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Date May 12, 1988
ABSTRACT

The effects of endogenous somatostatin on gastrin and insulin release were studied by passive immunization with a somatostatin monoclonal antibody, SOMA 10, in the isolated perfused rat stomach and pancreas, respectively. Previous passive immunization studies with somatostatin antiserum in the isolated perfused stomach have yielded conflicting results. The differences in results could be due to the differences in the binding characteristics of the antisera used, and the accessibility of the antiserum to the tissue. In previous studies in vivo and in isolated islets, somatostatin antiserum caused an increase in insulin release. Previous attempts at immunoneutralizing somatostatin in the isolated perfused pancreas of mammals have yielded negative results. However, the isolated perfused pancreas is an ideal model for passive immunization studies, because hormonal and central nervous system influences are eliminated, and the microanatomy of the islet is preserved.

This study differed from previous studies in that a monoclonal antibody to somatostatin, which is more specific than somatostatin antiserum, was used in an attempt to neutralize endogenous gastric and pancreatic somatostatin. Fab fragments of SOMA 10 were made by papain digestion and purification on protein A-sepharose. These fragments are advantageous for passive immunization, since they contain the somatostatin binding site, and are much smaller than the intact antibody. Therefore they should more readily penetrate into the interstitium and neutralize endogenous somatostatin.

SOMA 10 was purified by ammonium sulphate precipitation, in conjunction with hydroxylapatite chromatography. Purity was checked by gel filtration and affinity HPLC and determined to be 93%. Scatchard analysis calculated the binding capacity of SOMA 10 to be 8.3 µg/mg, and the dissociation constant to be 2.2 nM. Both SOMA 10 and the Fab fragments were shown to inhibit the effect of exogenously administered somatostatin in the isolated perfused stomach and in gastric fistula rats.
Single passage of SOMA 10 in the isolated perfused stomach at 100 µg/ml caused a significant decrease in basal gastrin release. Recirculation of the antibody in the stomach caused an increase in cumulated gastrin release in comparison to controls in which perfusate without the antibody was recirculated. Infusion of the Fab fragment at 15 and 66 µg/ml caused an increase in basal gastrin release suggesting that somatostatin inhibits basal gastrin release. Immunocytochemical staining of the perfused stomachs revealed that the Fab fragments but not the intact antibodies had penetrated into the interstitium.

In the pancreas, infusion of 45 µg/ml SOMA 10 and 30 µg/ml Fab fragments inhibited insulin secretion in response to 8.8 mM glucose. There are several explanations for the unexpected decrease in gastrin release and insulin release, observed when SOMA 10 was infused. The antibody and fragment could be binding to the somatostatin receptor and mimicking its effects on gastrin and insulin. A change in conformation of the somatostatin molecule due to binding of the antibody could change the affinity for the receptor. The increased secretion could be a result of neutralization of somatostatin, which inhibited a previously unknown inhibitor of gastrin and insulin release. Immunocytochemical staining revealed that both SOMA 10 and its Fab fragment had entered into the interstitium, implying that endocrine and paracrine effects of SOMA 10 could not be distinguished.

In summary, the increase in gastrin release observed when Fab fragments were infused and when SOMA 10 was recirculated suggested that somatostatin exerts a continuous restraint on gastrin release. Both SOMA 10 and Fab fragments were found in the pancreatic interstitium implying that large molecules can pass through the vascular walls, and that endogenous and paracrine effects of the antibody cannot be distinguished in this model.
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INTRODUCTION

Somatostatin was isolated in 1973 from ovine hypothalamic extracts on the basis of its ability to inhibit growth hormone secretion (Brazeau et al., 1973). The isolation of somatostatin was a milestone in that it was the first hormone found to have an inhibitory action on the anterior pituitary. At the time only releasing hormones had been isolated.

Further actions of somatostatin were soon identified. Somatostatin administration was shown to inhibit growth hormone secretion induced by L-dopa in man (Siler et al., 1973) and in dogs (Lovingier et al., 1974), and meal-stimulated gastrin secretion in man (Bloom et al., 1974). In 1975, Gomez-Pan et al. demonstrated that somatostatin inhibited gastric acid and pepsin secretion in response to pentagastrin and food stimulation in cats. Koerker et al. (1974) found that somatostatin caused hypoglycemia, and inhibited insulin and glucagon release. Somatostatin is now known to have several other central and peripheral actions. In the gut, somatostatin suppresses motor activity, nutrient absorption, blood flow and the secretion of all known peptides. These peptides include gastrin, cholecystokinin, vasoactive intestinal peptide (VIP), secretin, insulin and glucagon. It also inhibits the release of exocrine secretions, including pancreatic exocrine and gastric acid secretion (McIntosh, 1985).

Biologically active somatostatin exists in three major forms, with 14 (somatostatin-14), 25 (somatostatin-25) and 28 (somatostatin-28) amino acids. Both of the larger forms are extended at the N-terminus. In mammals, the amino acid sequence of somatostatin-14 is completely conserved. Two forms with regions of homology are found in anglerfish. Originally, somatostatin-28 was thought to be merely a precursor of somatostatin-14. However, somatostatin-14 has been shown to be formed directly from prosomatostatin, a 116 amino acid residue protein, without the intermediate formation of somatostatin-28 in the rat pancreas (Patel, 1983). The relative proportions of somatostatin-14 and -28 are cell and organ specific. Using radioimmunoassay with gel filtration chromatography, Penman et al. (1983) found higher concentrations of somatostatin-14 than -28 in the antrum, duodenum and pancreas, but lower concentrations of somatostatin-14 than -28 in the corpus, jejenum, ileum and colon in man.
The forms of somatostatin display differences in action. Somatostatin-28 is more potent at inhibiting insulin secretion than the 14 amino acid residue form (Mandarino et al., 1981), but only somatostatin-14 affected mesenteric circulation, oxygen uptake and intestinal motility in dogs at the concentrations tested (Konturek et al., 1981). On a molar basis, somatostatin-14 and -28 were found to be equipotent in causing inhibition of peptone-meal stimulated acid secretion (Seal et al., 1982). Somatostatin-28 has a plasma half life of less than 3.6 minutes, while that of somatostatin-14 has always been found to be less than 2 minutes (Polonsky et al., 1982) depending on the species. Nevertheless, the half life of somatostatin-28 has always been found to be longer than the half life of the 14 amino acid residue peptide. The longer half life of somatostatin-28 may influence the potency on a specific biological activity compared to somatostatin-14.
Somatostatin in the Stomach

In the stomach, somatostatin is located in endocrine D cells in the corpus and in the antrum. In the guinea pig, somatostatin is also located in gastric neurons. In the intestines of all animals studied, somatostatin is located in endocrine cells in the lamina propria, and in neurons in the myenteric plexus, and especially the submucous plexus, where it innervates the circular muscle, mucosa and submucosa (McIntosh, 1985).

Somatostatin is thought to be a physiological regulator of both acid secretion and gastrin release, because it is a strong inhibitor of both, and because of the proximity of the D cells to parietal cells in the corpus and to G cells in the antrum. Electron microscopy (Larrson et al., 1979) and double immunocytochemical staining techniques (Wolfe et al., 1984) have revealed long cytoplasmic processes from D cells abutting on parietal and G cells, suggesting paracrine relationships. The proportion of D cells to G cells is reasonable for such a relationship. The number of parietal cells is much greater than the number of D cells, suggesting that this type of relationship is unlikely. However, parietal cells may be electrically coupled or in subgroups, such that somatostatin could inhibit them in a paracrine manner. Somatostatin may also inhibit only a proportion of parietal cells by a paracrine route. An inhibition of acid secretion by blood-borne somatostatin has also been suggested.

There is substantial evidence for a functional linkage between gastrin and somatostatin. In earlier studies, exogenous somatostatin was shown to inhibit gastrin release. Hayes et al. (1974) demonstrated that somatostatin could eliminate the biphasic response of gastrin to arginine stimulation in perfused pieces of rat antrum. Infusion of somatostatin inhibited liver extract-stimulated gastrin release in dogs in vivo (Konturek et al., 1976), and basal and carbamylcholine-induced gastrin release from the isolated perfused canine stomach (Lefebvre et al., 1981).

The regulation of gastrin and somatostatin secretion is mediated by both cholinergic and non-cholinergic mechanisms. Evidence for a cholinergic component in the regulation of gastrin and somatostatin comes from a variety of preparations. For example, in the anaesthetized pig (Oleson et al., 1987), rat
antral tissue culture (Wolfe et al., 1984; Lucey et al., 1985) and isolated perfused rat stomach (Saffouri et al., 1980,1984a; McIntosh et al., 1981), stimulation of the vagus or stimulation with cholinergic agonists produced inhibition of somatostatin release and the stimulation of gastrin release. The gastrin response to the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) was completely abolished by hexamethonium, a nicotinic receptor antagonist, but only partially inhibited by atropine, suggesting that the intramural neurons were of a cholinergic and a non-cholinergic nature (Schubert and Mahklouf, 1982). In the isolated perfused rat stomach tetrodotoxin, an axonal blocker, completely reversed the inhibition of somatostatin secretion and the stimulation of gastrin secretion in response to a peptone solution, suggesting that these responses were mediated by intramural neurons. These responses were only partially blocked by atropine, a muscarinic receptor antagonist, supporting the involvement of both cholinergic and non-cholinergic neurons (Saffouri et al., 1984a).

The most prominent candidate for the stimulatory regulation of gastrin and somatostatin secretion is gastrin-releasing peptide (GRP). Schubert et al. (1983) found that bombesin antiserum, the amphibian counterpart to GRP, inhibited neurally mediated gastrin secretion. Stimulation of the vagus caused an increase in GRP and gastrin release, but reduced somatostatin secretion (Nishi et al., 1985). GRP is a neuropeptide present in gastric mucosal nerve terminals, and has been shown to stimulate somatostatin and gastrin release. GRP infusion during vagal blockade elevated postprandial gastrin secretion (Greenberg, 1987).

The secretion of somatostatin and gastrin are not always coupled. Pederson et al. (1981) observed that vagotomized rats exhibited basal hypergastrinaemia without concomitant changes in somatostatin secretion. Martindale et al. (1982) found that a concentration of bombesin required to stimulate somatostatin secretion had no effect on gastrin release. Atropine and hexamethonium inhibited bombesin-stimulated somatostatin release, but not bombesin-stimulated gastrin secretion, suggesting that somatostatin and gastrin secretion are not always functionally linked.

The secretion of gastric somatostatin and gastrin is also influenced directly by luminal chemicals. Microvilli on the cell membrane are exposed to
the lumen, and provide the means by which changes in the gut contents, especially hydrogen ion concentration, can be detected. Perfusion of the lumen in the isolated perfused rat stomach with an alkaline perfusate increased gastrin release in the basal state (Saffouri et al., 1984). Perfusion with an acidic perfusate caused the release of less gastrin and more somatostatin in the unstimulated state (Saffouri et al., 1984) and in response to vagal stimulation (Alino et al., 1986).

Peptides such as vasoactive intestinal peptide (VIP), secretin, gastric inhibitory polypeptide (GIP), the opioid peptides, substance P and galanin are known to affect gastrin and somatostatin secretion. Infusion of glucagon, secretin and VIP in the isolated perfused rat stomach caused a concentration-dependent increase in somatostatin secretion with a simultaneous concentration-dependent decrease in gastrin secretion, suggesting that the suppression of gastrin secretion induced by the hormones was mediated, at least in part, by somatostatin (Chiba et al., 1980). A study by Wolfe et al. (1983), using somatostatin antiserum on secretin-induced inhibition of carbachol-stimulated gastrin release in cultured rat antral mucosa concurred with Chiba et al. (1980) that secretin inhibition of gastrin release is mediated by somatostatin. In contrast, the infusion of VIP caused a transient increase in gastrin secretion, but a sustained increase in somatostatin secretion. Saffouri et al. (1984b) concluded from the simultaneous infusion of VIP and somatostatin antiserum that VIP participates in the regulation of somatostatin, but not of gastrin secretion. GIP increases somatostatin release and inhibits gastrin release only when the vagus is not stimulated (McIntosh et al., 1983), or when gastric acid secretion is high (Holst et al., 1983a). GABA induced stimulation of gastrin release and inhibition of somatostatin release is thought to be through the stimulation of antral postganglionic cholinergic neurons (Harty and Franklin, 1986). It has been suggested that opioid peptides at least partially mediate vagally-induced inhibition of somatostatin secretion (McIntosh et al., 1983), and that they may be involved in the vagal regulation of GRP and gastrin (Nishi et al., 1985). Substance P inhibited both basal somatostatin and somatostatin release stimulated by GIP or isoproterenol in the isolated perfused rat stomach (Kwok et al., 1985), but had no effect on gastrin release (Kwok, unpublished). Galanin suppressed somatostatin and gastrin release dose-dependently in the isolated perfused rat stomach, suggesting that it is involved in the neural
regulation of gastric endocrine secretions (Kwok et al., 1988).

The role of the sympathetic nervous system in the control of gastrin and somatostatin secretion is still unclear. Koop et al. (1982) found that infusion of isoproterenol into the isolated, perfused rat stomach caused the release of somatostatin. However, McIntosh et al. (1981) found that splanchnic nerve stimulation increased somatostatin secretion only when atropine was infused concomitantly. Direct stimulation of somatostatin secretion by sympathomimetics is unaffected by atropine, suggesting that there may be a cholinergic component within the splanchnic nerve inhibiting somatostatin secretion. The infusion of atropine, but not alpha and beta receptor blockers in humans inhibited the rise in postprandial somatostatin, suggesting that cholinergic, but not adrenergic mechanisms are important modulators of somatostatin (Lucey et al., 1985).
Somatostatin in the Pancreas

The interaction between islet hormones is complex. Somatostatin inhibits both insulin and glucagon secretion. Insulin inhibits both somatostatin and glucagon secretion, and glucagon stimulates both insulin and somatostatin release. In addition, insulin, glucagon and somatostatin inhibit their own secretions (Weir and Bonner-Weir, 1985). However, stimulation of the pancreatic islet with different substrates does not yield the same responses of these hormones. Stimulation with glucose causes an increase in insulin and somatostatin secretion, but a decrease in glucagon secretion. Stimulation of the pancreas with amino acids causes the release of all three hormones. Pancreatic polypeptide is present in the islets in specific cells (PP cells). It has been shown to affect insulin and glucagon release, but only at pharmacological concentrations.

The secretions of insulin, glucagon and somatostatin are pulsatile (Goodner et al., 1982; Stagner et al., 1982) with periods of 10 minutes, 8.6 minutes and 10 minutes, respectively, in the canine pancreas. This intermittent secretion is thought to increase the efficiency of glucose disposal (Bergman et al., 1985) by preventing receptor desensitization. Stagner and Samols (1985 a, b) suggested that the pacemaker lies in the pancreatic ganglia, and simultaneously innervates many islets randomly distributed throughout the pancreas.

In man, the isolated perfused rat and canine pancreas, and in isolated rat islets, exogenous somatostatin administration inhibited the first and second phase of insulin release stimulated by arginine and glucose, and inhibited glucagon release (Alberti et al., 1973; Curry et al., 1974; Efendic et al., 1974, 1976). Studies by Curry and Bennett (1976) indicated that the first phase of insulin release was 25 to 50 times more sensitive to somatostatin inhibition than the second phase. These studies suggested that somatostatin is a physiological regulator of insulin and glucagon secretion. Somatostatin modulates postprandial release of these hormones and of glucose levels (O'Shaughnessy et al., 1985).

There is increasing evidence that somatostatin affects glucagon
secretion much more than it does insulin secretion (Gerich et al., 1975; Itoh et al., 1980). Since the A cell is ~50 times more sensitive to somatostatin inhibition than the B cell, the inhibition of glucagon may be a more important function of somatostatin than the inhibition of insulin (Mandarino et al., 1981). Furthermore, there is parallel distribution of A and D cells. Fewer contacts exist between B and D cells supporting the suggestion that somatostatin has a greater effect on glucagon than on insulin secretion (Orci and Unger, 1975).

The secretion of somatostatin is influenced not only by metabolic substrates and the secretion of other pancreatic peptides, but also by neuropeptides and the autonomic nervous system. VIP, secretin, substance P, cholecystokinin, gastrin, neurotensin and prostaglandin E2 stimulate somatostatin secretion. Endorphins depress somatostatin secretion (Reichlin, 1983). Vagal stimulation or stimulation of the rat and pig pancreas with cholinergic agonists inhibited somatostatin release (Samols et al., 1978; Uvnas-Wallensten et al., 1980; Holst et al., 1983b, Ahren et al., 1986). In the canine pancreas, vagal stimulation or acetylcholine increased somatostatin secretion (Ahren et al., 1986). Alpha adrenergic agonists or adrenergic nerve stimulation inhibited somatostatin secretion in some studies (Samols et al., 1978, Holst et al., 1983b), but had no effect in others (Lucey et al., 1985; Nishi et al., 1987). Beta adrenergic agonists stimulate somatostatin secretion (Samols et al., 1978, Holst et al, 1983b).

Exocrine secretion of the pancreas is also influenced by islet secretion. Glucose stimulated islets receive a disproportionate volume of the pancreatic circulation in comparison to the exocrine tissue (Jansson and Hellerstrom, 1983). Bonner-Weir and Orci (1982), using methacrylate corrosion casts, found that a proportion of the efferent capillaries from the islets serve the exocrine tissue before converging into venules. Therefore this tissue is subjected to high concentrations of islet hormones, especially somatostatin (Kawai et al., 1982), which influence exocrine secretion.

The organization of the islets of Langerhans varies with the species. In the rat, the Chinese hamster and mouse, somatostatin-containing D cells and glucagon-secreting A cells lie in the periphery of the islet surrounding an insulin-secreting B cell core. In man, A and D cells are grouped together against capillary walls (Orci and Unger, 1975).
Using antisera against insulin, glucagon, somatostatin and pancreatic polypeptide to stain successive serial sections of the pancreas, Baetem et al. (1979) found that two different types of islets exist in rat. Glucagon-rich, pancreatic polypeptide-poor islets are found mostly in the dorsal lobe of the pancreas, while pancreatic polypeptide-rich, glucagon poor islets are found in the ventral lobe.

The islet microanatomy appears to be important to the normal functioning of cell secretion. In dispersed islet cells, in which the direct communication and hormonal secretion from other cells is lost, the secretion of insulin and glucagon were not as responsive to glucose and arginine as intact islets (Dunbar and Walsh, 1982; Hopcroft et al., 1985). Direct communication between B cells occurs by gap junctions, which were found to increase in number during B cell secretory activity (Meda et al., 1979). Major islet secretory products are too large to pass through these junctions, however electric charge and smaller molecules are continually exchanged. Gap junctions may participate in the regulatory system by which stimulated B cells adjust their level of activity in relation to need. The compartmentalization of islet secretory products is partially achieved by tight junctions between homologous and heterologous cells. However, recent evidence suggests that these junctions occur as a result of the experimental isolation procedure, as an adaptive mechanism to protect the islet microdomains, and that they do not play a role in normal islet cell function (Veld et al., 1984).

Somatostatin has been proposed to be a paracrine inhibitor of insulin and glucagon secretion. There is much evidence in support of this hypothesis. After somatostatin depletion by oral administration of cysteamine, isolated rat islets released more insulin in response to glucose stimulation than did control islets (Kanatsuka et al., 1984). Efendic et al. (1980) found in fasted rats that the concentration of somatostatin required to inhibit arginine and glucose stimulated insulin and glucagon secretion increased the plasma somatostatin concentration to 1500 pg/ml. Since plasma somatostatin is normally ~100 pg/ml, they suggested that such a high concentration of somatostatin release would only occur locally. Further evidence for the paracrine action of somatostatin arose from studies with static incubated islets in which somatostatin antiserum increased insulin (Taniguchi et al., 1977) and glucagon
release (Barden et al., 1977; Itoh et al., 1980). Under these experimental conditions, it is possible that the antiserum is neutralizing the high concentration of somatostatin that accumulated in the incubation media, and not the somatostatin in the islet interstitium. Support for a local role of somatostatin was provided by Taborsky (1983) who infused a nonimmunoreactive analog of somatostatin, and observed a suppression of somatostatin release and an increase in glucagon and insulin release in anesthetized dogs. Evidence against an endocrine action of somatostatin in the pancreas was provided by Bonner-Weir and Orci (1982). Using serial paraffin sections of islet perfused with India ink and immunocytochemically stained alternately for insulin, glucagon, somatostatin and pancreatic polypeptide, they found that the afferent arteriole entered the islet at discontinuities of the mantle of non-B cells. Upon entry to the B cell core, the arteriole diverged. Therefore somatostatin, glucagon and pancreatic polypeptide could have very little influence on insulin secretion via the circulation, unless the hormone was released upstream from the islet.

Alternatively, somatostatin may affect insulin and glucagon secretion via an endocrine route. Schusdziarra et al. (1980) found that injection of somatostatin antiserum in dogs increased postprandial insulin release compared to control dogs in which nonimmune serum was injected, suggesting that somatostatin is an endocrine inhibitor. Antibodies are large proteins and are assumed not to leave the circulation. In contrast to these findings, administration of somatostatin antiserum in starved rats (Tannenbaum et al., 1978), conscious baboons (Steiner et al., 1978) and in earlier dog studies by Schusdziarra et al. (1978) increased growth hormone, but not insulin release. These results suggest that somatostatin is an endocrine inhibitor of growth hormone but not of insulin. The differences in the results could be due to the accessibility of the antibody to the pancreas, since an increase in the frequency of antiserum administration in the later dog studies (Schusdziarra et al., 1980) gave positive results.

Other evidence suggesting that somatostatin is an endocrine inhibitor is that administration of exogenous somatostatin, at a concentration of 10 to 20% of the pancreatic vein somatostatin concentration, inhibited both insulin and glucagon release in the perfused canine pancreas. Since such relatively low
concentrations of somatostatin affect the islet cells, somatostatin probably acts through the circulation (Kawai et al., 1982). Immunocytochemical staining of D cells in the rat pancreas showed that they possessed long cytoplasmic projections extending to capillaries (Aponte et al., 1985). In unstimulated conditions, 54% of the secretory granules were distributed in the capillary end of the cell. Under stimulated conditions the secretory granules polarized in this region, suggesting the release of somatostatin into the circulation.

Passive Immunization Studies in the Stomach and Pancreas

Passive immunization or immunoneutralization is the conferment of resistance to an antigen, by administration of antibodies made in another individual or animal. It is an important tool in investigating the endogenous effect of a hormone. The results from passive immunization studies with somatostatin antiserum are conflicting, although consideration must be given to the differences in the amount and the binding characteristics of the antisera used, and the accessibility of the antiserum to the tissue.

Schusdziarra et al. (1980) found that repeated injection of somatostatin antiserum into dogs increased postprandial gastrin, insulin and pancreatic polypeptide concentrations, and concluded that somatostatin acts as a true hormone in the stomach and pancreas. In anaesthetized rats, somatostatin antiserum did not change plasma concentrations of gastrin, but increased growth hormone secretion (Chiba et al., 1981). In the isolated, perfused rat stomach, infusion of the antiserum had no effect on gastrin release in some studies (Chiba et al., 1981) and caused an increase in gastrin release in others (Saffouri et al., 1979; Short et al., 1985). Incubation of somatostatin antiserum with rat antral mucosa in culture increased basal gastrin release (Chiba et al., 1981). These increases in gastrin release suggest that somatostatin exerts a continuous restraint on gastrin secretion. The differences in the results could be due to the accessibility of the antiserum to the tissue. If somatostatin inhibits gastrin by a paracrine route, the antibody must enter the gastric interstitium before it can neutralize somatostatin.

Studies on the effect of passive immunization on pancreatic endocrine
secretion have been performed in vivo, with isolated perfused pancreas and isolated islets. In vivo studies in the baboon (Steiner et al., 1978), rat (Tannenbaum et al., 1978) and dog (Schusdziarra et al., 1978) in which endogenous somatostatin was neutralized by somatostatin antiserum revealed that growth hormone secretion was increased, but there was no increase in insulin or glucagon secretion.

The passive immunization of endogenous somatostatin in isolated rat islets has consistently yielded positive results. Addition of somatostatin antiserum to the incubation media caused an increase in insulin and glucagon secretion in response to glucose stimulation (Barden et al., 1977; Taniguchi et al., 1977; Itoh et al., 1980), and in response to amino acid stimulation (Taniguchi et al., 1979; Itoh et al., 1980). These experiments provide evidence that endogenous somatostatin is a physiological regulator of both insulin and glucagon secretion. However, as discussed earlier, there is a strong argument against the use of this preparation to assess the paracrine or endocrine nature of somatostatin secretion.

The preparation of choice for passive immunization studies is the isolated perfused pancreas, because it eliminates the complicating effect of circulating factors, yet maintains the integrity of the islet anatomy. Attempts at increasing insulin release by passively immunizing against somatostatin in the isolated perfused pancreas of mammals have so far yielded negative results (Sorenson et al., 1980). Possibly, the antisera concentrations were too low, or the antibodies were inaccessible to endogenous somatostatin. In the chicken pancreas, Honey et al. (1981) found that somatostatin neutralization caused a stimulation of both glucose stimulated insulin and glucagon release, suggesting that somatostatin continuously inhibits A and B cells. It is questionable whether these conclusions can be extrapolated to the mammalian pancreas.
Digestion of Antibody with Papain and Pepsin

H = Heavy chain
L = Light chain

Digestion of the antibody with papain yields 2 Fab fragments and an Fc fragment. Digestion of the antibody with pepsin yields an F(ab')2 and an Fc fragment. The Fab fragment contains the antigen binding site of the antibody. The Fc fragment does not have any antigen binding capacity, but can bind complement.
Monoclonal Antibodies and Fab Fragments

Monoclonal antibodies are antibodies which are derived from a single cell, and are therefore directed against one antigenic site on a molecule. Because of this, they are much more specific to that molecule than antisera, which are polyclonal. Other advantages of monoclonal antibodies are that they are relatively easy to purify, reasonably easy to produce, the cell line is immortal and to produce them, pure antigen is not required. The disadvantages of monoclonal antibodies are that they are expensive to produce, and the cell lines are relatively fragile. Monoclonal antibodies have been used as basic research tools in radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), and immunocytochemistry, as reagents in diagnostics, in the purification of antigens by affinity chromatography and in therapeutics.

Four monoclonal antibodies against somatostatin are available: SOMA 3, 8, 10 and 20 (Buchan et al., 1985). SOMA 3 is used in radioimmunoassays. SOMA 8 and 10 are used in immunocytochemistry. SOMA 20 has not been used for any specific purpose as of yet.

Antibodies are composed of 2 light chains (25 kd) and 2 heavy chains (50 kd) linked by disulfide bonds (fig. 1). The light chain and half of the heavy chain make up the Fab fragment (antigen binding). The Fab fragment is the portion of the antibody which contains its antigen binding property, and there are 2 Fab fragments per antibody. The other portion of the antibody, composed of the two remaining halves of the heavy chain is the Fc (crystallizable) fragment. It has the property of binding complement, for foreign cell lysis.

The antibody binding regions can be separated from the Fc region by digestion with papain or with pepsin, which cleave at the hinge region. Digestion of the antibody with papain yields two separate Fab fragments, while digestion with pepsin gives two attached antibody binding regions, F(ab')2. The advantages of the Fab and F(ab')2 fragments over the intact antibody are that they have a lower nonspecific binding due to the removal of the Fc region, and they are also smaller molecules, ~50 kd and 100 kd respectively, compared to the intact antibody which is ~150 kd.
Rationale

There is controversy as to whether somatostatin exerts a continuous restraint on gastrin release. In some situations, there is a functional relationship between the two hormones: increases in somatostatin parallel a decrease in gastrin release, and decreases in somatostatin are associated with increases in gastrin release. As previously mentioned, gastrin is thought to be a paracrine effect of somatostatin. However, there is conflicting data as to this relationship in the basal state. Saffouri et al. (1979), infused somatostatin antiserum into the isolated perfused rat stomach and found an immediate increase in gastrin release, supporting the hypothesis that somatostatin inhibits basal gastrin release. Short et al. (1985), using a similar model and procedure found only a significant increase after a 45 minute infusion of antiserum, while Chiba et al. (1981) did not find any effect of the antiserum. The differences in results could be due to the differences in the binding capacity of the antiserum used as well as the accessibility of the antisera to the tissue.

In the present study, monoclonal antibodies directed against somatostatin were infused into the isolated perfused rat stomach in an attempt to neutralize endogenous somatostatin, and the effects on gastrin release observed. Secondly, Fab fragment of the monoclonal antibody, SOMA10, were made and infused in similar experiments. These fragments, being approximately one-third the size of the intact antibody should more readily penetrate into the interstitium of the stomach and neutralize endogenous somatostatin. In the isolated perfused pancreatic preparation, SOMA 10 and its Fab fragment were infused in an attempt to neutralize endogenous pancreatic somatostatin. The present experiments differ from previous attempts in that monoclonal antibodies and Fab fragments were used. For these studies, high purity antibodies and Fab fragments were prepared and characterized.
METHODS

I. Antibody Purification Methods

A. Ammonium Sulphate Precipitation

1. Rationale

Ammonium sulphate precipitation is a method of crudely purifying antibodies. It is based on the precipitation of proteins due to the interaction of the solvent molecules with the salt ions. As this interaction increases, the protein-solvent interaction decreases. The interaction of protein with other protein molecules increases, due to the ionic atmosphere surrounding the protein, and precipitation of protein occurs as the complexes increase in size. Antibodies precipitate between 25 and 50% saturation of ammonium sulphate.

2. Procedure

SOMA 10 (Buchan et al., 1985), a mouse anti-somatostatin monoclonal antibody, was produced by conjugation of cyclic somatostatin-14 to keyhole limpet haemocyanin, and injection into BIO.BR SgSn mice. Spleen cells from a mouse giving a good antibody response were fused with NS1 cells and the hybrids were grown. Positive clones were selected and cloned out by limiting dilution. The cell line was grown as an ascites tumour in irradiated outbred mice.

Ammonium sulphate was added to ascites fluid containing the monoclonal antibody, SOMA 10, to a final concentration of 25%. This mixture was centrifuged at 4000 rpm for 15 min, the pellet was discarded and the supernatant taken to 50% saturated ammonium sulphate. The mixture was left overnight at 4 °C and again centrifuged. The precipitate was reconstituted in 10 mM phosphate, pH 6.5, the starting buffer for hydroxylapatite chromatography, and dialyzed overnight.
B. Hydroxyapatite Chromatography

1. Rationale

Hydroxyapatite chromatography (Stanker et al., 1985) was chosen as the method of purification of SOMA 10 because it is a simple, rapid and cost-effective method of processing a large volume of ascites fluid. Hydroxyapatite chromatography is thought to purify monoclonal antibodies on the basis of light-chain composition variations (Juarez-Salinas et al., 1984).

2. Procedure

The dialyzed ammonium sulphate-purified antibody was applied to a hydroxyapatite column (2.5 x 15 cm), in 10 mM phosphate, 0.02% sodium azide, pH 6.8. The antibody was desorbed by increasing the molarity to 0.5 M phosphate. The flow rate was 2 ml/min, and 1 min fractions were collected. An approximation of the protein content was made measuring absorbance at 280 nm (Pye Unican SP8-100 spectrophotometer). Fractions with absorbances exceeding 0.040 were pooled and tested for immunoreactivity with somatostatin by enzyme-linked immunosorbant assay (ELISA).

C. Enzyme-linked Immunosorbant Assay (ELISA)

The ELISA is a solid phase assay which provides a quick and sensitive determination of immunoreactivity and of quantification of protein (Voller et al., 1976). Ninety-six well Falcon 3912 microtitre plates (Becton Dickinson, California) were coated with 100 µl of somatostatin at a concentration of 1 µg/ml in carbonate-bicarbonate buffer, pH 9.6 (0.3 M NaHCO₃, 0.1 M Na₂CO₃, 0.02% NaN₃). The plates were incubated overnight at 4 °C.

The plates were washed with phosphate buffered saline containing 0.5% Tween 20 (PBS-Tween), pH 7.4. The PBS contained 137 mM sodium chloride, 1.5 mM monobasic potassium phosphate, 7.2 mM dibasic sodium phosphate, 2.7 mM potassium chloride, and 0.02% sodium azide. A 100 µl aliquot of test monoclonal antibody, appropriately diluted in PBS-Tween, was added to each well, and the plates were incubated for 1.5 h at room temperature. A second mouse monoclonal antibody, anti-vasoactive intestinal peptide (VIP) antibody
(Sikora et al., 1986), at a dilution of 1:1000 in PBS-Tween was used as a control. All studies were performed in duplicate.

After washing with PBS-Tween, 100 μl alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (RAMIg), diluted to 1:3000 was applied to the plate. After incubation for a further 1.5 h at room temperature, the plate was washed with PBS-Tween and developed with 100 μl of alkaline phosphatase substrate solution (Sigma 104-105) at a concentration of 5 mg in 5 ml 10% diethanolamine buffer (10% diethanolamine, 0.02% sodium azide and 0.1% MgCl₂·6 H₂O, pH 9.8). The reaction was allowed to proceed at room temperature for 15 to 45 min, and the yellow colour development was monitored at 405 nm using a MR-580 Microelisa reader (Dynatech Laboratories, Virginia). Immunoreactive fractions were then dialyzed using Spectrapor 2 membrane tubing (Spectrum Medical Industries Inc., Los Angeles) against PBS, without sodium azide. This tubing has a molecular weight cutoff between 12000 and 14000 d.

D. High Performance Liquid Chromatography (HPLC)

1. Rationale

HPLC is a technique for purifying and analyzing the purity of proteins. Its advantages over conventional chromatographic methods are rapidity of separation, reproducibility and resolution. This is a result of the high pressure that can be developed and the passage of the sample through an extremely tight packing matrix. The components of an HPLC system are a pump, sample injector, column and detector (fig 2).

2. HPLC Equipment and Buffers

Samples were applied using a Waters U6K injection system (Waters Associates Inc., Mississauga). Protein outflow absorbance was measured by a Waters Model 450 Variable Wavelength detector. For affinity and gel chromatography, absorbance was measured at 225 nm and at 280 nm, respectively. The absorbance profiles were recorded on a Hewlett Packard Integrator 3380A (Hewlett Packard, California).
FIGURE 2
Components of an HPLC System

Solvent and sample are pumped under high pressure through the column. The solvent composition can be varied using a programmed gradient maker. Elution of protein from the column is detected by absorbance with a variable wavelength detector.
All buffers were made up in HPLC-grade distilled water (Milli-Q Reagent Water system, Millipore, Mississauga), filtered and degassed under vacuum. Gradients were generated with a Waters 660 solvent programmer controlling the speed of two pumps (Pump A - Waters Model M-45; Pump B - Waters Model 6000A) to maintain a constant flow rate while solvent concentrations were varied.

3. **Affinity HPLC**

a. **Rationale**

Affinity chromatography is based on the specific and reversible binding of a compound of interest to an immobilized ligand. The ligand used to bind SOMA 10 was somatostatin. The compound can be desorbed by lowering the pH of the eluent, using chaotropic agents, by denaturing agents or by increasing the concentration of the ligand in the elution buffer. These agents weaken the non-covalent bonds between the immobilized ligand and the protein of interest.

b. **Derivitization of the Column**

Five milligrams of somatostatin (Peninsula) were dissolved in 10 ml of 1 M phosphate, pH 7. The solution was recycled through an Ultraffinity-EP (Waters) column (4.6 x 50 mm) overnight at a flow rate of 0.2 ml/min. The column matrix was an epoxy base. The column was then washed with 1 M phosphate buffer for 2 h. The effluent was tested for somatostatin content by radioimmunoassay and found to be undetectable for somatostatin-like immunoreactivity.

c. **HPLC Buffers**

The sample was applied to the column in a solution of 0.02 M phosphate, 0.2 M sodium chloride pH 7. The sample was desorbed from the column with water, pH adjusted to 2 with hydrochloric acid. The effluent was immediately neutralized with sodium hydroxide. The flow rate was 0.5 ml/min.
d. **Sample Application**

All samples were dissolved in PBS, without sodium azide. Not more than 50 μl of sample (0.3 mg) was applied to the column at a time. The Ultraffinity-EP column has a sample capacity of 10 to 20 mg of protein.

4. **Gel Filtration HPLC**

a. **Rationale**

Gel filtration is a technique used to separate molecules according to molecular size. The column contains a matrix of a particular pore size. Proteins larger than this size cannot enter into the pores, and travel in the void volume of the column, whereas smaller proteins are retained within pores and their passage through the matrix is impeded by the stationary phase. Proteins are therefore eluted in decreasing molecular size.

b. **Procedure**

The column used was the Protein Pak 300SW (Waters). The fractionation range of this column is 10,000 to 500,000 daltons. Elution with 0.1 M phosphate pH 7 was performed at a flow rate of 1 ml/min.

c. **Sample Application**

All samples were dissolved in PBS, without sodium azide. Not more than 50 μl (0.3 mg) of sample was applied to the column at a time.

II. **Antibody Characterization**

A. **Binding Region on Somatostatin Molecule**

1. **Rationale**

Somatostatin radioimmunoassays were used to determine the antibody binding site on the somatostatin molecule. The radioimmunoassay (RIA) is a highly sensitive competitive binding assay for measuring somatostatin concentration. It is based on the displacement of radioactively-labelled somatostatin from its specific antibody by unlabelled somatostatin, either by standards or samples. As the concentration of unlabelled somatostatin
increases, the amount of $^{125}$I-somatostatin binding to the antibody decreases. Separation of the somatostatin-antibody or $^{125}$I-somatostatin-antibody complexes from unbound or free somatostatin and antibody is achieved by charcoal adsorption of the unbound molecules.

Standard curves of somatostatin-14, and its analogues were compared. If an amino acid position is important for antibody binding, substitution of the amino acid would cause a decrease in binding affinity of the antibody.

2. Procedure

The assay procedure was performed according to the method described by McIntosh et al. (1978) using monoclonal antibodies to somatostatin (Buchan et al. 1985), SOMA 3, 8, 10 and 20.

a. Assay Buffer

A stock solution of somatostatin RIA buffer containing 23.8 mM sodium barbital, 3.9 mM sodium acetate, 43.6 mM sodium chloride and 0.24 mM merthiolate, pH 7.4, was prepared and stored at 4 °C. For use in the assay, 0.5% bovine serum albumin (BSA, Miles) and a proteolytic enzyme inhibitor, 1% aprotinin (Miles), were added.

b. Standards

Synthetic cyclic somatostatin-14 (Peninsula) was dissolved in 0.1 M acetic acid containing 0.05% BSA. Aliquots of 5 µg were lyophilized and stored at -20 °C. On the day of the assay, an aliquot was dissolved in assay buffer, and serially diluted to obtain the standards of 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8 and 3.9 pg/ml for assays using SOMA 3, 8 and 20. For assays in which SOMA 10 was used as the antibody, serial dilutions were made to obtain the standards of 250, 125, 62.5, 31.25, 15.625, 7.8, 3.9 and 1.95 ng/ml.

Synthetic analogues of somatostatin-14, synthethized by Drs. D.M. Coy and W. Murphy (Tulane University, Louisiana), and somatostatin-28 (Peninsula) were dissolved in 50 µl of 0.05 M acetic acid, made up to 1 µg/ml in assay buffer, and then serially diluted to obtain the standards as for
somatostatin-14. The synthetic analogs of somatostatin used in the RIA were as follows:

DES-ALA GLY Somatostatin-14  
GLU-4 Somatostatin-14  
PHE-4 Somatostatin-14  
LEU-4 Somatostatin-14  
THR-5 Somatostatin-14  
D-PHE-6 Somatostatin-14  
D-PHE-7 Somatostatin-14  
D-TRP-8 Somatostatin-14  
L-5-ME-TRP-8 Somatostatin-14  
PHE-9 Somatostatin-14  
GLU-9 Somatostatin-14  
THR-9 Somatostatin-14  
PHE-10 Somatostatin-14  
D-THR-10 Somatostatin-14  
D-PHE-11 Somatostatin-14  
D-THR-12 Somatostatin-14  
LEU-13 Somatostatin-14  
D-CYS-14 Somatostatin-14

c. Antibodies

Crude ascites fluid containing SOMA 3 was thawed from storage at -20 °C, filtered through 0.45 µm filters (Millipore Corp., Bedford, Massachusetts), diluted 1 : 1 in a solution of 0.9% sodium chloride, 0.5% sodium azide and 0.1% BSA, and stored at 4 °C. The antibody concentration was approximately 0.4 mg/ml. On the day of the assay, this was diluted in assay buffer to a final dilution of 1 : 10^6. This dilution of antibodies was shown to be sensitive to a range of somatostatin-14 concentrations between 20 and 250 pg/ml.

Filtered crude ascites fluid containing SOMA 8 (approximately 32 mg/ml) or SOMA 20 (approximately 29 mg/ml), stored at 4 °C, were diluted 1 : 1 in a solution of 0.9% sodium chloride, 0.5% sodium azide and 0.1% BSA. On the day of the assay, SOMA 8 was diluted in assay buffer to a final dilution of 1 : 2 x 10^6, and SOMA 20 to a dilution of 1 : 3 x 10^6. The dilutions of SOMA 8 and SOMA 20 were shown to be sensitive to a range of somatostatin-14
concentrations between 25 and 250 pg/ml and concentrations between 20 and 200 pg/ml, respectively.

Hydroxylapatite purified SOMA 10 (1.7 mg/ml) was stored in PBS containing 0.5% azide and 0.1% BSA. On the day of the assay, it was diluted to a final dilution of 1 : 1000. This dilution was shown to be sensitive to a range of somatostatin-14 concentrations between 20 and 200 ng/ml.

d. Label

i. Iodination of Somatostatin

Ten microlitres of 0.5 M phosphate, pH 7.5, 10 μl Na\(^{125}\)I in NaOH (1 mCi, Amersham) and 10 μl chloramine T (2 mg/ml in 0.05 M phosphate, pH 7.5) were added to 5 μg synthetic tyr-1 somatostatin-14 (Serono) dissolved in 10 μl of distilled water. The reaction was allowed to proceed for 30 sec before it was terminated with 10 μl sodium metabisulphite (5 mg/ml in 0.05 M phosphate buffer, pH 7.5). One millilitre of hormone-free plasma and 20 mg of microfine silica (QUSO G32, Philadelphia Quartz Co.) were added and the mixture centrifuged. The supernatant was removed and the pellet resuspended in 1 ml water. This centrifugation and resuspension was repeated twice to remove the unreacted \(^{125}\)I. The pellet was then resuspended in 1 ml of a mixture of acetic acid / acetone / water (0.1 : 3.9 : 4; v : v : v) and centrifuged. The supernatant contained the \(^{125}\)I-somatostatin. Percentage incorporation was measured by measuring the radioactivity in 10 μl aliquots of the supernatants and the pellet.

\[
\text{% Incorporation} = \frac{\text{\(^{125}\)I-somatostatin (final supernant) counts}}{\text{total counts}}
\]

Percent incorporation was usually about 30%. \(^{125}\)I-somatostatin was then diluted to 500,000 cpm/10 μl with 0.1 M acetic acid containing 0.5% BSA (RIA grade, Sigma). Aliquots of 100 μl were lyophilized and stored at -20 °C.

ii. Purification of \(^{125}\)I-Somatostatin

On the day of the assay, lyophilized samples of \(^{125}\)I-somatostatin were dissolved in 0.002 M ammonium acetate, pH 4.6, and applied to a CM-cellulose
(CM-52, Whatman Ltd, Maidstone, England) column (0.7 x 8 cm) previously equilibrated in the same buffer. $^{125}$I-somatostatin was desorbed from the column with 0.2 M ammonium acetate, pH 4.6. The flow rate was 1 ml/min, two millilitre fractions were collected and radioactivity measured in a gamma-spectrometer. The peak fractions and those following were neutralized with sodium hydroxide, and diluted with assay buffer for use at 3000 to 3500 cpm/100µl.

e. **Assay Protocol**

One hundred microlitres each of assay buffer, standard, antibody and $^{125}$I-somatostatin were allowed to incubate for 72 h at 4 °C. Nonspecific binding (NSB), the amount of $^{125}$I-somatostatin bound in the absence of the antibody, was determined for each set of standards. NSB and standard tubes were set up in triplicate.

Antibody-bound and free $^{125}$I-somatostatin were separated by adsorption to dextran-coated charcoal. One millilitre of a mixture of 0.25% dextran T-70 (Pharmacia), 1.25% activated charcoal (Fisher) and 1% charcoal-extracted human plasma in 0.05 M phosphate buffer, pH 7.5 was added to each tube. Tubes were vortexed and allowed to sit for 15 min at 4 °C. Following centrifugation at 3000 rpm for 30 min, the supernatant was decanted, and the pellet counted for 3 min in a gamma spectrometer (Searle Model 1285).

Charcoal-extracted plasma was prepared from outdated human blood (Red Cross, Vancouver, B.C.). After centrifugation of the blood, the serum was filtered through sharkskin filter paper (Shleicher and Schuell, Inc., Keene, N.H.). One percent activated charcoal was added to the filtrate. After continuous stirring for 1 h at 4 °C, the mixture was centrifuged at 10,000 rpm for 30 min. The supernatant was filtered twice more, and this hormone-free plasma was separated into 10 ml fractions and stored at -20 °C.
f. **Calculations**

Percentage of bound $^{125}\text{I}$-somatostatin was calculated by the following formula:

\[
\%B = \frac{\text{TC} - \text{sample counts}}{\text{TC} - \text{NSB counts}}
\]

\[
\text{TC} \quad \text{TC}
\]

where: $\%B =$ percent bound

TC = total counts

B. **Specific Activity from Self Displacement Curves**

a. **Rationale**

Specific activity is a measurement of the radioactivity in comparison to mass. The specific activity of $^{125}\text{I}$-somatostatin was used to calculate the amount of somatostatin in the radioactive tracer. This value was necessary for Scatchard analysis. To calculate specific activity, increasing amounts of labelled antigen are incubated with a constant amount of antibody under conditions similar to those employed in the RIA. Specific activity is then determined by comparing the ratio of bound to free tracer (B/F) at each increment of tracer with B/F ratios for a normal RIA standard curve. The amount of standard having a similar B/F ratio at a given cpm of label gives an estimate of the specific radioactivity.

b. **Procedure**

Similar materials and procedures for a somatostatin RIA standard curve were followed. In addition, increasing amount of $^{125}\text{I}$-somatostatin ranging from 1000 cpm/100 μl to 100,000 cpm/100 μl were incubated with a constant amount of antibody. NSB was calculated for each concentration of label, and all concentrations were measured in triplicate.
C. Scatchard Plots

The antigen-antibody interaction can be studied by measuring the binding of radioactive ligand to antibody. At equilibrium:

\[
K_D = \frac{[\text{Ab}][\text{Ag}]}{[\text{Ab-Ag}]}
\]

where: \(K_D\) = dissociation constant

\(\text{Ab}\) = free antibody

\(\text{Ag}\) = free antigen

\(\text{Ab-Ag}\) = antibody-antigen complex

This is represented as a hyperbolic function when \([\text{Ab-Ag}]/[\text{Ab}]\) is plotted against \([\text{Ag}]\); as the concentration of antigen increases, the complexed to free ratio of antibody increases asymptotically to approach the total antibody concentration. When \(B/F\) is plotted against \(B\), a linear relationship is obtained. The negative reciprocal of the slope gives the dissociation constant \(K_D\), and the \(X\)-intercept gives the maximum bound antigen. \(B\) was calculated by multiplying the \(\%B\) with the total somatostatin, i.e. standard plus \(125\)I-labelled somatostatin.

D. Immunodiffusion Gels

1. Rationale

The immunodiffusion or Ouchtelony gel is a method of determining immunoreactivity qualitatively. The interaction of soluble antigen with antibody results in the formation of a lattice which precipitates when it grows in size and becomes insoluble. These precipitation reactions can be carried out in various semi-solid media, eg. agarose gels. Wells, cut in the gel, are filled with solutions of antigen and antibody and these proteins diffuse through the gel surrounding the wells. Immunoreactive components form complexes, which
precipitate and present themselves as opaque streaks in the gel. The immunodiffusion gel was used to classify antibodies.

All antibody molecules have a common structure consisting of four polypeptide chains, two large or heavy chains and two small or light chains (fig 1). Antibodies have been assigned to five classes based on the primary structure of their respective heavy chains. These classes are IgG, IgA, IgM, IgD, and IgE. IgG has several subclasses.

2. Procedure

Agarose (1%) was dissolved in 0.01 M barbituric acid, pH 8.2. Approximately 3 ml was placed on a slide and allowed to cool. When the gel was set, 7 wells (6 wells surrounding a centre well) were cut and removed. The surrounding wells were filled with 7 μl of anti-mouse IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, or anti-IgM (ICN Immunobiological). The centre well was filled with a dilution of either SOMA 3, 8, 10, or 20. A precipitation line developed between 24 and 48 h later. The time required for the development of the precipitation line depended on the concentration of the proteins. When the precipitation line had formed, the slides were dried.

The slides were stained in 0.25% Coomassie Blue in a mixture of methanol/water/acetic acid (5:5:5, v:v:v) for 10 to 15 min. After a water rinse, the gels were destained in 10% glacial acetic acid for preservation of the gels.

III. SOMA 10 Fab Fragment Production

A. Production

1. Rationale

An antibody has two binding sites. The binding sites can be cleaved off by papain, yielding the two separate binding sites, and another fragment, the Fc (crystallizable) fragment. Papain cleaves at the hinge region of the antibody, between the two sets of disulphide bonds. The antibody can also be cleaved by pepsin, yielding two connected binding sites and the Fc fragment (fig 1). Fab fragments are approximately one-third the size of the whole antibody (antibody: approximately 150,000 d; Fab: approximately 50,000 d). These antigen-specific
fragments have low non-specific binding characteristics due to the removal of the Fc region.

2. **Procedure**

   SOMA 10 was found, by immunodiffusion gels, to be of class IgG1. This class of mouse antibody does not usually bind well to protein A, the purification column. To ensure that during Fab fragment purification, all Fc regions bound to the column, SOMA 10 was initially applied to a protein A column (Fab purification). The fraction of SOMA 10 that bound to the column was desorbed, dialyzed against PBS, and concentrated using polyethylene glycol (PEG) to a concentration greater than 1 mg/ml.

   The fraction of SOMA 10 binding to protein A was dialyzed overnight against 0.1 M phosphate, 0.004 M EDTA, pH 7.5. 2-mercaptoethanol (final concentration 0.01 M) was added immediately before the addition of papain. 2-mercaptoethanol was required to activate the papain, as well as to reduce the disulphide bonds between the two heavy chains of the antibody. Mercuripapain (18.2 mg/ml, 19 units/mg protein, Sigma) in the form of a crystallized suspension in 70% ethanol, was added to the solution in an amount equivalent to 2% of the weight of SOMA 10. The tube was flushed with nitrogen, capped and placed in a 37 °C water bath. This was mixed continuously during the digestion period. After 4 h, the reaction was terminated with 0.3 M iodoacetamide (Sigma) in 1.5 M glycine, 3 M sodium chloride, pH 8.9. Fifty microlitres of this solution were added per millilitre of reaction mixture, bringing the final concentration of iodoacetamide to 0.014 M. Because this reaction is light sensitive, it was maintained in the dark. The mixture was held on ice for 30 min, then dialyzed against 1.5 M glycine, 3 M sodium chloride, pH 8.9, the starting buffer for Fab purification.

B. **SOMA 10 Fab Fragment Purification**

1. **Rationale**

   Purification of Fab fragments from the digestion mixture was performed by affinity chromatography on protein A. Protein A is a protein produced by staphylococci with the unique property of binding the Fc region of antibodies.
Thus, the Fab fragments can be purified by adsorption of the Fc region of undigested and partially digested (one binding site removed) SOMA 10, and of free Fc fragments.

2. Procedure

The purification procedure was similar to that described in Pharmacia Separation News (Vol. 13.5). The digestion mixture was applied to a protein A-conjugated Sepharose CL-4B (Pharmacia, Sweden) column (9 x 90 mm), which was previously equilibrated with a solution of 1.5 M glycine, 3 M sodium chloride, pH 8.9. The column had a maximum capacity of 17 mg mouse antibody. The buffer was pumped at a rate of 0.8 ml/min. Two millilitre fractions were collected, and monitored for protein content by measuring absorbance at 280 nm in a spectrophotometer. The effluent that was not absorbed onto the column contained the Fab fragments. When absorbance dropped below 0.040, the Fc fragments and undigested antibody were eluted using 0.1 M citric acid, pH 6. This methodology was advantageous, as it did not expose the antibodies or fragments to extreme pHs, which may be detrimental. The column was regenerated with 0.1 M citric acid, pH 3. When not in use, the column was stored in 0.5% sodium azide at 4 °C.

IV. Characterization of Fab Fragments

A. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Rationale

SDS-Polyacrylamide gel electrophoresis is a method of determining the purity and the molecular weight of a denatured, reduced protein. Polyacrylamide serves as an inert matrix preventing turbulence and mixing of proteins during electrophoresis, and as a molecular sieve. Acrylamide molecules cross-link forming pores. The greater the amount of cross-linking, the smaller are the pores of the matrix. Low cross-linking of acrylamide is required for separation of high molecular weight proteins, and for stacking gels. The purpose of the stacking gel is to allow the proteins to align before entering the resolving gel. Greater cross-linking is required for separation of proteins of lower molecular weight. SDS, an ionic detergent, denatures proteins. Proteins
which are highly charged or contain a large carbohydrate component, such as antibodies, may run slower than proteins of a similar molecular weight.

2. **Materials**

For Resolving Gel (12%):
- 16 ml acrylamide-bisacrylamide (30 : 0.8, Biorad)
- 13.4 ml distilled water
- 10 ml 1.5 M tris base, pH 8.8
- 0.4 ml 10% SDS (Biorad)
- 0.2 ml 10% ammonium persulphate (Biorad) freshly made in water
- 20 μl TEMED (Biorad)

For Stacking Gel (4%):
- 1.3 ml acrylamide-bisacrylamide
- 6.1 ml distilled water
- 2.5 ml 0.5 M tris base, pH 6.5
- 0.1 ml 10% SDS
- 0.05 ml 10% ammonium persulphate freshly made in water
- 10 μl TEMED

3. **Preparation of Gel**

The Biorad gel apparatus was set up using 0.75 mm shims. A 12% resolving gel was prepared. The acrylamide, water and tris buffer were combined, and degassed under vacuum. SDS, ammonium persulphate and TEMED were added and the mixture gently stirred. Water-saturated isobutanol was gently layered on the gel, the gel was covered and allowed to polymerize. The excess isobutanol was poured off and the gel top surface was washed thoroughly with distilled water before being blotted dry with filter paper. The 4% stacking gel was prepared similarly to the resolving gel. Fifteen well combs were placed in between the glass plates and the stacking gel was poured. The gel was allowed to set for over 1 h, the combs were removed and the gel placed in the reservoir tank. The tank was filled with cooled reservoir buffer, containing 0.3% tris base, 1.44 % glycine, 0.1% SDS, pH adjusted to 8.3 with hydrochloric acid.
4. **Samples and Standards**

Samples were diluted to approximately 0.3 mg/ml with water. To this was added a volume of sample buffer (2x) equivalent to half the volume of the sample. Sample buffer contained 0.25 M tris-HCl, 20% glycerol, 4% SDS, 0.2 M dithiothreital (DTT) and 0.1% bromophenol blue. DTT reduces disulphide bonds. The samples were boiled in a waterbath for 10 min to denature the proteins. A blank containing PBS and sample buffer was given similar treatment. Pharmacia Low Molecular Weight Standards were used:

- Phosphorylase B 94,000 d
- BSA 67,000 d
- Ovalbumin 43,000 d
- Carbonic anhydrase 30,000 d
- Soybean Trypsin Inhibitor 20,100 d
- Lysozyme 14,400 d

The standards, approximately 100 µg of each, were dissolved in 250 µl water and 250 µl sample buffer (2x) and boiled for 10 min.

5. **Running the Gel**

The samples and standards were applied to separate wells using a Hamilton microsyringe. Constant voltage at 80 mV was run through the gel. Cold water continuously flowed through the tanks to keep the reservoir buffer cold and the reservoir buffer was continuously stirred to maintain the buffer temperature constant in all areas. When the gel front reached the bottom, the current was turned off and the gel was removed from the apparatus.

6. **Staining**

The gel was immediately stained in 0.25% Coomassie blue stain in methanol/water/acetic acid (5:5:5; v:v:v) for 1 h at 60 °C. After a brief rinse in water, the gel was destained at room temperature with 10% glacial acetic acid.
B. **Assessment of Binding of Antibody to $^{125}$I-Somatostatin by Chromatography on Sephadex G-100**

1. **Rationale**

Sephadex G-100 (Pharmacia, Sweden) has a fractionation range between 4,000 and 100,000 d. $^{125}$I-somatostatin and SOMA 10 or SOMA 10 fragments were incubated and chromatography of the complexes was performed on Sephadex G-100. SOMA 10 complexes are larger than Fab complexes and should elute earlier.

2. **Preparation of Gel**

The appropriate weight of gel was suspended in excess somatostatin RIA buffer, without BSA or aprotinin, at room temperature for several hours. The fines were decanted, and the gel was degassed under vacuum. The gel was poured into a 0.9 x 30 cm column, and connected to a pump.

3. **Samples**

$^{125}$I-somatostatin was purified on CM-52 as described earlier. The peak fraction was neutralized and diluted to approximately 50,000 cpm/100 μl. One hundred microlitres of SOMA 10 (25 μg/ml), SOMA 10 Fab fragments (17 μg/ml) or of the Fc fraction (25 μg/ml) were added to 100 μl of $^{125}$I-somatostatin, and the volume made up to 1 ml in somatostatin RIA buffer, without BSA or aprotinin. Other samples containing $^{125}$I-somatostatin, antibody or fragments, and somatostatin (3.5 μg or 7 μg) were prepared to check the displacement of $^{125}$I-somatostatin with somatostatin. The samples were allowed to equilibrate overnight at 4 °C before chromatography.

4. **Chromatography**

Elution was performed with somatostatin RIA buffer, without BSA or aprotinin, at a flow rate of ~0.4 ml/min. Half millilitre fractions were collected and counted on a gamma-spectrometer.
V. **Protein Quantification**

1. **Rationale**

   BCA Protein Assay Reagent (Pierce Chemical Company, Illinois) was used to determine protein concentration. When protein is reacted with copper$^{2+}$ in alkaline conditions, it produces copper$^{1+}$. This is reacted with the key component of the BCA Protein Assay reagent, bicinchoninic acid, to form a water-soluble, purple product. Detection of this product and therefore the protein quantity can be made by spectrophotometric measurement.

2. **Procedure**

   a. **Standard Protocol**

      To determine protein concentration in the range of 100 µg/ml to 1200 µg/ml, the following protocol was used. Bovine serum albumin (5%, Sigma) was dissolved in distilled water and stored at -20 °C. On the day of the assay, it was thawed and diluted in PBS, without sodium azide, to concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mg/ml. Samples were diluted in the same buffer to a concentration between 0.4 and 1.0 mg/ml. One hundred microlitres of standard or sample, in duplicate, were pipetted into a test tube. BCA reagent A contained sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 N sodium hydroxide. BCA reagent B contained copper sulphate. The working reagent was prepared by mixing reagent A and B in a ratio of 50 : 1. Two millilitres of this working reagent were added to each tube, vortexed and incubated at 37 °C in a shaking waterbath for 30 min.

   b. **Micro Protocol**

      This micro protocol determined protein concentration in the range of 5 µg/ml to 250 µg/ml. On the day of the assay, the 5% BSA stock was thawed and diluted in PBS, without sodium azide, to concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25 mg/ml. Samples were diluted in the same buffer to a concentration between 0.1 and 0.2 mg/ml. One hundred microlitres of standard or sample, in duplicate, were pipetted into a test tube. Two millilitres of the working reagent
were added to each tube, vortexed and incubated at 60 °C in a shaking waterbath for 30 min.

3. **Protein Determination**

Tubes were cooled to room temperature after incubation. Absorbance of the colour developed was read at 562 nm against PBS, without sodium azide. The background absorbance of BCA working reagent was subtracted from the absorbance, and a standard curve was prepared by plotting net absorbance at 562 nm against protein concentration.

VI. **Bioassays**

A. **Acid Secretion**

1. **Rationale**

An in vivo preparation for measuring gastric acid secretion was used to test the biological activity of SOMA 10 Fab fragments. Its biological activity was based on its ability to reverse the inhibition of liver extract-stimulated gastric acid secretion by exogenously-administered somatostatin.

2. **Apparatus**

Double-barrelled gastric cannulae were made in which fluid could be infused and drained from the same opening. A tuberculin syringe, with flared ends served as the drainage tube. The flaring prevented the removal of the cannula once it was secured. The inner tube was removable, so that particles blocking fluid flow could be removed easily (fig 3). Cannulae were secured in place by stabilizing them on the table top. The cannula inflow was connected to a tube secured on a bar to prevent movement of the cannula during flushing of the stomach.

Acid secretion was measured by titration of each sample with 0.1 M sodium hydroxide using an automatic titrator (Radiometer, Copenhagen).

Infusion of drugs was performed by means of Harvard infusion pumps (Harvard Apparatus Co., South Natick, Mass.).
FIGURE 3

Double-barrelled Gastric Cannula

The cannula was constructed such that fluid could be infused and drained from the same opening. The opening was inserted into an incision made in the fundus for measurement of gastric acid secretion. The flared end of the cannula was removeable, so that particles blocking fluid flow could be easily dislodged.
3. **Animals**

Male Sprague-Dawley rats (obtained from Animal Care, U.B.C.) weighing between 150-200 g were used in all experiments. The animals were housed in metal cages (5 - 6 rats/cage) in a light controlled room (12 h cycle) with free access to laboratory rat chow and water. The rats were fasted 24 h prior to experimentation.

4. **Surgical Procedure**

Animals were anaesthetized with 25% urethane at 1.25 g/kg body weight. A neck incision was made, a tracheotomy performed and the oesophagus ligated. The right jugular vein was cannulated with PE-50 tubing (Clay Adams, Parsippany, N.J.) filled with heparinized saline such that there was easy withdrawal of blood. Through a small midline abdominal incision, the fundus was located, a gastric cannula inserted and secured. The duodenum was ligated 3-4 mm distal to the pylorus, and the abdomen was sutured to prevent excess moisture loss.

5. **Experimental Protocol**

The stomachs were flushed with 30 ml saline to remove the remaining contents. The rats were allowed to equilibrate for a minimum of 1.5 h post-surgery.

After equilibration, the stomachs were flushed with 10 ml saline, followed by 10 ml air. At this point, experimental animals were administered 100 μg SOMA 10 Fab fragments, followed by an injection of a small volume of heparinized saline through the jugular cannula. Flushing of the stomach was repeated every 10 min for 30 min for collection of basal gastric secretion. In all animals, 2 μg kg⁻¹h⁻¹ somatostatin-14 (Peninsula Laboratories, Belmont, Calif.) prepared in saline and 0.01% BSA were infused for the remaining experimental period. The stomach was stimulated by a 5% liver extract (T.E.C. Chemical, Inc., Myerstown, Pa.) meal in saline, pH adjusted to 5.5 with 0.1 M sodium hydroxide. Every 10 min for 90 min, the stomach was flushed with 10 ml liver extract, of which 3 ml were retained to form the next meal. Samples were collected and acid content was determined by titrating with 0.1 M sodium
hydroxide to pH 5.5. The concentration of SOMA 10 Fab fragment and somatostatin were chosen based on previous experiments using the intact antibody (Seal et al. 1987).

B. Isolated Perfused Rat Stomach and Isolated Perfused Rat Pancreas

1. Rationale

The isolated, vascularly-perfused stomach and isolated, vascularly-perfused pancreas were used to test the effects of passive immunization with SOMA 10 antibody and Fab fragments on gastrin and insulin release, respectively. These preparations have advantages over whole animal preparations and isolated cell preparations. They preserve the anatomy of the organs, i.e. the intrinsic innervation, microvasculature, and cell to cell contacts. However, they remove many of the complicating factors present in an intact animal, such as the influence of the central nervous system, and of circulating factors.

In the stomach preparation, perfusate entered through the coeliac artery, and was collected from the portal vein. In the pancreas preparation, perfusate entered through the coeliac and superior mesenteric arteries and was collected from the portal vein.

2. Apparatus

The apparatus used in these studies is shown in figure 4. Perfusate was continuously stirred and gassed with water-vapour saturated 95% oxygen/5% carbon dioxide to maintain the pH at 7.4. Perfusate was pumped (Masterflex, Cole Palmer) at 2 ml/min for stomach preparations and at 3 ml/min for pancreatic preparations through a servo-controlled heated water bath, which maintained perfusate temperature at 37 °C, and into a bubble trap in which the temperature probe was inserted. The perfused organ was maintained at a constant temperature of 37 °C by a heating pad and a 60 watt desk lamp. The preparation was covered to prevent moisture loss. Venous effluent samples were collected in chilled test tubes in a fraction collector (FRAC-100, Pharmacia, Sweden).
Perfusate was continuously gassed with 95% O₂/5% CO₂. It was pumped through a heated water bath, which maintained the organ temperature at 37 °C, into a bubble trap to which the temperature probe and pressure monitor were attached. Perfusate entered the organ via the coeliac artery, in the stomach preparation, and via the coeliac and mesenteric arteries, in the pancreatic preparation. The venous effluent was collected from the portal vein. Drugs were infused via a sidearm at the level of the aortic cannula.
3. **Somatostatin, SOMA 10 and the Fab Fragment Administration**

Somatostatin (Peninsula), SOMA 10 and the Fab fragments were infused through a sidearm infusion pump (model 940; Harvard Apparatus Co., Inc., Millis, Mass.). They were made up in 10 ml syringes, and delivered via PE-90 polyethylene tubing (Clay Adams, Parsippany, N.J.) into the rubber bulb on the aortic cannula. For stomach preparations, substances were made up at 20 times the final concentration, and infused at 0.206 ml/min causing a 10% change in final volume. For pancreatic preparations, substances were made up at 30 times the final concentration, and infused at 0.103 ml/min causing a 3% change in final volume.

4. **Preparation of Perfusate**

The perfusing solution contained:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>4.4 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1.2 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>120 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25 mM</td>
</tr>
<tr>
<td>BSA (RIA grade, Sigma)</td>
<td>0.2%</td>
</tr>
<tr>
<td>Dextran (Sigma)</td>
<td>3%</td>
</tr>
<tr>
<td>Glucose: dextrose (Fisher Scientific)</td>
<td>4.4 mM (80 mg%), 4.4 mM, 8.8 mM (160 mg%), 16.6 mM (300 mg%)</td>
</tr>
<tr>
<td>Glucose: liquid glucose</td>
<td>4.4 mM, 8.8 mM (160 mg%), 16.6 mM (300 mg%)</td>
</tr>
</tbody>
</table>

Stock Krebs' solution, containing potassium chloride, calcium chloride, magnesium sulphate and potassium phosphate was mixed and stored at 4 °C. The night before the experiment, BSA and dextran were dissolved in 0.9% saline. For stomach perfusions, dextrose was also dissolved. On the day of the experiment, Krebs' solution and bicarbonate were added. For pancreatic perfusions, liquid glucose was added to give the required concentration. The solution was made up to volume with saline. The final glucose concentration of
the perfusate was measured using a Beckman Glucose Analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

5. Animals

Male Wistar rats (250 - 350 g) were housed in metal cages (5 - 6 rats) in a light-controlled room. They were given free access to laboratory rat chow and water. Rats were overnight fasted (16-18 h) prior to experimentation.

6. Surgical Procedure

a. Isolated Perfused Stomach

The animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). A midline abdominal incision was made, and the abdominal aorta located. The left renal artery and vein were doubly ligated and sectioned in between. The superior mesenteric artery was doubly ligated and cut in between. A loose ligature was placed around the portal vein and one placed more rostrally around the portal vein, hepatic artery and bile duct. Connective tissue connecting the spleen and stomach was cleared, and the pancreatic tissue attached to the stomach was ligated and detached ensuring that the gastroepiploic artery was left intact on the stomach side. A duodenal drainage tube was inserted 1 to 1.5 cm distal to the pyloric sphincter. The colon was sectioned at the rectum to allow removal of the intestines, spleen and pancreas.

The aorta was cannulated with heparinized saline-filled PE-160 tubing, such that the tip was adjacent to the coeliac artery branch from the aorta. Care was taken to prevent introduction of air into the animal. The cannula was secured, and the ligature above the coeliac artery was tightened to interrupt blood flow to the stomach. Three millilitres of heparinized saline were injected via the aortic cannula, causing a blanching of the stomach. The animal was hemi-sectioned. The right renal artery and the inferior vena cava were ligated, and a cannula was inserted into the portal vein for collection of venous effluent. The aortic cannula was connected to the pump and the animal was perfused with oxygenated perfusate. The period of anoxia was less than 60 sec. Any leaks in the preparation were immediately halted by clamps or ligatures.
b. **Isolated Perfused Pancreas**

The surgical preparation of the isolated, perfused pancreas was similar to the preparation of the isolated, perfused stomach with the following exceptions: ligatures were tied between the spleen and the pancreas, sparing the pancreatic tissue as much as possible, and the spleen was removed. A duodenal cannula was placed distal to the level of the pancreas adjacent to the ligament of Treitz for drainage. A ligature was tied around the intestines from the drainage tube to the cecum, and this isolated piece of gut was removed. The stomach and pancreas were separated by ligation and sectioning on the stomach side, ensuring that the gastroepiploic artery was preserved. A ligature was placed around the pyloric sphincter and the antrum was severed. The oesophagus, including the gastric artery, was doubly ligated and cut to remove the stomach.

The aorta was cannulated with heparinized saline-filled PE-160 tubing, such that the tip was adjacent to the superior mesenteric artery branch from the aorta. The cannula was secured, and the ligature above the coeliac artery was tightened to interrupt blood flow to the pancreas.

**VII. Statistical Analysis**

In all perfusion experiments, significance was tested using Student's T test for either paired or unpaired groups. Significance was set at $p < 0.05$. In line graphs, each point represented the accumulated data during the time period previous to the point. In column graphs, each column represented the accumulated data during 6 or 8 min.

**VIII. Peptide Quantification by Radioimmunoassay**

A. **Gastrin RIA**

1. **Assay Buffer**

   The assay buffer was made by dissolving sodium barbital (0.02 M) and BSA (5 %, RIA grade, Sigma) in distilled water, and adjusting the pH to 8.4 with hydrochloric acid.
2. **Standards**

Synthetic human gastrin (Peninsula) was stored in 200 μl fractions at a concentration of 100 ng/ml. For use in the assay, one aliquot was diluted with assay buffer to 25 ml. Serial dilutions were made achieving concentrations of 400, 200, 100, 50, 25, 12.5, and 6.25 pg/ml. These standards were stored at -20 °C in 1 ml fractions. On the day of the assay, one aliquot of each concentration was thawed and used.

3. **Antiserum**

Antiserum, L-2 (a gift from Dr. G. Dockray, Liverpool University), was diluted 1 : 5000 in assay buffer, and stored in 1 ml aliquots at -20 °C. On the day of the assay, one aliquot was thawed and further diluted in assay buffer to achieve a final concentration of 1 : 200,000. L-2 binds gastrin-17 and 34 equally well.

4. **125I-Gastrin**

a. **Iodination of Gastrin**

Five microlitres of synthetic human gastrin I (Peninsula), dissolved in 10 μl 0.4 M phosphate, pH 7.4 were incubated with 0.2 mCi Na$^{125}$I in sodium hydroxide, and 10 μl chloramine T (0.5 mg/ml in 0.04 M phosphate buffer, pH 7.4) for 1 min at room temperature. The reaction was terminated by the addition of 10 μl sodium metabisulphite (0.5 mg/ml in 0.04 M phosphate buffer, pH 7.4). One millilitre of 0.05 M imidazole buffer, pH 7.5 was added.

b. **Purification of 125I-Gastrin**

The iodination mixture was purified on a DEAE-Sephadex A25 column (0.9 x 13 cm), which was previously equilibrated with 0.05 M imidazole buffer, pH 7.5. The iodination mixture was eluted at a flow rate of 2.0 ml/min with a gradient of 1 M sodium chloride in the imidazole buffer (total volume = 55 ml). One millilitre fractions were collected and the radioactivity measured. Standard curves and controls were prepared using the $^{125}$I-gastrin fractions. Fractions yielding the best binding and displacement were diluted in assay buffer to approximately 1,000,000 cpm/ml, separated into 2 ml fractions, and stored at
-20 °C for use in the assay. On the day of the assay, an aliquot was diluted to give approximately 2000 cpm/100 μl.

5. Controls

Pooled venous effluent from an isolated perfused stomach experiment was diluted with assay buffer to a gastrin concentration of 100 pg/ml, and stored at -20 °C as 1 ml fractions. These were used to monitor intra- and inter-assay variability.

To ensure that the presence of SOMA 10 or its Fab fragment in the sample did not interfere with the assay, 100 μl of 100 μg/ml SOMA 10 or Fab in perfusate were added to the standards. No change in the standard curve occurred.

6. Assay Protocol

One hundred microlitres each of standard, sample or control, antiserum and 125I-gastrin were added to each tube, and the volume made up to 1 ml with assay buffer. Non-specific binding tubes were made up similarly, but without antiserum. The assays were incubated for 48 h at 4 °C.

7. Separation

Antibody-bound and free labelled-gastrin were separated using dextran-coated charcoal. Dextran T-70 (0.25%) and activated charcoal (1.25%) were made up in 0.04 M phosphate buffer, pH 7.5, and 6.5% charcoal-extracted plasma added. Two hundred microlitres were added to each test tube. The assays were vortexed, allowed to sit for 15 min, centrifuged at 3000 rpm for 30 min at 4 °C, decanted and counted.

8. Calculations

The percentage of 125I-gastrin bound (%B) in each tube was calculated, by the formula used in the somatostatin RIA. A computer program was used to produce a log-logit plot of %B versus standard concentration in pg/ml. The gastrin concentration of the unknown samples was determined using the calculated %B. Concentrations per minute were corrected for volume and expressed in pg/min.
B. **Insulin RIA**

1. **Assay Buffer**

   Insulin assay buffer was made by adding 5% charcoal-extracted plasma to 0.04 M phosphate buffer, pH 7.5.

2. **Standards**

   Rat insulin (21.3 U/mg, NOVO) was dissolved in assay buffer to achieve a concentration of 4260 µU/ml. One millilitre aliquots were stored at -20 °C. One aliquot was further diluted (1 : 26.6) in assay buffer to achieve a concentration of 160 µU/ml, divided into 2 ml fractions, and stored at -20 °C. On the day of the assay, the 160 µU/ml standard was serially diluted in assay buffer to give standard concentrations of 160, 80, 40, 20, 10, 5, and 2.5 µU/ml.

c. **Antiserum**

   Insulin antiserum was raised in guinea pigs by injection of unconjugated porcine insulin emulsified in Freund's adjuvant. The antiserum (GP01) was diluted (1:10), separated into 100 µl fractions, lyophilized and stored at -20 °C. These were reconstituted in assay buffer to achieve a dilution of 1: 5000, separated into 1 ml fractions and stored at -20 °C. On the day of the assay, the antiserum was further diluted to achieve a final dilution of 1 : 10^6. This dilution of antibody was sensitive to insulin in the range of 5 to 160 µU/ml.

4. **125I-Insulin**

   a. **Iodination of Insulin**

      Porcine insulin (Novo) was dissolved in 10 µl 0.1 N hydrochloric acid, and then diluted to a concentration of 5 µg/10 µl with 0.2 M phosphate buffer, pH 7.5, in a siliconized test tube. Ten microlitres of Na^125I (1 mCi) in sodium hydroxide, 10 µl of 0.2 M phosphate buffer, pH 7.5, and 25 µl of chloramine T (4 mg/ml in 0.2 M phosphate buffer) were added, and the oxidative reaction was allowed to proceed for 10 sec. Sodium metabisulphite (100 µl at 2.4 mg/ml in 0.2 M phosphate buffer) was added and gently mixed for 45 sec to stop the reaction.
b. **Purification of $^{125}$I-Insulin**

One percent potassium iodide (50 μl at 10 mg/ml in 0.2 M phosphate buffer) was added to the iodination mixture, and 0.04 M phosphate buffer was added to make up to a volume of 2 ml. Ten milligrams of microfine silica (QUISO G-32) were added to adsorb the iodinated insulin. The mixture was vortexed and centrifuged for 15 min at 3000 rpm. The supernatant was decanted and the pellet resuspended in 3 ml distilled water. The mixture was vortexed and centrifuged as before. The supernatant was decanted and the pellet resuspended in 3 ml acid ethanol (1500 ml 95% ethanol, 500 ml water, 30 ml concentrated HCl, stored at 4 °C), vortexed, and centrifuged. The supernatant, containing the $^{125}$I-insulin, was decanted into a container, 1.5 ml water and 2 ml acid ethanol were added, and the label was stored at -20 °C. Incorporation of the $^{125}$I was determined by counting aliquots of the supernatants and the silica and correcting for volume. Percent incorporation was calculated as follows:

$$\text{% incorporation} = \frac{\text{label counts + pellet counts}}{\text{total counts}}$$

For use in the assay, the $^{125}$I-insulin was diluted to approximately 10,000 cpm/100μl in assay buffer.

5. **Controls**

Insulin controls were made from perfusate from a rat pancreas stimulated with 17.8 mM glucose and 10 mM arginine for 30 min. The total venous effluent was pooled and the insulin content determined by RIA. The effluent was then diluted in assay buffer to achieve a concentration of 60 μU/ml, and stored in 1 ml aliquots at -20 °C.

To ensure that SOMA 10 or the Fab fragments in the sample did not interfere with the determination of insulin, 50 μg/ml of the antibodies diluted in perfusate, were added to the standard curve test tubes. No change in the standard curve occurred.
6. **Assay Protocol**

Samples were diluted in assay buffer to concentrations between 25 and 100 μU/ml. One hundred microlitres of each standard, control or sample and antibody were added to a test tube. Assay buffer was added to make up a volume of 0.9 ml, and the mixture was incubated at 4 °C for 24 h. The following day, \(^{125}\text{I}\)-insulin was prepared by dilution with assay buffer to ~10,000 cpm/100 μl, and 100 μl were added to each tube. The assay was allowed to incubate for a further 24 h at 4 °C. Nonspecific binding (NSB) was determined in the absence of antibody.

7. **Separation**

Antibody-bound and free \(^{125}\text{I}\)-insulin were separated by means of dextran-coated charcoal. Two hundred microlitres of 0.5% dextran T-70 and 5% activated charcoal in 0.04 M phosphate buffer, pH 7.5, were added to each test tube. The mixture was vortexed, allowed to sit for 15 min and centrifuged at 3000 rpm for 30 min. The supernatant was decanted, and the pellet, containing the free \(^{125}\text{I}\)-insulin was counted for 2 min.

8. **Calculations**

The percentage of \(^{125}\text{I}\)-insulin bound (%B) in each tube was calculated, using the formula used in the somatostatin RIA. A computer program was used to produce a log-logit plot of %B versus standard concentration in μU/ml. Insulin concentration of the unknown samples was determined using the calculated %B. Concentrations per minute were expressed in μU/min by multiplication by volume, to account for fluctuations in perfusion flow rate.

IX. **Immunocytochemistry (ICC)**

A. **Rationale**

ICC was used for two purposes: 1) to ensure that the SOMA 10 Fab fragments were immunoreactive and; 2) to locate the distribution of SOMA 10 and its Fab fragment in the stomach and pancreas of perfused animals.
B. Antibody Localization in Tissue

1. Preparation of Tissue sections

Tissue was excised and fixed in Bouin's solution (25% formaldehyde, 75% picric acid) for 1 - 2 h. The solution was replaced with 70% ethanol for 1 h. The tissue was stored in fresh 70% ethanol.

The fixed tissue was cut into smaller pieces, and placed in cassettes for automatic processing (Histomatic Tissue Processor Model 166, Fisher). Processing involved dehydration of the tissue in 80%, 90%, and 100% ethanol for 30 min each, defatting the tissue with two washes of xylene for 1 h each and finally, infiltration of the tissue with paraffin wax. The tissue was embedded in wax. The wax blocks were frozen overnight, 5 μm sections were cut with a microtome (1130/Biocut, Reichert-Jung), mounted, and placed on a heating block (37 - 40 °C) overnight to fix them onto the slide.

a. Procedure for Testing Antibody and Fragment Immunoreactivity

Slide sections of rat pancreas were placed in xylene for 10 min to remove the wax, and in petroleum ether for 2 min to remove the xylene. Normal horse serum (Dimension Laboratories, Mississauga, Ontario), at a concentration of 1 : 100 was applied to decrease non-specific binding of the antibodies. After 30 min, this was removed with 3 washes in PBS with 0.004% azide (PBS-azide) The antibodies to be tested, SOMA 10, the Fab fragments and the Fc fraction were diluted in PBS-azide (PBS with 0.004% azide) to 10 μg/ml, and placed onto the slide to cover the entire tissue section. The antibodies were allowed to incubate overnight at 4 °C. The slides were washed 3 times in PBS-azide, and normal horse serum (1 : 100 in PBS-azide) was applied for 30 min to decrease nonspecific binding. The slides were washed again. Biotin-spacer-affinity purified goat anti-mouse immunoglobulin (Jackson Immunoresearch Lab., Westgrovve, PA) was diluted 1 : 3000 and applied onto the section. This second antibody was allowed to incubate for 1 h before it was washed off. Fluorescein avidin D (Vector Laboratories, Inc., Burlingame, Calif.) was diluted 1 : 2000 and 1 : 3000 in PBS-azide and applied to the sections. The third layer was allowed to incubate for 1 h. After washing 3 times, the slides were protected by coverslip, and viewed under a fluorescence
microscope (Axiophot, Zeiss, W. Germany). Photographs of the tissues were taken using Fujichrome film.

b. Procedure for Locating Antibodies in Perfused Organs

Tissue sections of perfused organs (stomach and pancreas) were subjected to the same treatment as non-perfused organs (section VIII.B.1.a.) with the exception that the first antibody (SOMA 10 or Fab fragments) application was omitted, and the second antibody, the biotin-spacer-AP goat anti-immunoglobulin, was allowed to incubate for 2 h.

C. Assessment of Nonspecific Binding to Collagen

1. Rationale

It was found that there was a large amount of Fab fragment binding to structures in the perfused tissue that contained collagen. The possibility that Fab fragments bind nonspecifically to collagen was assessed.

2. Procedure

Two millilitres of rat tail collagen (approximately 5 g/l, pH 3) were incubated in a 35 mm culture plate for 3 h at room temperature. The fluid was decanted, and 1 drop of 1 M sodium hydroxide was placed on the cover of the inverted plate to neutralize the collagen. The plate was allowed to dry. SOMA 10 and Fab fragments at 1 μg/ml were incubated on the plates overnight at 4 °C. Mouse anti-VIP (1 μg/ml) and PBS-azide were used as controls. The plates were developed by the method described in section IX.B.2., protected by coverslip and viewed under a fluorescence microscope.
APPENDIX TO METHODS

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N.B. Unless otherwise indicated, chemicals were reagent grade.
RESULTS

I. Purification of SOMA 10

The elution profile of ammonium sulphate precipitated SOMA 10 from hydroxylapatite is shown in figure 5. SOMA 10 eluted in the second peak. Pooled fractions from each peak were tested by ELISA, and only the second peak was found to contain immunoreactivity to somatostatin. From one millilitre of ascites, 2 to 3 mg of purified SOMA 10 was extracted.

II. Assessment of Purity of SOMA 10

A. Affinity Chromatography

Purity of SOMA 10 at various stages of purification, as crude ascites, after saturated ammonium sulphate precipitation (SAS), and following hydroxylapatite purification (HAP), was assessed by affinity HPLC on an Ultraffinity-EP column. The sample was applied to the affinity column with 0.02 M phosphate, 0.2 M NaCl, pH 7, and the antibody was desorbed using water, pH adjusted to 2 by hydrochloric acid (fig 6a - c). Chromatography of 30 μl (0.09 mg) HAP SOMA 10, 15 μl (0.09 mg) SAS SOMA 10, and 9 μl (0.26 mg) crude ascites was performed on the Ultraffinity-EP column.

The integrated area under the peaks gave an indication of the proportion of protein in the peaks. An approximation of the SOMA 10 content of the crude ascites, SAS SOMA 10, and HAP SOMA 10 was 52%, 75%, and 93% respectively.

The immunoreactivity of half millilitre fractions of the effluent from the Ultraffinity-EP column was tested by ELISA on plates coated with somatostatin. Activity was found in the second peak eluting from the column, while no activity was found in the first peak.

B. Gel Filtration Chromatography

Similar SOMA 10 samples were applied to a Protein Pak 300SW column. Peaks eluted at 9.3, 12, 14.5 and 22 min. The peak containing the SOMA 10 immunoreactivity, determined by ELISA, eluted at 14.5 min (fig 7
FIGURE 5
Purification of Ammonium Sulphate Precipitated SOMA 10 on Hydroxylapatite

Fifteen millilitres (~100 mg) of ammonium sulphate precipitated SOMA 10 was applied to a hydroxylapatite column (2.5 x 15 cm) with 10 mM sodium phosphate, 0.02% sodium azide, pH 6.8. Purified SOMA 10 was desorbed by increasing the molarity to 0.5 M phosphate. The protein content in the second peak is not truly represented by the absorbance at 280 nm, because the maximum absorbance was reached.
FIGURE 6
Affinity HPLC Elution Profile and ELISA Activity of SOMA 10 at Various Purification Steps

SOMA 10 was applied to the Ultraffinity-EP column in 0.02 M phosphate, 0.2 M NaCl, pH 7 and desorbed from the column with water in which the pH was decrease to 2 by HCl. The flow rate was 0.5 ml/min. Protein elution was detected at 225 nm. The effluent was collected as 0.5 ml fractions, neutralized with NaOH and applied to ELISA plates coated with somatostatin. ELISA readings were measured at 405 nm and are represented by squares.

a. Nine microlitres of ascites fluid containing SOMA 10 (0.26 mg) were applied to the column.

b. Thirteen microlitres of SAS SOMA 10 (0.09 mg) were applied to the column.

c. Fifty microlitres of HAP SOMA 10 (0.09 mg) in PBS were applied to the column.
a. 0.02 M phosphate 0.2 M NaCl, pH 7

b. 0.02 M phosphate 0.2 M NaCl, pH 7

PROTEIN ELUTION at 225 nm

ELISA
c.

![Graph showing absorbance over time](image)

- Time (min): 0, 10, 20, 30
- Absorbance at 405 nm
- 0.02 M phosphate, 0.2 M NaCl, pH 7
- H₂O pH 2
- 0.2 AUFS
- Time scale: 5 min
- Line graphs for:
  - Protein elution at 225 nm
  - ELISA
FIGURE 7

Gel Filtration HPLC Elution Profile and ELISA Activity of SOMA 10 at Various Purification Steps

SOMA 10 was applied onto the 300 SW column in 0.1 M phosphate, pH 7, and eluted with the same buffer. The flow rate was 1.0 ml/min. Protein elution was detected at 280 nm. The effluent was collected as 1.0 ml fractions and applied to ELISA plates coated with somatostatin. ELISA readings were measured at 405 nm and are represented by squares.

a. Nine microlitres of ascites fluid containing SOMA 10 (0.26 mg) were applied to the column.

b. Thirteen microlitres of SAS SOMA 10 (0.09 mg) were applied to the column.

c. Fifty microlitres of HAP SOMA 10 (0.09 mg) in PBS were applied to the column.
0.1 M phosphate pH 7

- PROTEIN ELUTION at 280 nm
- ELISA

Absorbance 405 nm

Time (min)
a - c). The negative deflection in peaks eluting at 22 min may be due to changes in the refractive index, as a result of the salts in the sample.

An approximation of the SOMA 10 content of the crude ascites, SAS SOMA 10, and HAP SOMA 10 was 38%, 89%, and 92% respectively. The activity of SOMA 10 at various purification stages was measured by ELISA. The concentration giving half maximum reading for crude ascites was 25 μg/ml, for SAS SOMA 10 and HAP SOMA 10 it was ~10 μg/ml (figure 8).

III. Characterization of Antibodies

SOMA 3, 8, 10, and 20 were characterized according to the region of the somatostatin to which they bind, the antibody classification, and by determining their dissociation constants and maximum binding capacities.

Figure 9 shows the amino acid sequence of somatostatin-14. Analogs of somatostatin-14 with amino acid substitutions were used as standards in the somatostatin radioimmunoassay. The affinities of somatostatin analogs to the antibody relative to somatostatin-14 were calculated by comparing the concentration of somatostatin analogue giving 50% displacement in a somatostatin RIA standard curve to that of somatostatin-14 (table 1). The important positions of the molecule, i.e. the positions at which changing the amino acid reduced binding, represent the antibody binding region on the somatostatin molecule. For SOMA 3 and SOMA 8 the important amino acids are in position 4 and, 6 to 12 (fig 10a and b). For SOMA 10, the binding region is at amino acid position 5 to 12 (fig 10c), and for SOMA 20, it is at position 4 to 12 (fig 10d).

SOMA 3, 8, 10 and 20 were classified using an immunodiffusion gel, and antibodies against the various antibody classes, i.e. anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3 and anti-IgM. All the SOMA antibodies were of the class IgG1. The impurities of the ascites fluid made it difficult to classify SOMA 3, 8 and 20. However, the strongest band formed against anti-IgG1.

The specific activity of the $^{125}$I-somatostatin, determined from self-displacement curves, was found to be 880 pCi/pg or 1.52 x $10^6$ pCi/pmole. The theoretical $^{125}$I-somatostatin specific activity, assuming $^{125}$I specific activity to be 13 μCi/μg was 939 pCi/pg or 1.62 x $10^6$ pCi/pmole. The experimental
FIGURE 8

Comparison of SOMA 10 Activity on ELISA
FIGURE 9

Amino Acid Sequence of Somatostatin-14

1 2 3 4 5 6 7 8 9 10 11 12 13 14

ALA - GLY - CYS - LYS - ASN - PHE - PHE - TRP - LYS - THR - PHE - THR - SER - CYS
TABLE 1

Relative Affinities of Somatostatin Analogs to Somatostatin-14

(average of at least 2 experiments)

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FIGURE 10

Relative Affinity of Somatostatin Analogs to Somatostatin-14

Somatostatin radioimmunoassays were performed using somatostatin-14 and somatostatin analogs as standards. The molar values of the analogs giving 50% displacement were compared, and expressed as percentages of the somatostatin-14 molar value giving 50% displacement.
**RELATIVE AFFINITY (%)**

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specific activity is equivalent to 2442 cpm/pg, assuming 80% counting efficiency. This value was used to calculate the amount of somatostatin in the 125I-somatostatin for radioimmunoassays. The total counts for the somatostatin radioimmunoassay were an average of 3200 cpm. The somatostatin contributed by the label was 1.3 pg. This value was used for calculation of the total amount of somatostatin, necessary for Scatchard analysis.

The results from the Scatchard plots are shown in table 2. \( K_D \) was calculated by taking the negative reciprocal of the slope of B/F versus B. Maximum binding capacity was calculated by taking the X-intercept and dividing by the antibody concentration.

**IV. Fab Production**

Hydroxylapatite purified SOMA 10 was initially applied to protein A to select antibodies with the ability to bind to protein A (fig 11). Fractions with absorbance at 280 nm greater than 0.040 were pooled, dialyzed against PBS and the protein concentration measured. The first portion that did not bind to the column, peak A, was saved. Between 47 and 55% of the protein bound to the column. This portion, peak B, was digested with papain.

Peaks A and B were compared to SOMA 10 by RIA (fig 12). Similar concentrations of sample antibody were used. SOMA 10, peak A and peak B gave zero bindings of 33%, 13% and 32%, respectively. The standard curve using peak B as the antibody was similar to that using HAP SOMA 10. Peak A gave a standard curve which was relatively flat.

Binding of SOMA 10, protein A-purified peak A and B were also compared by ELISA (fig 13). The concentrations required for half maximum absorbance at 405 nm for SOMA 10, peak A and peak B were 10 µg/ml, 25 µg/ml and 5 µg/ml, respectively.

Papain-digested SOMA 10 peak B was purified on a protein A column (fig 14). The first peak contained the Fab fragments. The second contained Fc fragments, undigested and partially digested SOMA 10. Between 58 and 65% of the protein was recovered as Fab fragments. Therefore, there was an average of 26% yield of Fab fragments from the original HAP SOMA 10.
FIGURE 11
Elution Profile of HAP SOMA 10 from Protein A

HAP SOMA 10 was applied to a Protein A column with a solution of 1.5 M glycine, 3 M NaCl, pH 8.9, and desorbed with 0.1 M citric acid, pH 6. Peak A contained 44% of the original protein. Peak B contained 51% of the original protein. Five percent of the original protein was not recovered.
FIGURE 12

Comparison of SOMA 10 RIA Standard Curves

Somatostatin standard curves were produced using HAP SOMA 10 (open square), Protein A-purified SOMA 10 peak A (filled diamond) and B (filled square) as antibodies.
FIGURE 13
Comparison of Protein A-purified SOMA 10 Activity on ELISA
Figure 14

Elution Profile of Papain-digested SOMA 10 from Protein A

HAP SOMA 10, peak B was digested with mercuripapain for 4 hours. The digestion mixture was applied to a Protein A column with a solution of 1.5 M glycine, 3 M NaCl, pH 8.9, and desorbed with 0.1 M citric acid, pH 6. The peaks containing the Fab fragments and the Fc fraction contained 58% and 21% of the original protein, respectively. Twenty-one percent of the original protein was not recovered.
<table>
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<th>SOMA</th>
<th>Binding Capacity</th>
<th>Dissociation Constant (KD)</th>
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<td>3</td>
<td>2.58 ng/mg</td>
<td>3.2 x 10^{-12} mol/l</td>
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<tr>
<td>8</td>
<td>1.78 ng/mg</td>
<td>1.7 x 10^{-12} mol/l</td>
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<td>10</td>
<td>8.3 μg/mg</td>
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<tr>
<td>20</td>
<td>1.98 ng/mg</td>
<td>3.1 x 10^{-12} mol/l</td>
</tr>
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V. Identification of Fab Fragments

A. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Samples of SOMA 10, protein A-purified SOMA 10 peak A and B, the papain digest post-reaction, and the purified Fab fragments and Fc fraction were denatured and reduced, and electrophoresis on a SDS-polyacrylamide gel was performed (fig 15). The gel was stained with Coomassie Blue stain, and destained in a mixture of acetic acid and methanol. Bands were found corresponding to the molecular weights shown in table 3.

B. Chromatography of Incubation Mixtures of Antibody and $^{125}$I-Somatostatin on Sephadex G-100

Chromatography of an incubation mixture of SOMA 10 and $^{125}$I-somatostatin was performed on Sephadex G-100. The elution profile is shown in figure 16a. The first peak, containing the complexes of SOMA 10-$^{125}$I-somatostatin, was maximal at 9 ml, and the second, containing unbound $^{125}$I-somatostatin was maximal at 25 ml. $^{125}$I-somatostatin was partially displaced by 3.5 µg/ml somatostatin. Complete displacement of $^{125}$I-somatostatin required 7 µg/ml somatostatin.

The elution profile of the incubation mixture containing Fab fragments and $^{125}$I-somatostatin is shown in figure 16b. The peak elution of the complexes was at 15 ml, the unbound $^{125}$I-somatostatin eluted at 25 ml, and 3.5 µg/ml of somatostatin completely displaced the $^{125}$I-somatostatin.

Figure 16c shows the elution profile of the incubation mixture containing the Fc fraction and $^{125}$I-somatostatin. This Fc fraction contained Fc fragments, undigested and partially digested (one binding site removed) SOMA 10, and eluted between 9 and 20 ml. The second peak, unbound $^{125}$I-somatostatin, peaked at 25 ml. 3.5 µg/ml of somatostatin completely displaced the radioactively-labelled somatostatin. $^{125}$I-somatostatin alone was applied to the column and eluted at 25 ml.

On the same column, chromatography of immunoglobulin and BSA was performed as standards to ensure that the antibody complexes were not being
FIGURE 15

SDS-Polyacrylamide Gel Electrophoresis of SOMA 10 and Fragments

12% Resolving gel

Lane

1  HAP SOMA 10
2  Protein A-purified SOMA 10, peak A
3  Protein A-purified SOMA 10, peak B
4  Papain
5  Low molecular weight standards (94000 d, 67000 d, 43000 d, 30000 d, 20100 d, 14400 d)
6  Papain-digested SOMA 10, post-digestion
7  Fab fragments
8  Fc regions
9  Low molecular weight standards
TABLE 3
Electrophoresis of SOMA 10 and Fragments on SDS-Polyacrylamide Gel

<table>
<thead>
<tr>
<th>Sample</th>
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<tr>
<td>Protein A-purified HAP SOMA 10 Peak A</td>
<td>75 kd, 69 kd, 57 kd, 53 kd</td>
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<tr>
<td>Protein A-purified HAP SOMA 10 Peak B</td>
<td>57 kd, 26 kd</td>
</tr>
<tr>
<td>Papain-digested SOMA 10 Post-reaction</td>
<td>31 kd, 25 kd</td>
</tr>
<tr>
<td>Fab fragments</td>
<td>25 kd</td>
</tr>
<tr>
<td>Fc fraction</td>
<td>31 kd, 26 kd</td>
</tr>
</tbody>
</table>
FIGURE 16

Chromatography of Incubation Mixtures of Antibody and $^{125}$I-labelled Somatostatin on Sephadex G-100

$^{125}$I-labelled somatostatin was incubated with either SOMA 10 (a), Fab fragments (b) and the Fc fraction (c) and chromatography of the mixture was performed on Sephadex G-100 (open squares). Labelled somatostatin was displaced from the antibody or fragments with cold somatostatin at concentrations of 3.5 μg/ml (filled diamonds) and 7 μg/ml (filled squares).
retained by the column. Immunoglobulin (150 kd) and BSA (67 kd) eluted at 10 and 12 ml, respectively. SOMA 10 and the Fab complexes eluted at 9 and 15 ml, respectively. This indicated that the SOMA 10 complexes and Fab complexes were not retained by the column.

C. Immunocytochemical Characterization of Fab Fragments

Figure 17a - c shows the localization of somatostatin with HAP SOMA 10, Fab fragments and the Fc fraction on rat pancreas, respectively. These pictures were taken in consecutive sections of the pancreas. The apple-green colour represents specific staining of D cells in the periphery of the islet. HAP SOMA 10 gave the brightest fluorescence. The Fc fraction gave the second brightest fluorescence. This fraction contained Fc fragments, as well as undigested and partially digested SOMA 10. Fab fragments gave the least fluorescence.

VI. Acid Secretion

SOMA 10 was previously tested for biological activity on the basis of its ability to neutralize exogenously-administered somatostatin (Seal et al. 1987). SOMA 10 dose-dependently reversed the somatostatin (2 µg/kg/hr)-induced inhibition of liver extract-stimulated acid secretion. Concentrations of SOMA 10 greater than 100 µg completely reversed the inhibition.

Figure 18 shows the effect of Fab fragments on somatostatin-induced inhibition of gastric acid secretion, stimulated by 5% liver extract meal in gastric fistula rats. Acid secretion from Fab fragment infused rats (6 µEq/10 min) was consistently higher than from control rats (4.4 µEq/10 min). These experiments were performed in collaboration with Dr. A. Seal. In previous control experiments in which acid secretion was measured in response to liver extract alone (9 µEq/10 min), acid secretion was much higher than when somatostatin was also infused.
FIGURE 17

Comparison of immunoreactivity of SOMA 10 Fab Fragment by Immunocytochemistry

250 x

Five micrometre serial sections through pancreatic rat islets were immunocytochemically stained with HAP SOMA 10 (a), Fab fragments (b) and the Fc fraction (c) to test for immunoreactivity of the antibody and fragments. The apple-green colour represents specific staining of somatostatin in pancreatic D cells.
FIGURE 18

Effect of SOMA 10 Fab Fragments on Somatostatin-induced Inhibition of Acid Secretion

In experimental animals, 100 μg of SOMA 10 Fab fragments were injected as a bolus into the jugular vein (filled diamonds). In control animals (n = 4), no antibody was injected (open squares). Basal acid secretion was measured for 30 min. A five percent liver extract meal was flushed into the stomach to stimulate acid secretion. An infusion of 2.0 μg/kg/hr of somatostatin inhibited acid secretion.
VII. Isolated Perfused Stomach Experiments

Gastrin Release

i. Effect of SOMA 10 and Fab Fragments on Somatostatin-Induced Inhibition of Gastrin Release

These experiments were performed in order to investigate whether SOMA 10 and Fab fragments of SOMA 10 could reverse inhibition of gastrin release by somatostatin. After an equilibration period of 20 - 30 min, basal samples were collected for 10 min. Somatostatin was infused via a sidearm for 26 min at the following concentrations (nM): 60, 6, 1.5, 0.6, and 0.15. At 20 min, 10 min into the somatostatin infusion, an infusion of SOMA 10 (20 µg/ml) or SOMA 10 Fab fragments (15 µg/ml) was introduced. These concentrations of SOMA 10 and of Fab fragments were approximately 130 nM and 300 nM, respectively. Since the intact antibody is divalent, the concentrations were similar with respect to the number of binding sites. Control experiments were performed in which no peptide or antibody were infused.

Infusion of 60 nM somatostatin caused an 85% decrease in basal gastrin release (fig 19). Infusion of 20 µg/ml SOMA 10 had no effect on the inhibition. When the somatostatin infusion was terminated, gastrin release increased again to a level not significantly different from basal (time = 2 - 10 min).

Infusion of 6 nM somatostatin produced a 52% decrease in basal gastrin release (fig 20). Infusion of 20 µg/ml SOMA 10 caused a small, but insignificant increase in gastrin release. The onset of the reversal of somatostatin-induced inhibition of gastrin release was delayed. When the somatostatin infusion was terminated, gastrin release increased again to a level not significantly different from basal (time = 2 - 10 min).

Infusion of 1.5 nM somatostatin produced a 32% decrease in basal gastrin release (fig 21). Infusion of 20 µg/ml SOMA 10 increased gastrin to a level not significantly different from basal. When the SOMA 10 infusion was terminated, gastrin release decreased back to pre-SOMA 10 levels. When the somatostatin infusion was terminated, gastrin release increased to levels not significantly different from basal.
FIGURE 19 - 23
Effect of SOMA 10 on Somatostatin-induced Inhibition of Gastrin Release

n = 6

For figures 19 to 23, the following protocol was followed.

a. Basal samples were collected for 10 min. Somatostatin was infused for 26 min (filled diamonds). Ten minutes into this infusion, an infusion of 20 μg/ml SOMA 10 was superimposed for 6 min. Effluent was collected for a further 10 min, after the termination of the somatostatin infusion. In control experiments (n = 6), buffer alone was perfused for 46 min (open squares). Immunoreactive gastrin (IRG) was measured by RIA as described in methods.

b. The integrated gastrin release during period 4 to 10 min, 14 to 20 min, 22 to 28 min, 30 to 36 min, and 40 to 46 min is shown here. The asterisks represent significance (p ≤ 0.05) compared to basal levels (2 to 10 min).
FIGURE 19

Effect of SOMA 10 on Somatostatin-induced (60 nM) Inhibition of Gastrin Release

n = 6
FIGURE 20

Effect of SOMA 10 on Somatostatin-induced (6 nM) Inhibition of Gastrin Release

n = 6
FIGURE 21

Effect of SOMA 10 on Somatostatin-induced (1.5 nM) Inhibition of Gastrin Release

n = 6
FIGURE 22
Effect of SOMA 10 on Somatostatin-induced (0.6 nM) Inhibition of Gastrin Release

n = 6
FIGURE 23

Effect of SOMA 10 on Somatostatin-induced (0.15 nM) Inhibition of Gastrin Release

n = 6
Infusion of 0.6 nM somatostatin produced an 18% decrease in gastrin release (fig 22). Infusion of 20 µg/ml SOMA 10 caused a reversal of gastrin release to a level similar to basal gastrin release. Termination of the somatostatin infusion returned gastrin release to a level not significantly different from basal.

Infusion of 0.15 nM produced an 18% decrease in gastrin release (fig 23). Infusion of 20 µg/ml SOMA 10 produced a small, but insignificant reversal of gastrin release to a level similar to basal gastrin release.

In studies on the effect of Fab fragments on the inhibition of gastrin release by 1.5 nM somatostatin, somatostatin produced a 37% decrease (fig 24). Infusion of 15 µg/ml Fab fragments reversed the inhibition to a level similar to basal gastrin release. After termination of the Fab fragment infusion, gastrin release returned to levels which were not significantly different from basal gastrin release. As when SOMA 10 was infused, the onset of the reversal of somatostatin-induced inhibition of gastrin release was delayed.

ii. Effect of SOMA 10 and Fab Fragments on Basal Gastrin Release

The effect of a single passage of the antibody on basal gastrin release was studied. After a 20 to 30 min equilibration period, basal samples were collected for 20 min. 100 µg/ml and 20 µg/ml final concentrations of SOMA 10 were infused via a sidearm for 6 min, and samples were again collected for 20 min. In control experiments, no antibody was infused.

SOMA 10 at 20 µg/ml caused no significant change in gastrin release compared to control experiments (fig 25). SOMA 10 at 100 µg/ml caused a significant decrease in gastrin release 2 min into the infusion, compared to control values at the same time, and compared to pre-SOMA 10 gastrin release (fig 26). Basal gastrin release was approximately 170 pg/min and SOMA 10 caused it to decrease to 110 pg/ml. After termination of the SOMA 10 infusion, gastrin release returned to basal levels within 2 min. There was a small, but insignificant, rebound in gastrin release following the termination of SOMA 10.
FIGURE 24

Effect of SOMA 10 Fab Fragments on Somatostatin-Induced (1.5 nM) Inhibition of Gastrin Release

n = 6

a. Basal samples were collected for 10 min. Somatostatin at a concentration of 1.5 nM was infused for 26 min (filled diamonds). Ten minutes into this infusion, an infusion of 15 μg/ml SOMA 10 Fab fragments was superimposed for 6 min. Effluent was collected for a further 10 min, after the termination of the somatostatin infusion. In control experiments (n = 6), perfusate alone was perfused for 46 min (open squares). Immunoreactive gastrin (IRG) was measured by RIA as described in methods.

b. The integrated gastrin release during period 4 to 10 min, 14 to 20 min, 22 to 28 min, 30 to 36 min, and 40 to 46 min is shown here. The asterisks represent significance (p < 0.05) compared to basal levels (2 to 10 min).
FIGURE 25 and 26

Effect of SOMA 10 on Basal Gastrin Release

n = 6

For figures 25 and 26, the following protocol was followed.

Basal samples were collected for 20 min before the infusion of SOMA 10. SOMA 10 was infused at 20 min for 6 min (filled diamonds). After the termination of the infusion, samples were collected for a further 20 min. In control experiments (n = 6), perfusate alone was perfused for 46 min (open squares). Immunoreactive gastrin (IRG) was measured by RIA as described in methods.
FIGURE 25

Effect of SOMA 10 (20 μg/ml) on Basal Gastrin Release

n = 5
FIGURE 26

Effect of SOMA 10 (100 μg/ml) on Basal Gastrin Release

n = 6

The asterisks represent a significant difference (p<0.05) between gastrin release (IRG) in control and SOMA 10 infused animals.
The experiment was repeated using SOMA 10 Fab fragments instead of the whole antibody. The concentrations of Fab fragments used were 66 µg/ml and 15 µg/ml. These concentrations are approximately equivalent to 100 µg/ml and 20 µg/ml SOMA 10 with respect to the number of binding sites. Control experiments were performed in which no fragments were infused.

In figure 27, basal gastrin release was approximately 150 pg/min. Infusion of 15 µg/ml Fab fragments caused an immediate increase in gastrin release to approximately 270 pg/min. Following termination of the infusion, gastrin release returned to basal levels within 6 min. The increase in gastrin release was not significant.

Infusion of 66 µg/ml Fab fragments (fig 28) caused an immediate increase in gastrin release that was significantly different from control experiments, and from basal gastrin release at 20 min. Gastrin release remained elevated throughout the Fab fragment infusion. When the infusion was terminated, gastrin release slowly returned to basal within 6 min.

iii. Effect of Recirculation of SOMA 10 on Basal Gastrin Release

Normally in the isolated perfused stomach preparation, an infused antibody would have one passage through the stomach vasculature. To give the antibody more opportunity of neutralizing endogenous somatostatin, a system was set up such that perfusate containing antibody could be recirculated in the stomach vasculature. Basal samples were collected for 10 min, and gastrin concentrations were measured. Perfusate containing a final concentration of 20 µg/ml SOMA 10 was infused for 14 min. This was sufficient time to flush all perfusate without antibodies from both the tubing and stomach vasculature. For the last 4 of the 14 min, the venous effluent was collected and reoxygenated. This was used as a reservoir for recirculation of the antibody. The perfusate containing the antibody was recirculated for 20 min, and then non-circulated perfusate was perfused for a further 10 min. In control experiments, perfusate without the antibody was recirculated.
FIGURE 27 and 28

Effect of SOMA 10 Fab Fragments on Basal Gastrin Release

For figures 27 and 28, the following protocol was followed.

a. Basal samples were collected for 20 min before the infusion of SOMA 10 Fab fragments. Fab fragments were infused at 20 min for 6 min (filled diamonds). After the termination of the infusion, samples were collected for a further 20 min. In control experiments (n = 6), perfusate alone was infused for 46 min (open squares). Immunoreactive gastrin (IRG) was measured by RIA as described in methods.

b. The integrated gastrin release during period 16 to 20 min, 22 to 26 min, 28 to 32 min, and 34 to 38 min is shown here. The asterisks represent significance (p < 0.05) compared to controls.
FIGURE 27

Effect of SOMA 10 Fab Fragments (15 μg/ml) on Basal Gastrin Release

n = 6
FIGURE 28
Effect of SOMA 10 Fab Fragments (66 μg/ml) on Basal Gastrin Release

n = 6

The asterisks represent a significant difference (p<0.05) between gastrin release (IRG) in control and Fab fragment infused animals.
Perfusion of the stomach with 20 μg/ml SOMA 10 caused no significant change in gastrin release compared to controls. After recirculation of the perfusate for 20 min, cumulated gastrin release from SOMA 10-perfused animals was significantly higher than from control animals (fig 29). Gastrin release in both animals decreased to a level that was not significantly different from basal.

VIII. Isolated Perfused Pancreas Experiments

Insulin Release

i. Effect of SOMA 10 and Fab Fragments on Insulin Responses to a Glucose Gradient

Perfusate containing 4.4 mM glucose was administered for 5 min. A gradient of glucose ranging from 4.4 to 16.5 mM was then infused over the next 30 to 35 min. In experiments using the whole antibody, 16.5 mM glucose was perfused after termination of the glucose gradient. The effect of the antibody on basal insulin release was tested by sidearm infusion to a final concentration of either 5 μg/ml or 10 μg/ml SOMA 10, or 5 μg/ml SOMA 10 Fab fragments at time 5 min and continued throughout the glucose gradient stimulation. In control experiments, the pancreas was stimulated with a glucose-gradient, but was not subjected to the antibody. The gradient was generated by a gradient maker in which the chambers were filled with perfusate at concentrations of 4.4 mM and 16.5 mM glucose.

SOMA 10 and Fab fragments at the concentrations used (fig 30 and 31) had no effect on basal insulin release. Insulin release increased in response to the glucose gradient, reaching between 800 μU/min and 1000 μU/min at 16.5 mM glucose. Neither SOMA 10 nor the Fab fragments had a significant effect on glucose-stimulated insulin release in comparison to controls.

ii. Effect of Fab Fragments on Glucose (16.5 mM)-Stimulated Insulin Release

Perfusate containing 4.4 mM glucose was administered for 5 min. Insulin release was stimulated with 16.5 mM glucose for the remainder of the
FIGURE 29

Effect of Recirculation of SOMA 10 on Gastrin Release

n = 7

Basal immunoreactive gastrin (IRG) was measured for 10 min. In experimental animals, SOMA 10 at a final concentration of 20 μg/ml was infused for 14 min (filled diamonds). For the last 4 of the 14 min, the venous effluent was collected, oxygenated, and used as a reservoir for recirculation. Recirculation of the antibody continued for 20 min, upon which the animals were perfused with non-recycled perfusate for a further 20 min. In control animals (n = 9), the perfusate did not contain antibody (open squares).

The asterisks represent a significant difference (p<0.05) between gastrin release (IRG) from experimental animals and control animals.
FIGURE 30

Effect of SOMA 10 (5 and 10 µg/ml) on Glucose Gradient-Stimulated Insulin Release

Perfusate was collected for 10 min, after which a gradient of glucose from 4.4 mM to 16.5 mM was administered (total volume = 120 ml). After the gradient, glucose was maintained at 16.5 mM for 10 min. In control animals (n = 6, open squares), no antibody was infused. Immunoreactive insulin (IRI) was measured by RIA as described in methods.

In figure a, experimental animals (n = 6, filled triangles) were infused with 5 µg/ml SOMA 10 from the 5th minute to the end of the gradient.

In figure b, experimental animals (n = 6, filled triangles) were infused with 10 µg/ml SOMA 10.
a. 5 μg/ml SOMA 10

b. 10 μg/ml SOMA 10
FIGURE 31

Effect of SOMA 10 Fab Fragments (5 μg/ml) on Glucose Gradient-Stimulated Insulin Release

n = 6

Basal immunoreactive insulin (IRI) was collected for 10 min, upon which the pancreas was stimulated with a gradient of glucose from 4.4 mM to 16.5 mM (total volume = 120 ml). In experimental animals (filled triangles) Fab fragments were infused from the 5th minute to the end of the gradient. In control animals (n= 6, open squares) no fragments were infused.
perfusion. In experimental animals, 10 μg/ml Fab fragments were infused from the 16th to the 25th min. Insulin was collected for a further 10 min. In control experiments, no fragments were infused (fig 32).

The first insulin peak in response to the increase in glucose concentration in control pancreases did not correspond to that of the experimental animals. However, the shape and height of this peak is quite variable. The fragments caused a decrease in insulin release. Significance from control values was achieved only during the 20th to the 22nd min. After the termination of the Fab infusion, insulin release was not significantly different from controls.

iii. Effect of SOMA 10 and Fab fragments on Glucose (8.8 mM)-Stimulated Insulin Release

A similar protocol as above (section ii) was followed, with the exception that the pancreas was stimulated with 8.8 mM glucose. A lower concentration of glucose was used since the stimulation was thought to be too strong. A glucose concentration of 8.8 mM was chosen based on previous studies indicating that in the presence of a weak insulin stimulus, the effect of somatostatin on insulin release was greatest. Conversely, the effect of somatostatin was weaker when insulin release was strongly stimulated. In experimental animals, either 30 μg/ml Fab fragments or 45 μg/ml SOMA 10 were infused. The concentration of Fab fragments was chosen based on the results of the infusion of 10 μg/ml Fab fragments on 16.5 mM glucose-stimulated insulin release. The concentration of SOMA 10 was chosen as it is approximately equivalent to 30 μg/ml Fab fragments with respect to the number of binding sites.

Stimulation of the pancreas with 8.8 mM glucose caused a first phase increase in insulin release reaching 320 μU/min at 10 min (fig 33). Infusion of the Fab fragment (30 μg/ml) had no significant effect, except at the 22 min time period where insulin release decreased. Immediately after the termination of the infusion of the Fab fragments, insulin release significantly increased to 222 μU/min, and then slowly returned to control values within 3 min.
FIGURE 32

Effect of SOMA 10 Fab Fragments on Glucose (16.5 mM)-Stimulated Insulin Release

n = 6

Basal immunoreactive insulin (IRI) was measured for 5 min. The pancreas was then stimulated with 16.5 mM glucose for the remainder of the experimental period. In experimental animals (filled diamonds), 10 μg/ml SOMA 10 Fab fragments were infused during the 16th to 25th minute. In control animals (n = 6, open squares), no fragments were infused. The asterisks represent a significant difference (p≤0.05) between the control and experimental values.
FIGURE 33

Effect of SOMA 10 Fab Fragments on Glucose (8.8 mM)-Stimulated Insulin Release

n = 7

Basal immunoreactive insulin (IRI) was measured for 5 min. The pancreas was then stimulated with 8.8 mM glucose for the remainder of the experimental period. In experimental animals (filled diamonds), 30 μg/ml SOMA 10 Fab fragments were infused during the 16th to 23th minute. In control animals (n = 9, open squares), no fragments were infused. The asterisks represent a significant difference (p < 0.05) between control and experimental values.
In the experiments with the higher concentration of SOMA 10 (45 μg/ml), stimulation of the pancreas with 8.8 mM glucose caused an increase in insulin release between 190 and 270 μU/min at 10 min (fig 34). Infusion of SOMA 10 caused a significant decrease in insulin release 3 min into the infusion, in comparison to controls. The decrease remained significant up to 1 min after the termination of the SOMA 10 infusion. Insulin release then returned to values similar to controls.

iv. **Effect of Pre-surgical Immunoneutralization of Somatostatin with SOMA 10**

Experimental animals were injected with 0.5 mg of SOMA 10 into the vena cava just before the surgical procedure. This allowed for a 30 to 40 min period in which the antibody could neutralize somatostatin. The preparation was equilibrated for 10 min with perfusate (4.4 mM) not containing antibody. In experimental animals, 20 μg/ml SOMA 10 was infused for the entire experimental period. No antibody was infused in control experiments. Per fusate was collected for 5 min, and the pancreas was then stimulated with 8.8 mM glucose for the remainder of the experimental period.

The antibody caused a significant decrease in the first insulin peak; controls reached 276 μU/min, while in SOMA 10 infused animals, insulin release only reached 126 μU/min (fig 35). In the remainder of the experimental period, control and experimental insulin release was not significantly different.

IX. **Immunocytochemical Identification of Fab Fragments and SOMA 10 in Perfused Organs**

In vitro studies in which antibody and Fab fragments were incubated on collagen-coated plates (fig 36) showed that Fab fragments, and to a lesser extent SOMA 10 bound non-specifically to collagen. Controls, a monoclonal antibody to VIP and PBS, did not cause as much non-specific fluorescence as SOMA 10. During processing of the perfused tissues, in which SOMA 10 or Fab fragments were infused, the antibody would have been washed out if it had not bound to the tissue, i.e. to somatostatin in or on cells, or to collagen.
Basal immunoreactive insulin (IRI) was measured for 5 min. The pancreas was then stimulated with 8.8 mM glucose for the remainder of the experimental period. In experimental animals (filled diamonds), 45 μg/ml SOMA 10 was infused during the 16th to 23th minute. In control animals (n = 9, open squares), no antibody was infused. The asterisks represent a significant difference (p < 0.05) between control and experimental values.
In experimental animals, the vena cava was exposed and a 0.5 mg bolus injection of SOMA 10 in PBS was administered. The animals were equilibrated for 10 min after the preparation for the isolated-vascularly perfused pancreas. Basal immunoreactive insulin (IRI) was measured for 5 min. The pancreas was then stimulated with 8.8 mM glucose for the remainder of the experimental period. In experimental animals (filled diamonds), 20 μg/ml SOMA 10 was infused during the entire experimental period. In control animals (n = 9, open squares), no antibody was infused. The asterisks represent a significant difference (p < 0.05) between the control and experimental values.
FIGURE 36

Nonspecific Binding of SOMA 10 and Fab Fragments to Collagen

PBS (a), and 1.0 μg/ml of anti-VIP (b), SOMA 10 (c), and SOMA 10 Fab fragments (d) were incubated with collagen bound to Petri dishes to test for nonspecific binding of antibodies to collagen. The plates were then incubated with biotin-spacer-AP goat anti-immunoglobulin, followed by avidin-FITC. The plates were screened using epi-fluorescence and photographed.
Immunocytochemical staining of the Fab fragments and SOMA 10 was performed on the stomach and pancreas of perfused organs. Sections of stomach perfused with 100 µg/ml SOMA 10 or with recirculated antibody did not exhibit any fluorescence. However, sections of stomach perfused with 66 µg/ml Fab fragments demonstrated the infiltration of the Fab fragment into the interstitium. Apple-green fluorescence represents the specific staining of Fab fragments. In figure 37 (right), fluorescence is seen over the collagen matrix in the submucosa of Fab fragment infused (66 µg/ml) stomachs. On the left side, in which no antibody was infused, only intrinsic fluorescence from chief cells is seen (bright yellow). In the pancreas, both SOMA 10 and especially Fab fragments (fig 38) were found in the interstitium, binding to collagen-containing structures. Note that a halo surrounds the duct in the Fab fragment infused (30 µg/ml) pancreas, but not in the control pancreas. Intrinsic fluorescence (bright yellow) from zymogen granules is found in acinar tissue in both control and fragment infused pancreases.
FIGURE 37

Immunocytochemical staining of Fab Fragments in the Perfused Stomach

125 x

Five micrometre sections through the corpus of the rat stomach. On the left is a control section showing only the intrinsic fluorescence from the chief cells. On the right is a section after perfusion with 66 µg/ml SOMA 10 Fab fragments for 6 minutes. Note the presence of specific (apple-green) fluorescence over the collagen matrix in the submucosa.
FIGURE 38

Immunocytochemical Staining of Fab Fragments in the Perfused Pancreas

250 x

a) A five micrometre section through a control pancreas showing exocrine and endocrine elements. Note that the ductular tissue to the left of the islet is not surrounded by a halo of fluorescence.

b) A similar section from a pancreas perfused with SOMA 10 Fab fragments (30 µg/ml). Note the halo of positive immunofluorescence around the duct in the centre of the figure.

N.B. The bright yellow fluorescence on the acinar tissue is intrinsic to the zymogen granules.
DISCUSSION

Since high purity antibodies are important in animal studies, ascites containing SOMA 10 was purified, accessed, and characterized. Fab fragments of SOMA 10 were produced and characterized. SOMA 10 and its Fab fragment were then used in in vitro studies of the effect of somatostatin on gastrin and insulin release in the stomach and pancreas, respectively.

I. Purification and Characterization of SOMA 10 and Fab Fragments

The purification of SOMA 10 by ammonium sulphate precipitation, in conjunction with hydroxylapatite chromatography resulted in an antibody with purity of 93%. This indicates that hydroxylapatite chromatography is a good method of purifying mouse monoclonal antibodies. The yield of 2 to 3 mg of pure SOMA 10 per millilitre of ascites fluid is low compared to the yield from other monoclonal antibody producing cells. This is a result of the low production of antibody by SOMA 10 cells, and not of the purification procedure.

The purity of SOMA 10 at various stages of purification was determined by affinity and gel filtration HPLC. In the elution profile of SOMA 10 on the Ultraffinity-EP column, the first peak, which did not bind to the column, had no immunoreactivity to somatostatin, as determined by ELISA. Only the second peak, desorbed with water in which the pH was decreased to 2 by HCl, was immunoreactive to somatostatin. The increase in purity of SOMA 10 after each purification step is determined by the decrease in size of the first peak. On the gel filtration column, the SOMA 10 peak eluted at 14.5 min. As the purification of SOMA 10 proceeded, this peak became larger and more homogeneous. Although there were small differences in the results of the percentage purity of ascites and SAS SOMA 10 assessed by affinity and gel filtration HPLC, both methods were in agreement that HAP SOMA 10 was of very high purity.

When SOMA 10 samples, at various stages of purification, were tested for immunoreactivity to somatostatin on ELISA, ascites, ammonium sulphate precipitated, and hydroxylapatite purified SOMA 10 gave half maximum readings of 25 µg/ml, 10 µg/ml and 10 µg/ml, respectively. The decrease in the concentration giving half maximum reading for ammonium sulphate precipitated
SOMA 10 and HAP SOMA in comparison to that for ascites reflects the increase in purity of SOMA 10.

By immunodiffusion gels, it was determined that the four SOMA antibodies were of class IgG1. Under the usual conditions utilized for purification of antibodies, this class of antibody does not bind well to protein A (Goding, 1978). To assess the binding of SOMA 10 to protein A, hydroxylapatite SOMA 10 was applied to a protein A column. An average of 52% bound to the column. The fraction that did not bind to the protein A, peak A, was reapplied to a fresh protein A column, to confirm that the antibody did not bind to protein A, and not that the column was overloaded. Again this fraction did not bind to protein A, suggesting that this fraction did not contain SOMA 10, or that the antibody did not have the ability to bind to protein A.

SOMA 10, protein A purified peak A and the fraction binding to protein A (peak B) were compared by RIA, ELISA and on SDS-polyacrylamide gels. By RIA, the activity of SOMA 10 and peak B were similar. Peak A gave 39% of the maximum binding of SOMA 10 and peak B. The results from the ELISA indicated that peak A was 2.5 times and 5 times less immunoreactive to somatostatin than SOMA 10 and peak B, respectively. That peak A did not bind to protein A, does not imply that peak A did not contain SOMA 10, since it was slightly immunoreactive to somatostatin. Furthermore, hydroxylapatite purified SOMA 10 was determined by HPLC on the affinity column to be 93% pure. The 7% non-SOMA 10 could not account for the difference in the activity between peak A and peak B. Monoclonal antibodies, such as SOMA 10, should be homogeneous. There is speculation that there is differential glycosylation of the antibodies causing some to bind to protein A, and some to lose this ability. Alternatively, peak A antibodies may have been damaged during the hydroxylapatite purification, and lost the ability to bind to protein A.

Electrophoresis of hydroxylapatite SOMA 10 on an SDS-polyacrylamide gel revealed strong bands corresponding to molecular weights of 57 and 26 kd, which are the heavy and light chains of SOMA 10, respectively. Weaker bands appeared at 69, 53 and 31 kd. The 69 kd band is thought to contain albumin, the 53 kd band could be another contaminating protein, and the 31 kd band could be part of the heavy chain. The strongest band appearing on electrophoresis of protein A purified SOMA 10 peak A corresponded to a
molecular weight of 69 kd, with weaker bands at 75, 57 and 53 kd. The 75 kd band is thought to be a dimer or trimer of parts of the heavy chain and the light chain. Electrophoresis of peak B revealed only two bands at 57 and 26 kd.

Incubation of protein A purified SOMA 10 peak B with papain for 4 h achieved almost complete digestion of the antibody. Affinity chromatography on protein A was chosen as the method of purifying the Fab fragment from the digestion mixture, because Fab fragments purified by other mean, such as by ion exchange chromatography, may still contain undigested or partially digested SOMA 10, that have all or some of the Fc portion of the antibody attached.

The Fab fragments were identified on a reducing gel by SDS-polyacrylamide gel electrophoresis. The papain-digested SOMA 10 post-digestion sample gave bands corresponding to molecular weights of 31 kd and more prominently at 25 kd. Fab fragments eluted at a molecular weight corresponding to 25 kd. The fraction of the digestion mixture absorbed onto the protein A column, the Fc fraction, may have contained undigested and partially digested SOMA 10 and Fc fragments. Electrophoresis of this sample gave a strong band eluting at a molecular weight of 31 kd, and a weaker band at 26 kd. They represent parts of the heavy chain in the Fc fragment and the light chain. The lack of a 57 kd heavy chain gives support that SOMA 10 was almost completely digested by papain, although treatment of this sample with SDS and dithiothreitol may have damaged weakened bonds, so that intact heavy chains were broken down.

To assess the ability of the Fab fragments to bind somatostatin, chromatography of antibody-\(^{125}\)I-somatostatin complexes was performed on Sephadex G-100. The complexes were prepared by incubating SOMA 10, Fab fragments and the Fc fraction with \(^{125}\)I-somatostatin overnight. SOMA 10-\(^{125}\)I-somatostatin complexes eluted first, followed by Fab-\(^{125}\)I-somatostatin complexes. Fc fraction-\(^{125}\)I-somatostatin complexes eluted over a range, since this fraction may have contained intact and partially digested antibodies. Fc fragments, which have no somatostatin binding ability would not be recognized by monitoring radioactivity. Chromatography of \(^{125}\)I-somatostatin alone showed that it eluted last. When somatostatin was incubated with SOMA 10 and \(^{125}\)I-somatostatin, and the chromatography of the complexes performed,
the radioactively-labelled somatostatin was displaced from the antibody, as indicated by the decrease in the complex peak, and the increase in the $^{125}$I-somatostatin peak. $^{125}$I-somatostatin was completely displaced from SOMA 10 by 7 μg/ml somatostatin. With the Fab complexes, 3.5 μg/ml of unlabelled somatostatin was required to displace the $^{125}$I-somatostatin.

The immunoreactivity of the Fab fragments was also assessed by immunocytochemistry. SOMA 10, the Fc fraction and then the Fab fragments were found to cause fluorescence in decreasing order of intensity. It is tempting to suggest that the activity of the Fab fragments is less than that of the intact antibody. However the second antibody, goat anti-mouse immunoglobulin, may not bind equally to the Fab fragment and the intact antibody. The fluorescence caused by the Fc fractions suggests that this fraction still contained somatostatin binding ability.

II. Effect of SOMA 10 and Fab Fragments on Biological Activities

The reversal of the somatostatin-induced inhibition of liver extract-stimulated gastric acid secretion by the Fab fragments was the basis by which the biological activity was assessed. Over a 90 min period, the average control acid secretion was $40 \pm 4$ μEq. Fab fragment injected animals secreted $55 \pm 8$ μEq, suggesting that the Fab fragments could neutralize exogenously-administered somatostatin. Over a 60 min period, the acid secretion from Fab fragment injected animals (100 μg) was $37$ μEq, while that from SOMA 10 injected animals (100 μg) was $46$ μEq (Seal et al., 1987). Again this suggests that the activity of the fragment is less than the intact antibody.

A. Passive Immunization Studies in the Isolated Perfused Stomach

The biological activities of SOMA 10 and the Fab fragment were also assessed by their ability to reverse the inhibition of gastrin release by somatostatin. Somatostatin caused a decrease in gastrin release at the four highest concentrations used, 60 nM, 6 nM, 1.5 nM and 0.6 nM (fig 19-22), but failed to significantly inhibit gastrin release when 0.15 nM somatostatin (fig 23) was infused. An infusion of 20 μg/ml of SOMA 10 reversed the inhibition of gastrin release, except at 60 nM somatostatin. Gastrin release inhibited by 1.5 nM somatostatin was completely blocked by 20 μg/ml of SOMA 10.
Vale et al. (1978) determined that the binding site to the somatostatin receptor was located in the amino acid residues 6 to 11. Modification of these positions produced a drastic decrease in biological potency. Substitution of residues 7, 8 and 9 caused a complete loss of activity. The binding site of SOMA 10 was determined to be between residues 5 and 12. The binding site of the other SOMA antibodies was found to be between positions 4 and 12. This implies that the SOMA antibodies bind to the active site of the somatostatin molecule, and can therefore potentially neutralize the action of somatostatin.

The somatostatin receptor on isolated rat pancreas membranes was shown to have a $K_D$ of 0.1 nM, with a density of 1800 sites per cell (Reyl-Desmars and Lewin, 1982). Two somatostatin receptors have been found to be present on isolated rat gastric cells. The high and low affinity receptors had a $K_D$ of 0.08 nM with a density of 560 sites/cell, and 4.5 nM with a density of 4600 sites/cell, respectively (Reyl et al., 1979). These results suggest that somatostatin would preferentially bind to pancreatic somatostatin receptors rather than to SOMA 10, but that somatostatin would bind SOMA 10 preferentially to gastric somatostatin receptors because of the high density of low affinity somatostatin receptors.

The maximum binding capacity of SOMA 10, determined by Scatchard analysis, was found to be 8.3 μg/mg. Assuming the molecular weight of SOMA 10 to be 150 kd, the binding capacity in molar terms is 760 mmoles/mole of SOMA 10, i.e. each antibody molecule binds one somatostatin molecule. The maximum binding capacities of SOMA 3, 8 and 20 were 2.58, 1.78, and 1.98 ng/ml, ~1000 less than that of SOMA 10. The dissociation constants of these antibodies were 3.2, 1.7 and 3.1 pM, respectively, and that of SOMA 10 was 2.2 nM.

Assuming that the molecular weight of SOMA 10 is 150 kd, 20 μg/ml is equivalent to 130 nM; 130 nM SOMA 10 neutralized 1.5 nM somatostatin in the isolated perfused stomach. The reasons for the requirement of such a high concentration of SOMA 10 to neutralize exogenously-administered somatostatin in the isolated, perfused stomach, in comparison to the one to one binding of somatostatin and SOMA 10 as determined by Scatchard analysis could be 1) the greater affinity of somatostatin to the high affinity somatostatin receptor than to SOMA 10; 2) the neutralization of both endogenous and
exogenous somatostatin in this preparation and; 3) the limited access of the antibody to somatostatin.

Fab fragments at a concentration of 15 µg/ml were able to completely reverse the inhibition of gastrin release by 1.5 nM somatostatin in the isolated perfused stomach (fig 24). In comparison to the concentration of SOMA 10 required for complete blockage of inhibition, the Fab fragments have slightly less activity. Whether these fragments have a lower affinity to somatostatin or a lower binding capacity than the intact antibody was not determined.

SOMA 10 and its Fab fragments were then used in an attempt to neutralize endogenous somatostatin. An infusion of 20 µg/ml SOMA 10 had no effect on basal gastrin release (fig 25). When the concentration of SOMA 10 was increased to 100 µg/ml, a significant decrease in gastrin release occurred (fig 26). When the SOMA 10 infusion was terminated, there was a small but insignificant rebound in gastrin release. These results are contrary to what was expected, which was an increase in gastrin release. There are a number of possible explanations for these results.

The decrease in gastrin release could be due to SOMA 10 neutralizing somatostatin, which in turn inhibited an inhibitor of gastrin. This disinhibition of a previously unrecognized inhibitory mechanism has been previously suggested. Seal et al. (1987) found the acid secretion decreased in response to both 5% liver extract and saline, when gastric fistula rats were passively immunized with 200 µg SOMA 10.

A second explanation for the decreased gastrin release could be that the antibody is binding to somatostatin, and changing its conformation, such that it enhances its effects on the receptor. That is, SOMA 10 may increase the affinity or increase the binding time of somatostatin to its receptor. Several antibodies have been observed to enhance the effect of their antigens. Monoclonal antibodies to thyrotropin (Holder et al., 1987) and to growth hormone (Aston et al., 1986) have been shown to potentiate the production of thyroxine, and to potentiate the somatogenic and lactogenic activity of growth hormone, respectively. The enhancement of activity of somatostatin by SOMA 10 is unlikely since infusion of SOMA 10 reversed the somatostatin-induced inhibition of gastrin release.
The decrease in basal gastrin release observed when SOMA 10 was infused could be a nonspecific effect of the antibody, since such a large concentration was used. To test this possibility, mouse monoclonal antibodies not directed against a gastric peptide, or purified ascitic fluid from mice injected with myeloma cells could be used as control antibodies.

A final possible explanation for the observed decrease in gastrin release is that SOMA 10 is binding to the somatostatin receptor, and mimicking the effect of somatostatin. It should be noted that SOMA 10 binds to amino acid residues 5 to 12. The somatostatin receptor binds to amino acid residues 6 to 11.

In contrast to the present results, Saffouri et al. (1982), using somatostatin antiserum with a binding capacity of 19.2 ng/ml, found that infusion of the antiserum at both a two fold and a tenfold dilution, caused an immediate and significant increase in basal gastrin release in the isolated, perfused rat stomach. Short et al. (1985) infusing somatostatin antiserum (binding capacity = 2.86 μg/ml) at a 2000 fold dilution increased gastrin release in the isolated, perfused rat stomach, but only after 45 min of infusion. Chiba et al. (1981), using a similar preparation and an antiserum with a binding capacity of 8.7 μg/ml, found no increase in gastrin release with infusion of the antiserum at a twofold and hundredfold dilution.

In contrast to the results with the intact antibody, Fab fragments at concentrations of 15 μg/ml and 66 μg/ml (fig 27 and 28), corresponding to 20 μg/ml and 100 μg/ml SOMA 10, respectively, caused an immediate increase in gastrin release to 270 pg/min. Gastrin release remained greater than control for at least 5 min following the termination of the fragment infusion, indicating that the Fab fragments were not immediately removed from the stomach. The increase in gastrin release suggests that SOMA 10 could neutralize endogenous somatostatin, and that somatostatin inhibits basal gastrin release.

Immunocytochemical staining performed on the SOMA 10 perfused stomachs revealed no antibody in the interstitium of the stomach. Staining of the Fab fragments in the perfused organ (fig 37) revealed that the Fab fragments had entered into the interstitium, but were binding nonspecifically to collagen. In vitro, it was determined that the SOMA 10 and especially the Fab
fragments bound nonspecifically to collagen (fig 36), much more than another anti-mouse monoclonal antibody (anti-VIP; V31). Both antibodies and the Fab fragments produced more fluorescence than the background fluorescence produced by incubating PBS on the collagen-coated plates. Antibodies or Fab fragments in the interstitium could not be detected by immunocytochemistry unless they bound to something, because the vigorous processing and multiple washes of the tissue would have removed any unbound molecules.

Granger (unpublished) found that when radioactively-labelled gastrin antibody was perfused into the intestinal circulation, after one hour, the antibody could be detected in the lymphatic system. This demonstration suggests that the blood vessels are permeable to molecules even as large as 150 kd.

The difference in the gastrin release obtained when SOMA 10 and its Fab fragment were infused could be due to the permeability of the antibody. The intact antibody is ~150 kd, while the Fab fragment is only ~50 kd. The smaller size of the fragment would increase the probability of its penetration through the blood vessel into the interstitium. The detection of the Fab fragment, but not the intact antibody in the gastric interstitium may be due to the permeability differences of the molecules. Alternatively, the lack of detection of the intact antibody could be due to the lower nonspecific binding of SOMA 10 with respect to the fragment, or a combination of both the reduced permeability and decreased nonspecific binding to collagen. The antibody may have been detected in the interstitium if it were perfused for a longer period of time. If, however, SOMA 10 does not leave the blood vessel, the difference in the effect on gastrin release of SOMA 10 and the Fab fragment infusion could be the result of an endocrine and a paracrine effect of somatostatin, respectively.

In the above experiments, the antibody had only a single passage through the vasculature. In order to increase the opportunity of the antibody entering into the interstitium, a system was set up such that the antibody was recirculated (fig 29). After recirculation of the antibody for 20 min, the cumulated gastrin release from the SOMA 10 perfused stomachs was much greater than from the controls, suggesting that somatostatin tonically inhibits gastrin release. This is in agreement with results from the Fab infusion. An argument against the validity of these results is that metabolites which would normally be eliminated,
are collected and recirculated. However, the buildup of metabolites would be present in both the control and the SOMA 10 perfused animals.

Immunocytochemical staining of the SOMA 10 recirculated stomachs revealed no SOMA 10 in the interstitial space. The lack of detection may be due to the concentration of SOMA 10 binding nonspecifically to collagen, or that the antibody may have been broken down.

B. Passive Immunization Studies in the Isolated Perfused Pancreas

Most passive immunization experiments using somatostatin antiserum in perfused pancreas (Sorenson et al., 1980) and in vivo studies (Schusdziarra et al., 1978, Steiner et al., 1978, Tannenbaum et al, 1978) have yielded negative results. However, passive immunization studies in isolated islets have consistently been successful. Only Schusdziarra et al. (1980) showed that in vivo injections of somatostatin antiserum into dogs enhanced plasma concentration of insulin following a meal. Honey et al. (1981) demonstrated that the neutralization of endogenous somatostatin using somatostatin antiserum stimulated glucagon and insulin release from the isolated perfused chicken pancreas. They stress that these results were obtained in chicken and that the local effects of islet somatostatin in mammals may be different. In isolated rat islets, incubation with somatostatin antiserum increased insulin release stimulated by leucine (Taniguchi et al., 1979), arginine (Itoh et al., 1980) and glucose (Taniguchi et al., 1979, Itoh et al., 1980). Glucagon release was also increased by somatostatin antiserum in isolated islets (Barden et al., 1977, Itoh et al., 1980).

SOMA 10 has previously been shown to block the biological effect of exogenous somatostatin on GIP-stimulated insulin release (Takemura et al., unpublished). Simultaneous infusion of a glucose gradient from 4.4 to 16.5 mM glucose and of SOMA 10 at 5 or 10 µg/ml or Fab fragments at 5 µg/ml into the isolated perfused pancreas caused no change in insulin release compared to controls. The lack of response could be due to the low concentration of the antibody and fragments used.

Infusion of 10 µg/ml Fab fragments caused a slight decrease in insulin release stimulated by 16.5 mM glucose (fig 32). The minor effect of the Fab
fragment on insulin release could be attributed to the small effect of somatostatin on strongly stimulated insulin release (Taniguchi et al., 1977).

Reduction of the insulin stimulus to 8.8 mM glucose, and infusion of 30 μg/ml Fab fragments caused insulin release to be depressed slightly (fig 33). However, there was a large increase in insulin release observed following termination of the infusion.

When insulin release was stimulated with 8.8 mM glucose and a SOMA 10 (45 ng/ml) infusion, equivalent to 30 μg/ml Fab fragments, was applied, a more distinct decrease in insulin release was observed (fig 34). Termination of the infusion did not cause the prominent increase in insulin release observed when the Fab fragment infusion was terminated. During infusion of SOMA 10, and increase in insulin release may not have been observed, because of the 22 fold greater dissociation constant of the antibody in comparison to the rat pancreatic somatostatin receptor.

The results from the latter three experiments are contrary to what was expected, which was an increase in insulin release. Explanations for the inhibition of insulin release are similar to those for the inhibition of gastrin release by the infusion of SOMA 10. The decrease in insulin release could be a nonspecific effect of the antibody. SOMA 10 could be binding to somatostatin changing its conformation so that it becomes more suitable to its receptor, i.e. by increasing its affinity or increasing its binding time. Somatostatin neutralization could cause a disinhibition of a previously unknown inhibitor of insulin release. The decrease in insulin release may also be caused by SOMA 10 binding to the somatostatin receptor, mimicking the effects of somatostatin.

Immunocytochemical staining of the pancreata perfused with 30 μg/ml Fab fragments and 45 μg/ml SOMA 10 revealed that the fragment and antibody had permeated into the interstitial spaces of the pancreas, and had bound to collagen containing structures (fig 38). Since both antibody and fragment had left the circulation, endocrine and paracrine effects of somatostatin could not be distinguished.

Kviety et al. (1983) studied the permeability of capillaries in the isolated, blood-perfused canine pancreas with the double-indicator dilution technique.
They suggest that the fenestrations of the capillaries restrict the movement of solutes greater than 27 Å diameter, therefore allowing polypeptide hormones (less than 15 Å) to move freely across vessel walls, while restricting the leakage of plasma proteins (greater than 37 Å) from the vessel. However, the Krebs-bicarbonate buffer used in the present study to perfuse the pancreas contained dextran, which increases the size of the capillary pores, allowing the entrance of molecules larger than would be allowed through in a physiological situation.

Immunoneutralization of somatostatin with 0.5 mg SOMA 10 prior to surgery for the isolated perfused pancreas, followed by infusion of 20 μg/ml SOMA 10 caused the first phase of insulin release to decrease significantly (fig 35). No effect was observed on the second phase of insulin. These results are contrary to what was expected. However, since the first, but not the second phase of insulin was affected, somatostatin may have more of an effect on the first phase of insulin release than on the second. This concurs with findings by Curry and Bennett (1976), in which they found the first insulin phase to be 25 to 50 times more sensitive to somatostatin inhibition than the second phase.

III. Conclusions

Purification of mouse monoclonal antibody by ammonium sulphate precipitation, in conjunction with hydroxylapatite chromatography is a fast and reproduceable method of purification. Assessment of HAP SOMA 10 by affinity and gel filtration HPLC revealed that it was over 90% pure. SOMA 10 was found to bind to the somatostatin molecule at the same region as the somatostatin receptor, therefore neutralization of somatostatin activity can be achieved. SOMA 10 has a large binding capacity. These characteristics make SOMA 10 a valuable antibody for passive immunization.

From studies in which SOMA 10 recirculation and Fab infusion caused an increase in basal gastrin release, it can be concluded that somatostatin exerts a continuous restraint on gastrin release. Since in the perfused pancreas experiments, and possibly in the perfused stomach experiments, both SOMA 10 and the Fab fragment were entering into the interstitium, it is impossible to distinguish between endocrine and paracrine effects of somatostatin in these preparations. Results from studies in which SOMA 10
was infused into the isolated perfused stomach, and pancreatic studies were contrary to expected results, and further experiments need to be performed.

Better controls are needed for these experiments. As discussed earlier, purified ascites fluid from mice injected with myeloma cells could be used as control antibodies. Recirculation studies, similar to those performed in the stomach, need to be performed in the pancreas. Passive immunization studies in static incubated islets should also be performed, since previous immunoneutralization studies using this preparation have consistently been successful.

To access the binding of SOMA 10 to somatostatin in the perfused organs, SOMA 10 could be infused first to bind endogenous somatostatin and to saturate the system with SOMA 10. The system could then be titrated with somatostatin.

To test the hypothesis that SOMA 10 binds to the somatostatin receptor mimicking the effect of somatostatin, an anti-idiotypic antibody to SOMA 10 could be infused in similar gastric and pancreatic experiments. If an increase in insulin release occurs, this would support the hypothesis.

Somatostatin appears to have more influence on glucagon release than on insulin release (Itoh et al., 1980; Honey et al., 1981), partially because of anatomical considerations and because of greater A than B cell sensitivity to somatostatin (Mandarino et al., 1981). Future studies should investigate the effect of SOMA 10 on glucagon release. Future studies should also include the infusion of other SOMA antibodies, since SOMA 10 has a higher dissociation constant than the pancreatic somatostatin receptor and the low affinity gastric somatostatin receptor. SOMA 3, 8, and 20 have a lower dissociation constant than these receptors, so somatostatin should preferentially bind to the antibody and not the receptor.
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