrin secretory output during exposure to peptone remained relatively constant in the control cultures, decreasing somewhat from the levels measured during the basal period. In addition, peptone induced a prominent "off response," as medium gastrin levels increased 1.5–2-fold above control values during the posttreatment periods (periods 4 and 5). It can also be appreciated that abolishing the elevation of medium somatostatin concentration during the peptone treatment period by the addition of somatostatin antisera failed to influence the gastrin secretory response. This response, however, was

TABLE 2
Alterations in Gastrin Release from Antral Mucosal Organ Culture Explants

Group	n	Periodic changes in medium gastrin concentration[a]					
		1	2	3	4	5	6
Control	5	100	97 ±10	80 ±8	67 ±14	69 ±10	76 ±18
5% Peptone (2,3)[b]	5	100	53 ±5	52 ±9	108 ±19	140[c] ±13	118 ±18
5% Peptone (2,3)[b] + 1×10^{-5} *M* glucagon (4,5)[b]	4	100	95 ±23	46 ±9	59 ±9	94 ±13	131[c] ±16
5% Peptone (2,3)[b] + somatostatin antisera (2,3)[b]	5	100	102 ±25	63 ±8	132 ±34	135[c] ±28	112 ±20
Peptone (2,3)[b] + 20 μg/ml somatostatin (4,5)[b]	5	100	84 ±5	68 ±10	134[c] ±10	200[c] ±10	200[c] ±33

[a]Medium gastrin concentration expressed as a percentage of the medium hormone concentration measured during the basal period, period 1. The mean basal gastrin concentration is 344 pg/ml.

[b]Number in parenthesis represents period(s) that the test agent was added to the medium.

[c]Represents a significant difference in comparison to the hormone concentration of control cultures measured during the same period.

significantly diminished if glucagon was added to the incubation medium at high concentrations during the posttreatment periods. As indicated previously, somatostatin levels remained at control values during the periods the tissue was exposed to glucagon. It is also of interest that both gastrin and somatostatin levels displayed an "off response" to glucagon, significantly increasing upon its removal.

In a previous study it was determined that maintenance of an elevated somatostatin level during the peptone posttreatment period by supplementation of the medium with exogenous hormone failed to influence the gastrin secretory response (12). In those experiments a dose of somatostatin of 4 ng/ml was administered, since it was determined that this best mimicked the medium somatostatin levels recorded in the presence of peptone. It was initially thought that this negative result may be attributable to the low dose of somatostatin employed. For this reason, in the present study, an additional group of antral organ culture explants was exposed to a high dose of somatostatin (20 μg/ml) during the posttreatment periods. It, however, can be appreciated that the gastrin secretory response to the removal of peptone was not diminished, but in fact was enhanced slightly by the addition of exogenous somatostatin to the medium.

These results therefore strongly suggest that gastrin and somatostatin levels appear to change independently in organ culture, and a discernible negative correlation between the two antral peptides is not apparent. It is not clear why high concentrations of somatostatin failed to influence gastrin release in culture in contrast to findings reported in other systems (8,10). It is possible that a certain cholinergic tone is required for the inhibitory action of the peptide to be manifest, since it has been reported that somatostatin inhibits the release of acetylcholine from the myenteric plexus (7). Another possible explanation is that somatostatin may inhibit the release of another antral peptide (i.e, glucagon), which has even more pronounced inhibitory influence on gastrin release than somatostatin itself. This possibility was given some support by the finding that the addition of glucagon to the medium blocked the gastrin secretory response to peptone during the posttreatment periods.

ENRICHED G-CELL PREPARATION

In contrast to the gastrin secretory response in organ culture, peptone directly stimulated gastrin release from a preparation of isolated and enriched G cells (Table 3). It was determined that peptone as well as other products of proteolysis (i.e., a peptic digest of BSA) induced a 2–4-fold increase in medium gastrin levels over control values during a 5-min incubation period. The reason the same stimulant induced a prominent "on response" in this system and an "off response" in the organ culture system is unknown. It is possible, however, that the paracrine interactions of antral peptides that may play an important role in the regulation of gastrin release in intact antral

TABLE 3
Effect of Somatostatin on Peptone-Stimulated Gastrin Release from an Isolated and Enriched Preparation of Rodent G Cells

	n	Medium gastrin concentration[a]
Control	27	102 ±6
2.5% Peptone	28	306[b] ±22
2.5% Peptone + 1 μg/ml somatostatin	10	190[b] ±19

[a]Gastrin concentration expressed as a percentage of the medium hormone concentration of unincubated controls. The mean basal medium gastrin concentration is 5.5 ng/10^6 cells.

[b]See Table 1, footnote *c*.

mucosal organ culture explants may be reduced or eliminated in the isolated G-cell preparation because there is a decreased number of other endocrine cells (i.e., D and A cells) in the enriched G-cell preparation and also because of complete disruption of intimate intercellular contacts between neighboring antral endocrine cells (6). In addition, somatostatin induced a modest but significant reduction in the gastrin secretory response to peptone in the enriched G-cell preparation (Table 3). The reason somatostatin has a discernible inhibitory activity in this system, but not in organ culture, is uncertain. It may be attributable to the fact that the organ culture technique is diffusion limited, whereas this is not the case in the isolated G-cell preparation. Thus the putative somatostatin receptors on the basal membrane of G cells of the antral mucosal organ culture explants may not be accessible to the exogenous hormone even when somatostatin is added at high concentration to the medium. Alternatively, it is possible that the removal of other antral endocrine cells in the enriched G-cell preparation, which also may be influenced by somatostatin, unmasked the inhibitory activity of the peptide on gastrin release not readily apparent in the multicellular organ culture system.

In summary, we feel that gastrin release is under the paracrine influence of local antral peptides as well as extrinsic factors (intraluminal, neural, systemic). By nature these local interactions are difficult to investigate and establish, and it is our contention that information from various kinds of *in vitro* systems is needed to understand fully the importance of paracrine factors in the regulation of gastrin release.

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