

**RELEASE AND METABOLISM OF GASTRIC INHIBITORY  
POLYPEPTIDE**

by

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## ABSTRACT

This thesis reports methodology that was developed to isolate and enrich intestinal endocrine cell preparations by centrifugal elutriation and short-term culture to enable the study of the regulation of IRGIP secretion at the cellular level. Adherent canine epithelial cell cultures contained ~10% IRGIP cells. The release of IRGIP from these cells was significantly increased by incubation with depolarizing concentrations of  $K^+$ , glucose, somatostatin immunoneutralizing antibody, or GRP, in addition to pharmacological elevations of intracellular cAMP or  $Ca^{2+}$ . It is concluded that this method provides a means of further investigating the cellular mechanisms controlling GIP release.

Enteroendocrine tumor cell lines were also investigated as an alternate source of GIP cells. A cell line derived from intestinal tumors of transgenic mice (STC-1) was subcloned to produce a cell line with ~30% IRGIP (STC 6-14). HPLC of STC 6-14 extracts indicated that the tumor cell derived IRGIP eluted with synthetic porcine GIP 1-42. Release of IRGIP from STC 6-14 cells; increased in a concentration dependent fashion in response to glucose, was augmented by the addition of somatostatin neutralizing antibody, and attenuated by exogenous somatostatin. Immunoreactive somatostatin (IRSS) release was significantly increased by adding GIP to the incubation medium. It is concluded that this cell line represents a means of rapidly obtaining large numbers of GIP cells, and thus should be useful to investigate stimulus-secretion coupling in the GIP cell.

GIP secreted by STC 6-14 cells was metabolized by a serum constituent to biologically inactive GIP 3-42.  $^{125}I$ -GIP was purified by HPLC and used as a highly sensitive means to further investigate the degradation of GIP by serum. The removal of the N-terminal dipeptide by serum could be blocked by diprotin A, a competitive inhibitor of dipeptidyl peptidase IV (DPP IV). No GIP 3-42 was produced by incubation of GIP with serum from rats specifically lacking DPP IV. Infusion of  $^{125}I$ -GIP into rats, followed by HPLC analysis, indicated that 50% was metabolized to  $^{125}I$ -GIP 3-42 by ~1.5 min. It is concluded that DPP IV is a primary degradative and inactivating enzyme of GIP.

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## CHAPTER 1

### INTRODUCTION

#### 1.1 OVERVIEW

In 1902, Bayliss and Starling noted that water and bicarbonate secretion from the pancreas increased when weak acid was introduced into the duodenum, but not when administered intravenously. Furthermore, the intravenous injection of an acid extract of upper intestinal mucosa resulted in copious secretion from the pancreas. Thus, the presence of "secretin" was proposed. Bayliss and Starling also searched for a general name that would convey the meaning of "chemical messenger". It was W. B. Hardy that proposed the name "hormone" derived from the Greek word for "I arouse to activity", and although it did not suggest the property of messenger, it was adopted. The word first appeared in print in Starling's Croonian Lecture of 1905.

The original concept - that a chemical substance called a hormone, liberated by one kind of cell, and carried by the blood stream to act on a distant target cell - represented a major advance in physiological thinking. Hormones are principal players in the maintenance of physiological homeostasis, as perturbation of this equilibrium leads to their appropriate corrective secretion. These responses are monitored and feedback loops exist to inhibit further stimulation of hormone release in a regulatory fashion. To be effective, this feedback must be accompanied by the clearance of previously secreted hormone from the circulation through enzymatic inactivation and/or removal of the substance by the liver or kidneys.

Investigation of a hormone first requires isolation and identification of the active principle in order that the biological response be reproduced by administration of exogenous hormone. The demonstration of an endocrine-mediated biological response then allows examination of other components of the system. Important physiological questions are left outstanding without knowledge of stimulus-secretion coupling,

circulating hormone levels, and duration of action and metabolism in plasma. The goal of these investigations was to further understand the secretion and metabolism of the hormone GIP (Gastric Inhibitory Polypeptide).

## **1.2 DISCOVERY OF GIP AS AN ENTEROGASTRONE**

### ***1.2.1 ENTEROGASTRONE CONCEPT***

It was first reported in 1886 by Ewald and Boas that the addition of olive oil to a test meal produced an inhibition of gastric secretion in human subjects. In 1910, Pavlov demonstrated that fat added to a meal fed to dogs inhibited the secretion of acid and pepsin. In 1926, Farrell and Ivy made the observation that the acid response to a meal could be inhibited by injection of extracts of the duodenal mucosa and suggested that a hormonal mechanism must be involved. Kosaka and Lim (1930) proposed the term "enterogastrone" to describe a putative hormone which was secreted in response to fat or its digestive products in the intestinal lumen and inhibited gastric acid secretion.

### ***1.2.2 EVIDENCE FOR EXISTENCE OF GIP***

Two candidate intestinal hormones apparently satisfying the requirements for enterogastrone were secretin (Bayliss and Starling, 1902) which was isolated for its ability to stimulate pancreatic secretion, and cholecystokinin (CCK; Ivy and Oldberg, 1927), a potent stimulator of gall bladder contraction. Impure preparations of both were found to have acid inhibitory activity in the vagally denervated (Heidenhain) canine gastric pouch (Gillespie and Grossman, 1964). However, in apparent conflict with the described acid inhibitory effects of CCK, Magee and Nakamura (1966) observed that CCK preparations were capable of stimulating acid secretion under fasting conditions when administered intravenously. Brown and Pederson (1970a) tested the hypothesis that the gastric effects resulted from the actions of factors other than CCK present in the impure preparations. The acid secretory effects of two different preparations of CCK

designated 10% and 40% pure on the basis of gallbladder stimulating potency were compared in dogs prepared with Heidenhain pouches. The 40% pure preparation produced a greater stimulatory effect on acid secretion than the 10% pure preparation at doses that yielded comparable gallbladder activity. It was proposed that either a gastric stimulant had been concentrated or an inhibitor of acid secretion had been removed during the purification procedure. Support for the latter was provided in 1971 (Pederson) when it was demonstrated that the 10% pure preparation of CCK was a more potent inhibitor of pentagastrin stimulated acid secretion than the 40% pure preparation.

### *1.2.3 ISOLATION OF GIP*

The canine Heidenhain pouch was used as a bioassay model for the isolation of the putative inhibitor of acid secretion. Brown *et al.* (1969, 1970) purified the active substance from extracts of hog duodeno-jejunal mucosa to a degree of homogeneity suitable for amino acid analysis to be performed, and named it gastric inhibitory polypeptide, or GIP (Brown, 1971; Brown and Dryburgh, 1971). Pure GIP was shown to be a potent inhibitor of gastric acid and pepsin secretion in the dog (Pederson and Brown, 1972). The amino acid sequence of the porcine peptide, as determined by Brown and Dryburgh (1971) and later corrected by Jörnvall *et al.* (1981) is shown in Figure 1, along with that of human, bovine, and rat GIP. The high degree of conservation of the peptide among species suggests that GIP has an important physiological role.

---

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
PORCINE:	Tyr	Ala	Glu	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser	Ile	Ala	Met
HUMAN:	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BOVINE:	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RODENT:	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
PORCINE:	Asp	Lys	Ile	Arg	Gln	Gln	Asp	Phe	Val	Asn	Trp	Leu	Leu	Ala
HUMAN:	.	.	.	-His-	.	.	.	.	.	.	.	.	.	.
BOVINE:	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RODENT:	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	29	30	31	32	33	34	35	36	37	38	39	40	41	42
PORCINE:	Gln	Lys	Gly	Lys	Lys	Ser	Asp	Trp	Lys	His	Asn	Ile	Thr	Gln
HUMAN:	.	.	.	.	.	-Asn-	.	.	.	.	.	.	.	.
BOVINE:	.	.	.	.	.	.	.	.	-Ile-	.	.	.	.	.
RODENT:	.	.	.	.	.	-Asn-	.	.	.	.	.	-Leu-	.	.

---

**FIGURE 1:** Amino acid sequences of porcine, human, bovine, and rodent gastric inhibitory polypeptide (GIP). From Jörnvall *et al.*, 1981 (porcine); Moody *et al.*, 1984 (human); Carlquist *et al.*, 1984 (bovine) and Sharma *et al.*, 1992 (rat).

#### 1.2.4 GLUCAGON SUPERFAMILY

The sequence of GIP, as well as the structure of its precursor peptide (Takeda *et al.*, 1987) and the gene encoding it (Inagaki *et al.*, 1989) indicate that GIP belongs to the glucagon superfamily of peptides, which share sequence homologies. This family includes glucagon, glicentin, the glucagon-like peptides (GLP-I and GLP-II), secretin, vasoactive intestinal peptide (VIP), peptide histidine-methionine (PHM), peptide histidine-isoleucine (PHI), peptide histidine-valine (PHV), growth hormone releasing factor (GRF), pituitary adenylate cyclase activating peptides (PACAP-38 and PACAP-27), helodermin, helospectins (I and II), extendins (3 and 4), and somatoliberin (Arimura, 1992; Eng *et al.*, 1990; Parker *et al.*, 1984; Raufman *et al.*, 1991; Rai *et al.*, 1993; Robberecht *et al.*, 1991). It has been suggested that these hormones probably arose from a common ancestral gene (Bell, 1986; Campbell and Scanes, 1992).

### 1.3 ENTEROGASTRONE ACTIONS OF GIP

#### 1.3.1 EFFECT OF GIP ON GASTRIC SECRETION

Dogs surgically prepared with Heidenhain pouches of the body of the stomach were used to determine the acid inhibitory action of GIP and served as a bioassay for its isolation. Using this vagally and sympathetically denervated gastric pouch preparation, GIP dose-dependently inhibited pentagastrin-stimulated acid secretion, with the highest dose able to produce ~80% inhibition (Pederson and Brown, 1972). However, in the vagally innervated gastric remnant, the same authors observed only ~40% inhibition of acid secretion induced by insulin hypoglycemia. Subsequent studies in man (Maxwell *et al.*, 1980) and rat (El-Munshid *et al.*, 1980) also found GIP to be a weak inhibitor of pentagastrin-stimulated acid secretion when innervation was intact, and it was speculated that GIP may not be physiologically important as an enterogastrone.

In a comparative study in the dog stomach, Soon-Shiong *et al.* (1979) reproduced the profound inhibition of pentagastrin-stimulated acid secretion observed earlier in the Heidenhain pouch but also found GIP had only weak effects on the innervated gastric remnant. Yamagishi and Debas (1980) noted that if oleic acid was introduced into the duodenum, a GIP infusion was able to completely inhibit acid secretion in the innervated dog stomach in response to a meal. The possible involvement of a cholinergic mechanism antagonistic to the action of GIP on the stomach was suggested by the observation that the acid inhibitory effect of this hormone in the denervated gastric pouch of the dog could be blocked by the intravenous infusion of urecholine (Soon-Shiong *et al.*, 1979). It therefore seemed possible that GIP exerted its inhibitory effect on the parietal cell indirectly, via the release of an inhibitor under cholinergic control.

#### 1.3.2 MECHANISM OF GASTRIC ACTION OF GIP

In 1981, McIntosh *et al.* suggested that gastric somatostatin might mediate the acid inhibitory action of GIP. In testing this hypothesis, they found a strong stimulation

of immunoreactive somatostatin (IRSS) release from the perfused stomach by GIP. In addition, both acetylcholine and vagal stimulation potently inhibited the somatostatinotropic activity of GIP. These data accounted for the observation of weak acid inhibitory activity of GIP in the innervated stomach. McIntosh *et al.* (1981) proposed a model whereby GIP released by the presence of fat in the duodenum stimulated somatostatin secretion upon reaching the stomach. However, the cholinergically or neurally mediated inhibition of GIP-stimulated IRSS secretion in the rat was only partially blocked by atropine (McIntosh *et al.*, 1979). Naloxone partially reversed the vagally induced inhibition of GIP-stimulated IRSS release, suggesting that opioid peptides were released and also inhibited IRSS release during the period of vagal stimulation (McIntosh *et al.*, 1983). Subsequent studies have demonstrated that noncholinergic transmitters, including the opioid peptides (enkephalins, dynorphins) and the tachykinins (substance P, neurokinin A), also inhibit somatostatin secretion and reverse the actions of GIP on the stomach (Kwok *et al.*, 1988a; McIntosh *et al.*, 1989; 1990).

In order for GIP to act as a physiological inhibitor of acid secretion, a mechanism capable of antagonizing the parasympathetic influence would be required. McIntosh *et al.* (1981) suggested that in addition to stimulating GIP secretion, fat in the duodenum may activate sympathetic fibres which provide such control. Evidence for this hypothesis was supplied by the observation that catecholamines stimulate gastric IRSS release in the perfused stomach (McIntosh *et al.*, 1981; Goto *et al.*, 1981; Koop *et al.*, 1981). McIntosh *et al.* (1981) also observed a marked increase in IRSS with stimulation of preganglionic sympathetic nerves together with simultaneous infusion of atropine. Alternatively, Brown *et al.* (1989) suggested that the inhibitory effect of GIP may be most profound when the parasympathetic activity of the stomach is minimal, such as the interdigestive period, when GIP levels have been shown to remain elevated (Jorde *et al.*, 1980; Salera *et*

*al.*, 1983). While the mechanism of action is still not completely understood, GIP appears to fulfill the requirements of an enterogastrone.

### 1.3.3 INTERACTION WITH OTHER ENTEROGASTRONES

Sequencing of the proglucagon gene by two separate groups (Bell *et al.*, 1983; Lopez *et al.*, 1983) revealed that the precursor peptide contained, in addition to glucagon and glicentin, the sequence of two glucagon-like peptides (GLP-I and GLP-II). Mojsov *et al.* (1986) showed that post-translational processing of the prohormone yielded a C-terminal 30 residue form of GLP-I, with amino acids 7-36-NH<sub>2</sub> (tGLP-I). As a member of the glucagon superfamily (section 1.2.4), tGLP-I has a high degree of homology with GIP, sharing 11 amino acids. Like GIP, tGLP-I has also been shown to inhibit pentagastrin and meal stimulated acid secretion in man (Schjoldager *et al.*, 1989; O'Halloran *et al.*, 1990; Wettergren *et al.*, 1993). In a seeming paradox, tGLP-I stimulated H<sup>+</sup> production in an enriched preparation of isolated rat parietal cells (Schmidler *et al.*, 1991). It was suggested that *in vivo*, direct stimulation by tGLP-I of the parietal cells might be counterbalanced by indirect inhibitory mechanisms that are excluded in the *in vitro* cell system. Like GIP, tGLP-I may act indirectly via somatostatin release, as tGLP-I has been reported to stimulate somatostatin release in the isolated perfused rat stomach (Eissele *et al.*, 1990). It is quite likely, therefore, that GIP fulfills the enterogastrone role by acting in concert with other peptides, such as CCK, secretin, and tGLP-I, in addition to nervous inhibitory mechanisms that may be initiated during the digestive and absorptive process in the upper small bowel.

Nauck *et al.* (1992) recently questioned whether GIP and tGLP-I were in fact physiological enterogastrones in humans. These investigators examined the effects of intravenous human GIP and/or tGLP-I on pentagastrin-stimulated gastric volume and acid output. Pentagastrin significantly stimulated acid output, but neither GIP nor tGLP-I, either alone or in combination, reduced pentagastrin-stimulated gastric acid secretion.

It was therefore concluded that these hormones were not likely enterogastrones in man. However, these authors did not acknowledge the data from the previous decade which clearly indicated that the enterogastrone effects of these hormones are likely mediated by gastric somatostatin, which is also under neural control. Thus, in the absence of intraduodenal stimuli (i.e. not a physiologically relevant design), there may be no sympathetic activity to antagonize the parasympathetic inhibition of somatostatin release, and thus no reduction in gastric acid secretion (see section 1.3.2). Thus, to date, the role of GIP as an enterogastrone in man is unresolved.

## 1.4 DISCOVERY OF GIP AS AN INCRETIN

### 1.4.1 INCRETIN CONCEPT

In 1902, Bayliss and Starling speculated that signals arising in the gut after ingestion of nutrients may elicit endocrine responses and affect the disposal of carbohydrates, as well as stimulating pancreatic bicarbonate secretion. This came as a result of their discovery of secretin as a regulator of pancreatic secretion. Moore *et al.* (1906) postulated that the duodenum produced a "chemical excitant" for pancreatic secretion, the absence of which caused diabetes. Their attempted treatment of diabetes mellitus by injection of gut extracts was unsuccessful. Zunz and Labarre (1929) and Labarre and Still (1930) prepared an intestinal extract free of secretin activity which was able to produce hypoglycemia in dogs. Thus Labarre (1932) introduced the term "incretin" to describe humoral activity of the gut that might enhance the endocrine secretion of the pancreas.

Interest in the search for the active principle in the duodenum waned with the isolation of insulin, allowing the effective control of blood glucose in diabetes mellitus (Banting *et al.*, 1922). Also, studies in 1940 by Loew *et al.* questioned the existence of gastrointestinal factors with insulin-releasing potency. It was not until the development of a reliable radioimmunoassay for insulin in the 1960's by Berson and Yalow, allowing

measurement of circulating levels of this hormone, that interest was renewed. It became apparent that the direct effect of nutrients and their metabolites on the islets could not adequately account for the amount of insulin released. Absorption of nutrients from the gut seemed to be accompanied by the release of additional factors. The insulin response to intravenous glucose was much smaller than to either oral glucose (Elrick *et al.*, 1964) or intrajejunal infusion (McIntyre *et al.*, 1964), even though there was a greater increase in blood glucose levels with intravenous administration. It was concluded from these observations that a humoral substance was released from the jejunum during glucose absorption which acted to stimulate insulin release. Perly and Kipnis (1967) estimated that as much as half of the insulin secreted following an oral glucose load was a result of gastrointestinal factors.

#### *1.4.2 ENTEROINSULAR AXIS*

In 1969, Unger and Eisentraut named the connection between the gut and the pancreatic islets described above the "**enteroinsular axis**". While Labarre had noted an incretin or hormonal effect from the gut on insulin release (1932), he had provided earlier evidence that insulin could be released by vagal stimulation (Labarre, 1927). It became apparent that the gastrointestinal neural system might participate in the enteroinsular axis not only by direct stimulation of the islets, but also by regulating the release of an incretin into the blood. As it was clear that the pancreas played a major role in maintaining circulating glucose levels, it also seemed logical that nutrients, like glucose, were able to act directly on islets to control hormone release. Creutzfeldt (1979) therefore suggested that the enteroinsular axis encompass nutrient, neural and hormonal signals from the gut to the islet cells secreting insulin, glucagon, somatostatin, or pancreatic polypeptide. Furthermore, Creutzfeldt (1979) defined the criteria for fulfillment of the hormonal or incretin part of the enteroinsular axis as: 1) it must be released by nutrients, particularly

carbohydrates, and 2) at physiological levels, must stimulate insulin secretion in the presence of elevated blood glucose levels.

### 1.4.3 EVIDENCE FOR GIP AS AN INCRETIN

In 1966, Dupré and Beck demonstrated that a crude preparation of CCK possessed insulinotropic activity. In 1972, Rabinovitch and Dupré found that this insulinotropic action could be removed by further purification of the CCK. This resembled the loss of the acid inhibitory activity previously reported by Brown and Pederson (1970a) in the purification of GIP from CCK, and led Dupré to the hypothesis that GIP may have insulin-releasing capabilities. By 1973, Dupré *et al.* demonstrated that a purified preparation of GIP infused intravenously in humans in concert with glucose, resulted in the stimulation of insulin release and an improvement in glucose tolerance. The insulin response was sustained for the duration of the GIP infusion and was not observed in the euglycemic state.

While no specific GIP antagonists have been developed to further establish the physiological contribution of GIP to the postprandial insulin response, antisera to GIP have been produced which may suppress its biological activity. One of the first attempts at GIP immunoneutralization study was by Lauritsen *et al.* (1981). Insulin release was measured in rats given an oral glucose tolerance test (OGTT) and compared to an OGTT plus infusion of GIP antisera via the jugular vein. They found that the insulin release relative to the glycemic stimulus (the insulinogenic index) was significantly depressed by anti-GIP serum and was comparable to that observed in rats which received intravenous glucose. The GIP antiserum completely suppressed the incretin effect of the oral glucose, suggesting GIP to be the only incretin. However, the GIP antiserum used in these studies was prepared using a 40% pure GIP preparation which was derived from porcine CCK extracts. It is therefore probable that the antisera preparation also bound other potential incretin factors contained in the impure gut extract.

Ebert and coworkers (1979a) tested the effect of intravenous GIP antisera raised to a much purer form of GIP on intestinal stimuli for IRGIP release in rats. During intraduodenal acid administration along with intravenous glucose infusion, GIP antibodies strongly inhibited the initial increase in insulin levels, although after 20-30 min, circulating insulin levels were the same as in control rats. Similarly, in 1982, Ebert and Creutzfeldt found the incretin effect of GIP was strongest immediately after an intraduodenal glucose load, and the GIP antisera did not completely block the incretin effect. It was therefore concluded from these studies that GIP was not the exclusive incretin and that additional gut factors with insulinotropic activity existed. Since the nervous system also influences glucose-induced insulin secretion following an oral or intraduodenal glucose load (Porte *et al.*, 1973), the preserved incretin effect after GIP antiserum injection could be related to the neural part of the enteroinsular axis. To circumvent potential neural effects, Ebert *et al.* (1983) examined the insulinotropic potency of differently prepared gut extracts. They found that intravenous rat gut extracts exerted insulinotropic activity even after removal of GIP by immunoadsorption. Approximately 50% of the incretin activity of the gut extracts remained suggesting the existence of other hormonal gut factors with insulinotropic activity.

## **1.5 INSULINOTROPIC ACTIONS OF GIP**

### ***1.5.1 EFFECT OF GIP ON INSULIN SECRETION***

The observation by Dupré *et al.* (1973) that GIP was not insulinotropic under euglycemic conditions suggested glucose played an important role in regulating this action of GIP. It was also established that the insulinotropic action of GIP was glucose-concentration dependent *in vivo* in dog (Pederson *et al.*, 1975b), man (Elahi *et al.*, 1979) and in the perfused rat pancreas (Pederson and Brown, 1976) where a glucose threshold of approximately 5.5 mM for the insulinotropic action of GIP was observed. This prompted Brown and Pederson (1976a) to suggest that GIP be given the alternate

designation Glucose-dependent Insulinotropic Polypeptide. Thus while ingestion of fat has been shown to be a potent stimulus for IRGIP release (see section 1.10.3), no increase in insulin was observed unless intravenous glucose was administered as well (Brown, 1974; Cleator and Gourlay, 1975; Ross and Dupré, 1978). There have even been reports that GIP may attenuate the stimulation of insulin secretion under euglycemic conditions (Opara and Go, 1993). These observations demonstrate that the glucose-dependency of GIP-stimulated insulin secretion provides an important safeguard against hypoglycemia by preventing the inappropriate stimulation of insulin release during a high fat, low carbohydrate meal.

Not only was it shown that the insulinotropic action of GIP depended on the presence of a threshold glucose concentration, but it also appeared that glucose could potentiate the action of GIP on the  $\beta$ -cell. At a fixed GIP concentration, increased glucose concentrations stimulated insulin secretion in more than an additive manner (Pederson and Brown, 1976). In the perfused rat pancreas, the maximum potentiating action of glucose on GIP-stimulated insulin release was observed at approximately 16 mM (Brown, 1982). In the presence of 17.8 mM glucose, when GIP was delivered to the rat pancreas in a linear gradient from 0 to 200 pM, the insulinotropic effect of GIP was initiated at concentrations as low as 70 pM (Pederson *et al.*, 1982), concentrations within the physiological range (see section 1.9.2). It was also noted that the effect of GIP on insulin release from the perfused rat pancreas was dose-dependent (Pederson and Brown, 1976).

### *1.5.2 OTHER POTENTIAL INCRETINS*

Several other gastrointestinal hormones have been investigated as potential incretins involved in the enteroinsular axis, including gastrin, secretin, CCK, and tGLP-I (Creutzfeldt and Ebert, 1988; Brown, 1988). Kreymann *et al.* (1987) showed that tGLP-I was present in the small intestine of man, and observed a sustained increase in plasma

levels following an oral glucose load or ingestion of a mixed meal. At postprandial concentrations, Mojsov *et al.* (1987) showed potent insulinotropic actions of tGLP-I in the perfused rat pancreas. In humans, tGLP-I infusion at physiological concentrations in the presence of an intravenous glucose load significantly enhanced insulin release and significantly reduced peak plasma glucose concentrations. Furthermore, like GIP, the effectiveness of tGLP-I has been shown to be glucose-dependent (Weir *et al.*, 1989). It therefore appears that tGLP-I also fulfills the criteria of an incretin.

### 1.5.3 INTERACTIONS BETWEEN INSULIN SECRETAGOGUES

Rasmussen and his colleagues have suggested that interactions between various insulin secretagogues occur at the level of signal transduction with the  $\beta$ -cell (Zawalich and Rasmussen, 1990; Rasmussen *et al.*, 1990). Thus, agonists that act via distinct intracellular pathways tend to potentiate the action of each other on the  $\beta$ -cell, while those that act via the same intracellular pathway tend to have additive stimulatory effects. Evidence for this hypothesis is the observation of potentiating interactions between GIP and acetylcholine (Verchere *et al.*, 1991) as well as CCK (Sandberg *et al.*, 1988) in the perfused rat pancreas. GIP appears to act by stimulating cAMP production in the  $\beta$ -cell (see section 1.5.6), while acetylcholine and CCK act via the phosphoinositide pathway and mobilization of intracellular calcium (Prentki and Matschinsky, 1987). On the other hand, activators of cAMP, such as tGLP-I tend to exert additive effects with GIP on insulin release (Fehmann *et al.*, 1989).

In addition to interacting at the  $\beta$ -cell with other hormones and neurotransmitters, GIP stimulated insulin release is subject to modulation by nutrients such as arginine. In 1978, Pederson and Brown demonstrated that in the presence of a glucose concentration below the threshold for GIP-stimulated insulin release (2.7 mM), arginine (5 or 10 mM) was able to potentiate the insulinotropic action of GIP. At higher concentrations (20 mM) arginine attenuated the insulinotropic effect of GIP, suggesting that GIP and

arginine acted on the  $\beta$ -cell via a similar mechanism (Pederson and Brown, 1978). Support for this hypothesis has come from the observation of similar interactions of arginine and GIP on insulin release *in vivo* in man (Elahi *et al.*, 1982).

#### 1.5.4 RELATIVE CONTRIBUTION OF GIP vs tGLP-I

There have been numerous studies aimed at determining the relative importance of GIP and tGLP-I in the enteroinsular axis. While some studies indicated both peptides were equally effective (Schmid *et al.*, 1990; Kieffer *et al.*, 1993), other dose-response analyses of the insulinotropic activity of GIP and tGLP-I indicated that lower plasma concentrations of tGLP-I are necessary to augment insulin secretion, particularly at elevated glucose concentrations (D'Alessio *et al.*, 1989; Holst *et al.*, 1987; Mojsov *et al.*, 1987; Shima *et al.*, 1988; Weir *et al.*, 1989; Nauck *et al.*, 1989; Krarup *et al.*, 1987c; Kreyman *et al.*, 1987). On the other hand, while tGLP-I appears more potent than GIP, the rise in IRGIP following oral glucose is greater in magnitude than the increment in plasma immunoreactive tGLP-I after a similar load (Nauck *et al.*, 1986; Salera *et al.*, 1983; Kreyman *et al.*, 1987; Ørskov and Holst, 1987; Ørskov *et al.*, 1991; Nauck *et al.*, 1993). Nauck *et al.* (1993) examined the incretin effect of GIP and/or tGLP-I infused at doses chosen to produce plasma levels roughly comparable to values measured after oral glucose in human subjects (~450 pM and ~50-60 pM respectively). It was observed that the doses used were sufficient to augment the  $\beta$ -cell response to intravenous glucose, to values not significantly different from those after oral glucose. It was also noted that GIP made a major contribution to the incretin effect after oral glucose, while tGLP-I appeared to mediate a smaller proportion. Nauck *et al.* (1993) concluded that GIP and tGLP-I together were sufficient to explain the full incretin effect after oral glucose in normal individuals. Fehmann *et al.* (1989) and Suzuki *et al.* (1992) noted synergistic stimulatory incretin effects of tGLP-I and GIP on the rat pancreas and concluded that the hormones act "in concert" to guarantee adequate insulin responses to a meal. Kreymann *et al.*

(1987), found that when human volunteers consumed a test breakfast, plasma IRGIP levels rose more rapidly than tGLP-I, implying that GIP mediated the early insulin response to a meal while tGLP-I was more important in the late response.

#### *1.5.5 PARTICIPATION OF GIP AND tGLP-I IN AN ENTEROENDOCRINE LOOP*

The observed differences in IRGIP and tGLP-I secretion rates have been interpreted differently by different investigators. The two insulinotropic peptides GIP and tGLP-I have distinct patterns of distribution in the intestine. GIP cells are located mainly in the upper small intestinal mucosa, found in highest concentrations in the duodenum and, to a much lesser extent, in the ileum and colon (see section 1.8). tGLP-I-producing L-cells, on the other hand, are located predominantly in the ileum and colon, with 10- to 40-fold lower concentrations of proglucagon-derived peptides in the duodenum (Eissele *et al.*, 1992). As nutrients rarely reach the ileum before postprandial insulin responses are observed, the role of tGLP-I as a physiological incretin acting from this location has been questioned. In 1991, Roberge and Brubaker made the interesting observation that intestinal proglucagon-derived peptide secretion was stimulated equally by fat in the duodenum as compared to fat in the ileum. It was concluded that duodenal fat either stimulates the enteric nervous system or the secretion of a factor that, in turn, stimulates the release of intestinal proglucagon-derived peptides, including tGLP-I. Brubaker (1991) also reported on the regulation of intestinal proglucagon-derived peptide secretion by intestinal regulatory peptides using a fetal rat intestinal culture model. It was observed that GIP stimulated intestinal proglucagon-derived peptide secretion at physiological concentrations and in a dose-dependent fashion. This prompted the group to hypothesize an 'enteroendocrine loop', whereby GIP might stimulate the release tGLP-I before arrival of nutrients in the ileum, allowing tGLP-I to act as an incretin.

Recently, Roberge and Brubaker (1993) investigated this hypothesis. They found, as did Kreyman *et al.* (1987), that the rise in plasma IRGIP levels in response to

duodenal nutrients occurred slightly before intestinal proglucagon-derived peptide levels, suggesting a relationship between the two peptides. Intravenous infusion of GIP at a dose sufficient to yield concentrations similar to those observed after duodenal fat administration induced a 2-fold increase in plasma levels of intestinal proglucagon-derived peptides that were independent of glycemic levels. No increment in intestinal proglucagon-derived peptides was found in response to infusion of CCK. It was therefore concluded that this enteroendocrine loop between the duodenal peptide GIP and the ileal proglucagon-derived peptides may account for some of the early rises in secretion of tGLP-I observed in response to nutrient ingestion. Nauck *et al.* (1993) however questioned this hypothesis when intravenous GIP infusions failed to reproduce an increase in tGLP-I levels mimicking levels attained after oral glucose in human subjects.

#### *1.5.6 MECHANISM OF ACTION OF GIP ON THE $\beta$ -CELL*

The mechanism by which GIP stimulates insulin release from the  $\beta$ -cell is not fully understood. This has largely been due to the lack of homogenous preparations of  $\beta$ -cells, which have only recently become available. Using a hamster pancreatic  $\beta$ -cell line (In III), Amiranoff *et al.* (1984) showed that GIP produced a concentration-dependent increase in cAMP content in the cells which paralleled insulin release. The idea that GIP acted via stimulation of  $\beta$ -cell G-protein coupled adenylate cyclase was later substantiated by studies with cultured rat islets (Siegle and Creutzfeldt, 1985), the rat insulinoma cell line RINm5F (Gallwitz *et al.*, 1993), and human insulinoma tissue *in vitro* (Maletti *et al.*, 1987). The closely related peptide tGLP-I was also shown to increase cAMP levels in a rat islet cell line (Drucker *et al.*, 1987; Göke and Conlon, 1988; Gallwitz *et al.*, 1993). An alternative pathway for the augmentation of insulin secretion by GIP was suggested by Lardinois *et al.* (1990). Using neonatal rat islet cell cultures, they observed that GIP-stimulated insulin release could be suppressed by

inhibitors of the membrane associated enzyme phospholipase A2 and intracellular lipoygenase and cyclooxygenase. It was concluded that GIP exerted its influence in part by modulating membrane associated phospholipase A2 activity, and that the formation of intracellular lipoygenase products appeared to be a pivotal step in the insulinotropic action of GIP.

More recently, Wahl *et al.* (1992) investigated the potential for a role of ionic fluxes in pancreatic beta cells as a target for the action of GIP. Using mouse pancreatic islets, it was observed that the amplification of insulin release by GIP did not occur in the absence of  $\text{Ca}^{2+}$  in the extracellular medium, and did not involve changes in the cellular content of inositol triphosphate. It was therefore concluded that the amplifying effect of GIP on insulin release was due to an effect on  $\text{Ca}^{2+}$  uptake, and subsequent increase in electrical activity. It was also pointed out that an additional contribution of the other islet cell types ( $\alpha$  and  $\delta$ ) should also be taken into account, although the mouse islet contains more than 80%  $\beta$ -cells. A potential role for glucagon is suggested by the fact that GIP has been shown to increase glucagon release (Szecowka *et al.*, 1982b), and the observation by Pipeleers *et al.* (1985) that glucose-induced insulin release from isolated  $\beta$ -cells depended on the cAMP levels, which were markedly increased after addition of  $(\text{Bu})_2\text{cAMP}$ , glucagon, or pancreatic  $\alpha$ -cells.

Using a hamster  $\beta$ -cell line (HIT T15) Lu *et al.* (1993) found that both tGLP-I- and GIP-stimulated increases in cAMP resulted in increased extracellular  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels. Neither peptide altered phosphoinositide metabolism, further underlining the fact that the mobilization of intracellular  $\text{Ca}^{2+}$  from endoplasmic reticulum is not involved in the GIP and tGLP-I signal transduction pathways. Yada *et al.* (1993) also observed that tGLP-I potentiated rises in cAMP and resultant increases in the cytosolic free  $\text{Ca}^{2+}$  concentration in size selected rat pancreatic  $\beta$ -cells. It was determined that the  $\text{Ca}^{2+}$  influx resulted from an enhanced activity of L-type  $\text{Ca}^{2+}$  channels in the  $\beta$ -cell plasma membrane.

### 1.5.7 NATURE OF GLUCOSE-DEPENDENCY

The glucose-dependency of the insulinotropic action of GIP has been well-documented in rat, dog, man (Dupré *et al.*, 1973; Pederson *et al.*, 1975b; Elahi *et al.*, 1979; Pederson and Brown, 1976; Zawalich *et al.*, 1993, Siegel *et al.*, 1992), and in  $\beta$ -cell lines (Kieffer *et al.*, 1993; Lu *et al.*, 1993). Other insulin secretagogues such as CCK (Verspohl and Ammon, 1987; Zawalich *et al.*, 1987), acetylcholine (Garcia *et al.*, 1988; Verchere *et al.*, 1991) and tGLP-I (Weir *et al.*, 1989; Komatsu *et al.*, 1989) have also exhibited glucose-dependency. The mechanism by which GIP and other insulinotropic agents are glucose-dependent is not fully understood. D-glyceraldehyde, an intermediate of the glycolytic pathway, was able to potentiate the insulinotropic action of GIP in the absence of glucose from the perfused rat pancreas (Brown, *et al.* 1981). Mannoheptulose, which blocks glycolysis, abolished GIP-stimulated insulin secretion in the same preparation (Müller *et al.*, 1982). These studies strongly suggest that glucose metabolism is a prerequisite for GIP-stimulated insulin release to occur.

Rasmussen *et al.* (1990) proposed that GIP-induced increases in cAMP content leading to the conversion of latent to operative  $\text{Ca}^{2+}$  channels are ineffective in producing sufficient  $\text{Ca}^{2+}$  influx for insulin release unless the  $\beta$ -cell is partially depolarized by the actions of glucose. Postprandial changes in plasma glucose concentrations are thus suggested to primarily serve as conditional modifiers of insulin secretion. Calmodulin, activated by glucose-induced rises in intracellular  $\text{Ca}^{2+}$ , has been shown to stimulate adenylate cyclase activity in rat islets (Sharp *et al.*, 1980) and was proposed by Holz and Habener (1992) to be required for tGLP-I stimulated insulin release. Although this may also explain the glucose-dependency of GIP,  $\text{Ca}^{2+}$ -calmodulin does not always appear to activate adenylate cyclase activity in islets (Thams *et al.*, 1982). Furthermore, transgenic mice with elevated levels of  $\beta$ -cell calmodulin develop severe diabetes, even though their pancreatic  $\beta$ -cells contain reserve levels of insulin (Epstein *et al.*, 1992).

Another possible mechanism by which glucose may sensitize the  $\beta$ -cell to further stimulation by insulinotropic agents is through the expression of functional hormone receptors. The transport of glucose, and/or its metabolism resulting in the production of various intracellular messengers could be involved in the expression or allosteric activation of receptors for insulinotropic hormones such as GIP and tGLP-I. Thus in the absence of glucose, a reduction in the expression of functional hormone receptors would be expected and it would therefore be predicted that GIP or tGLP-I would not alter cAMP levels; an observation that was recently made using a hamster  $\beta$ -cell line (Lu *et al.*, 1993). If in fact glucose transport is necessary for 'correct' GIP receptor expression, then in cases where glucose transport is altered, a corresponding alteration in the insulinotropic action of GIP would be expected. Reduced expression of the  $\beta$ -cell high  $K_m$  glucose transporters (GLUT2) has been demonstrated in the non-insulin-dependent diabetes mellitus model, the obese Zucker rat (Johnson *et al.*, 1990; Orci *et al.*, 1990). This observation has also been observed in transformed  $\beta$ -cells, combined with the presence of a glucose transporter isoform with a lower  $K_m$  for glucose (GLUT1; Brant *et al.*, 1992) and alterations in the expression of various hexokinases (Visher *et al.*, 1987). In both the obese Zucker rat and the transformed  $\beta$ -cell line,  $\beta$ TC3, a significant reduction in the glucose threshold for GIP-stimulated insulin release has been observed (Chan *et al.*, 1984; Kieffer *et al.*, 1993). Examination of the hypothesis that altered GIP receptor expression may contribute to these observations may soon be possible with the recent cloning of the GIP receptor (Usdin *et al.*, 1993).

## 1.6 OTHER ACTIONS OF GIP

In addition to enterogastrone and insulinotropic actions, GIP has been suggested to have other functions, although whether they are physiological or not is uncertain. This list includes actions on gastrointestinal motility, mesenteric blood flow, intestinal secretion, and release of anterior pituitary hormones (Brown *et al.*, 1989). The recent

discovery of the GIP receptor in tissues outside the gut (brain; see section 1.11.2) suggests that there may be as yet undiscovered actions of this hormone. Potential anabolic actions of GIP are better documented. In the liver, GIP has been demonstrated to inhibit glucagon-induced lipolysis and diminish glucagon-stimulated hepatic glucose production (Hartmann *et al.*, 1986). GIP may also increase glucose uptake in adipocytes and other peripheral tissues such as muscle (Hauner *et al.*, 1988). These insulin-like actions are compatible with the insulinotropic effect of GIP in promoting glucose utilization. In adipose tissue, GIP has been shown to increase lipoprotein lipase activity, (Eckel *et al.*, 1979), inhibit glucagon-induced lipolysis (Dupré *et al.*, 1976), and potentiate the insulin-stimulated incorporation of fatty acids into triglycerides (Beck and Max, 1983). GIP has also recently been shown to stimulate fatty acid synthesis in adipose tissue (Oben *et al.*, 1991). A role for GIP in the control of fat metabolism is not surprising considering the potent stimulatory effect of fat on IRGIP release, which in the absence of glucose, is not insulinotropic (Beck, 1989).

## **1.7 GIP GENE**

### ***1.7.1 GENE STRUCTURE AND POST-TRANSLATIONAL PROCESSING***

The sequence of the human GIP gene was reported by Takeda *et al.* in 1987. The cDNA sequence coding the human GIP precursor (Figure 2) indicates that human preproGIP is a protein of 153 amino acids, with a predicted molecular weight of 17,107 daltons. The sequence indicates that proteolytic processing at single arginine residues at either end of the GIP sequence would yield GIP 1-42 (Figure 2). The structural organization of human preproGIP was reported by this group to be: putative signal peptide (21 amino acids), NH<sub>2</sub>-terminal peptide (30 amino acids), GIP (42 amino acids) and COOH-terminal peptide, (60 amino acids). Subsequently, Inagaki *et al.* (1989) revealed that the human GIP gene consists of six exons separated by five introns, with exons 3 and 4 encoding mature human GIP. Recently, rat preproGIP was reported to be



### 1.7.2 GENE REGULATION

The mechanism of GIP gene regulation is not completely understood. The promoter region of the human GIP gene contains potential binding sites for multiple transcription factors, including Sp1, AP-1 and AP-2, but the roles of these sites are unknown (Inagaki *et al.*, 1989). More recently, this same group investigated whether GIP gene expression is regulated by cAMP and glucose. Two cAMP response element (CRE) binding protein (CRE-BP) binding sites were identified in the promoter region (Someya *et al.*, 1993). CRE is an inducible enhancer of genes which can be transcribed in response to increased cAMP levels (Comb *et al.*, 1986). CRE-BP1 can dimerize with the *c-jun* protooncogene product (c-Jun) (Macgregor *et al.*, 1990), and interestingly, c-Jun represses the cAMP-induced activity of the insulin promoter (Inagaki *et al.*, 1992). A possible mechanism of this c-Jun action is by its ability to inhibit the binding of some CRE-BP's, such as CRE-BP1, to the CREs of the insulin gene by formation of a heterodimer. These results, and another observation that the level of c-Jun is dramatically increased by glucose deprivation in hamster insulinoma (HIT T15) cells (Inagaki *et al.*, 1992), suggest that glucose may regulate expression of the human insulin gene through CREs and c-Jun. Mutation analysis showed that the two CREs are required for basal promoter activity. Interestingly, the GIP promoter activity was repressed by c-Jun like the human insulin promoter, possibly through the CREs.

### 1.8 GIP CELL DISTRIBUTION AND CHARACTERIZATION

Proof of the existence of endocrine cells in the gastrointestinal mucosa came with the use of electron microscopy by Solcia *et al.* (1967). Several morphologically distinct cell types were soon identified and correlated with the production of members of the expanding family of gut endocrine peptides. Immunoreactive GIP (IRGIP) cells have been located in the upper small intestine of ruminants (Bunnett and Harrison, 1986), man, pig, dog (Buffa *et al.*, 1975) and rats (Buchan *et al.*, 1982). In the gastrointestinal tract of

dog and man, IRGIP is present in cells predominantly in the mid-zone of the glands in the duodenum, and to a lesser extent in the jejunum (Polak *et al.*, 1973). Other studies have found a few IRGIP cells as far as the terminal ileum in rat (Buchan *et al.*, 1982) and man (Ferri *et al.*, 1983). IRGIP cells are part of the amine precursor uptake decarboxylation (APUD) series (Pearse, 1968). Ultrastructural studies of human IRGIP cells indicated a characteristic appearance of the K-cell; intracellular secretory granules having a small electron-dense core surrounded by a concentric electron-lucent halo (Buchan, 1978). In the dog, however, IRGIP cells identified in the duodenum contained uniformly electron-dense secretory granules consistent with the cell type recognized as the I-cell of the endocrine cell classification (Usellini *et al.*, 1984). Thus some species specificity exists regarding the ultrastructure of the GIP cell.

Some immunocytochemical studies indicated that GIP was co-localized with glucagon in the pancreatic  $\alpha$ -cells in mammals (Smith *et al.*, 1977; Alumets *et al.*, 1978; Ahrén *et al.*, 1981). Other studies indicated, however, that the staining of the pancreatic  $\alpha$ -cells could be blocked by preincubation of the antisera with glicentin (Larson and Moody, 1980) or glucagon (Buchan *et al.*, 1978), suggesting that these results were due to cross-reactivity of the GIP antisera used with glucagon or the glucagon-like peptides and their precursors. Furthermore, studies using RNA blot analysis have only detected the presence of human preproGIP mRNA in human intestine, and not in the pancreas (Takeda *et al.*, 1987; Inagaki *et al.*, 1989; Sharma *et al.*, 1992). It is interesting to note that IRGIP has been localized to a distinct cell type in the islet organ of elasmobranchs, although its function there is not known (Hazelwood, 1989). It appears that GIP cells are not found phylogenetically in the gut mucosa until a distinct islet organ evolves upon which the peptide may act, i.e. the hagfish, suggesting the possibility of a functional enteropancreatic axis at this evolutionary level (Falkmer *et al.*, 1980).

## 1.9 MEASUREMENT OF GIP

### 1.9.1 DEVELOPMENT OF GIP RADIOIMMUNOASSAY

With the purification of GIP came the possibility of development of techniques for measuring endogenous hormone secretion. This was a prerequisite for the establishment of GIP as a hormone, in order to demonstrate the polypeptide in blood and tissues and release from tissues by physiological mechanisms. The development of a radioimmunoassay (RIA) for GIP was first achieved in 1974 by Kuzio *et al.* Antisera were produced in guinea pigs by subcutaneous immunization with GIP emulsified with Freund's adjuvant, followed by boosting with GIP conjugated to bovine serum albumin using the carbodiimide method. Antisera were shown to have no measurable cross reactivity with natural secretin, synthetic glucagon, synthetic human gastrin, pure porcine CCK, pure porcine motilin, and pure porcine vasoactive intestinal peptide. GIP was labeled with  $^{125}\text{I}$  by a minor modification of the chloramine-T technique, and purified on a Sephadex G-15 column. The RIA developed had a sensitivity range of 25 to 250 pg, and Kuzio *et al.* (1974) reported a mean fasting serum concentration of  $237 \pm 14$  pg/ml and  $>1200$  pg/ml after a meal.

### 1.9.2 PHYSIOLOGICAL LEVELS OF GIP

Subsequent to the development of the first GIP RIA, other assays followed (Moody and Lauritsen, 1977; Morgan *et al.*, 1978; McLoughlin *et al.*, 1979; Ebert *et al.*, 1979b; Sarson *et al.*, 1980; Burhol *et al.*, 1980a; Wolfe and McGuigan, 1982; Jorde *et al.*, 1983; Sheu *et al.*, 1987; Wishart *et al.*, 1992; Moody *et al.*, 1992). There appeared to be a general agreement on the pattern of IRGIP release after ingestion of nutrients, whereas the absolute values measured differed widely, ranging from 9 to 80 pmol/l in the fasting state, and from 35 to 700 pmol/l after meals. In 1983, Jorde *et al.* attempted to resolve this disparity by measuring fasting and postprandial IRGIP values in man with seven different antisera, including two of their own. Even under the same assay conditions,

mean fasting IRGIP levels ranged from 12 to 92 pmol/l, and the mean postprandial IRGIP values ranged from 35 to 235 pmol/l. It was concluded that this variability was a result of different cross reactivities of the antisera with human GIP. Porcine GIP (two amino acids different) of varying purities had been used as standards and to raise antisera for RIAs used to measure IRGIP in man.

Amland *et al.* (1984) measured fasting and postprandial IRGIP values in pigs, rats, dogs and man with five different antisera and porcine GIP standards and tracers. The mean IRGIP values in rats, dogs, and man varied considerably, depending on the antiserum used, whereas all the antisera recorded fairly similar IRGIP values in pigs. These findings again demonstrated the species differences in immunological properties of GIP, and thus the importance of using GIP from the appropriate species as standards. The sequencing of human GIP and its successful production by both chemical synthesis (Yajima *et al.*, 1985; Fujii *et al.*, 1986) and recombinant DNA techniques (Chow *et al.*, 1990) should have allowed the proper evaluation of IRGIP concentrations in man. Oddly enough, only two groups at present appear to have used human GIP in assays employed for determining plasma IRGIP levels in man. Kreymann *et al.* (1987) used synthetic human GIP as standards for the measurement of IRGIP in human subjects in response to oral glucose and a test meal. Basal values were  $47 \pm 16$  (mean  $\pm$  SEM) and peaked at  $174 \pm 24$ , 30 min after receiving oral glucose. The material used for tracer production was not stated. Using synthetic human GIP for tracer and standards, Nauck *et al.* (1992) measured basal IRGIP levels in human subjects as  $69 \pm 18$  pM, and peak IRGIP concentrations after oral glucose of  $340 \pm 39$  pM. It is not surprising that these peak levels are higher than others previously reported in response to oral glucose.

### *1.9.3 IMMUNOREACTIVE FORMS OF GIP*

In 1975, Brown *et al.* reported that the antisera used in the RIA developed by Kuzio *et al.* (1974) identified more than one molecular form of IRGIP in plasma and gut

tissue. In fact, all seven of the antisera tested by Jorde *et al.* (1983) recognized three molecular forms of IRGIP to variable degrees, likely contributing to the marked discrepancies in reported IRGIP levels. When subjected to gel filtration, one high molecular weight component eluted in the void volume of the column, while the two other components eluted at positions corresponding to molecular weights of approximately 8000 and 5000 daltons (Brown *et al.*, 1975). The 5000 IRGIP form is the major component of GIP in both blood and tissues and has been used for the establishment of RIAs (see Figure 1 for sequence). After gel filtration of extracts of porcine and human small intestine, a fourth IRGIP form was found eluting between 8000 IRGIP and void volume (Krarup and Holst, 1984). This fourth form has never been detected in plasma. Krarup and Holst (1984) concluded that since all five antisera tested reacted with this IRGIP form it may represent a prohormone to 5000 IRGIP.

No void volume IRGIP ( $V_0$ -IRGIP) has been detectable after gel filtration of extracts of gut mucosa (Bacarese-Hamilton *et al.*, 1984; Krarup and Holst, 1984). This component could be removed from plasma by alcohol extraction, indicating its association with high molecular weight constituents of plasma (Krarup, 1988). This suggestion had been made by Dryburgh (1977), when it was noted that pre-treatment of serum by boiling or by addition of 6 M urea diminished  $V_0$ -IRGIP and increased 5000 IRGIP. In addition,  $V_0$ -IRGIP does not appear to contribute to the increase in IRGIP in response to glucose, fat, or a meal (Krarup *et al.*, 1985; Krarup *et al.*, 1987b). Krarup (1988) concluded that  $V_0$ -IRGIP may represent large molecular weight proteins interfering in the assays.

While 5000 IRGIP was shown to increase in plasma 60 min after intraduodenal glucose or fat had been given in man, 8000 IRGIP showed small and inconsistent changes after both stimuli (Krarup *et al.*, 1985; Krarup *et al.*, 1987b). It was concluded by Krarup (1988) that 8000 IRGIP contributed little to the increase in IRGIP after glucose and fat, and only modestly to that observed after a meal. A partially purified 8000 IRGIP from

porcine gut did not stimulate insulin release even when used at pharmacological doses in an isolated porcine pancreas system (Krarup *et al.*, 1987a). Otte *et al.* (1984) found that the entire sequence of GIP was not contained in an 8000 IRGIP form extracted from pig gut and concluded that it was not a proform of GIP. Since 8000 IRGIP cross reacts with most GIP antisera (Jorde *et al.*, 1983; Krarup, 1988) there must be a degree of homology between this form and 5000 IRGIP. It is possible, therefore, that 8000 IRGIP is derived from a GIP precursor.

## **1.10 GIP SECRETION**

### ***1.10.1 RESPONSE TO A MIXED MEAL***

It is generally agreed that IRGIP levels increase approximately six times above basal in response to ingestion of a mixed meal, although absolute values vary significantly (Kuzio *et al.*, 1974; Morgan *et al.*, 1978; Jorde *et al.*, 1980; Jorde *et al.*, 1983; Amland *et al.*, 1984). Plasma levels of IRGIP remained elevated for up to six hours after a meal, and thus during a 24 h period, with three meals ingested, IRGIP was significantly high all day (Jorde *et al.*, 1980; Salera *et al.*, 1983; Jones *et al.*, 1985). Beck *et al.* (1984) observed that ingestion of a meal with a higher caloric level yielded a significantly greater IRGIP response, even though blood glucose levels were not significantly different. It was concluded that this was an adaptation to the greater amount of food ingested, and that the GIP producing cells have a rapid mechanism of adapting to the ingested caloric load. Service *et al.* (1983) also observed a highly significant association between meal size and post-prandial plasma IRGIP responses.

Oektedalen *et al.* (1983), compared the IRGIP response to a meal in previously fasted and fed individuals. It was observed that the IRGIP response to oral glucose of a test meal was augmented after food deprivation. It was suggested that the increased catecholamine levels associated with fasting may have caused the increased IRGIP release, as the adrenergic nervous system has been shown to modify the IRGIP release

(see section 1.10.5). Hampton *et al.* (1983) found the IRGIP response to a meal in rats fed a high fat diet for 4 days prior was greater than controls. Deschodt-Lanckman *et al.* (1971) showed that pancreatic lipase activity in the rat could be stimulated by a high fat diet. This increased lipase activity might result in an increase in the rate of absorption of fat, and thus a greater IRGIP response (section 1.10.3). Morgan *et al.* (1988), however, only noted a small but significant increase in IRGIP release by human subjects previously on a high fat diet for 35 days, suggesting that species differences are likely to exist.

### 1.10.2 RESPONSE TO CARBOHYDRATES

Evidence supporting the role of GIP as an incretin came with the first observation of its release in response to oral glucose, followed almost immediately by an increase in plasma insulin levels (Cataland *et al.*, 1974). A direct relationship between the glucose load ingested and the release of IRGIP was observed in dog (Pederson *et al.*, 1975b) and man (Falko *et al.*, 1980). The fact that luminal stimulation by glucose was necessary for IRGIP release was proven when no changes in circulating levels of this hormone were observed when glucose was administered intravenously (Cataland *et al.*, 1974; Pederson *et al.*, 1975b; Cleator and Gourlay, 1975; Andersen *et al.*, 1978).

The digestion of complex carbohydrates occurs in the gut through the action of pancreatic enzymes and brush border hydrolases such as sucrase and maltase. Final products of digestion, D-glucose, D-galactose, and D-fructose, can then be absorbed by enterocytes lining the upper third of the intestinal villi. The absorptive process occurs in the duodenum and jejunum and is generally complete before the chyme reaches the ileum. The pyranoses are absorbed by a two-stage process. Glucose and galactose enter epithelial cells by an energy requiring Na<sup>+</sup>-dependent transporter, SGLT1 (Wright, 1993). In contrast, fructose is absorbed down its concentration gradient by the facilitative glucose transporter, GLUT5 (Burant *et al.*, 1992). The second step is the downhill transport of sugar out of the enterocyte across the basolateral membrane into the blood,

by the facilitated sugar transporter GLUT2 (Thorens, 1993). During some point in this process, endocrine cells release GIP; the actual step that triggers this secretion is however not understood.

In an attempt to determine the carbohydrate specificity for GIP release, Morgan (1979) studied the IRGIP responses to the sugars glucose, galactose, sucrose, and fructose given as an oral carbohydrate tolerance test in human subjects, or perfused through the rat small intestine. In both models, IRGIP was released by glucose, galactose and sucrose, but not fructose. It was also noted that the response to sucrose was significantly delayed relative to the rise in response to glucose and galactose. The results indicated that IRGIP secretion was dependent on the active transport of monosaccharides. The delay in IRGIP response to sucrose was explained by the fact that sucrose must be hydrolysed into fructose and glucose by a brush border hydrolase prior to absorption. Similar results were reported by Sirinek *et al.* (1979; 1983) from studies of intraduodenal infusion of carbohydrates in dogs. Sirinek *et al.* (1983) suggested, on the basis of their data, that the structural integrity of the glucose molecule from the C-1 to C-4 carbon atom, a free aldehyde group on the C-1 carbon atom and a cyclic structure are all necessary for both the active transport of glucose and the release of GIP.

Further evidence for the necessity of active sugar transport of monosaccharides for IRGIP release has been supplied by studies in which transport is blocked. Morgan (1979) observed that reduction of the post-prandial rise in blood glucose by guar (a gum produced from the seeds of the Indian Cluster bean, *Cyanopsis tetraagonoloba*) resulted in a significant reduction in IRGIP release. Sykes *et al.* (1980) blocked the Na<sup>+</sup>-dependent carrier protein with phloridzin, a  $\beta$ -glucoside that binds but is not transported (Hopfer, 1987) and found that glucose-stimulated IRGIP release in rats was abolished. This group also provided further information on the minimal structural requirements for the release of IRGIP in response to carbohydrates. Of the numerous monosaccharides tested, only those with the structural requirements for active transport by the Na<sup>+</sup>-

dependent hexose pathway (Hopfer, 1987) were effective as stimulants of IRGIP release. Furthermore, their observations that  $\alpha$ -methylglucoside and 3-*O*-methylglucose stimulated significant IRGIP release indicated that transport out of the cell, or metabolism, was not necessary for IRGIP release, respectively. While similar results were obtained by Flatt *et al.* (1989) with mice, others have observed no IRGIP response to 3-*O*-methylglucose in rats (Fushiki *et al.*, 1992).

While the majority of data seem to suggest that the Na<sup>+</sup>-dependent glucose transporter was involved in the mechanism by which GIP cells 'sense' glucose, the evidence was not definitive. Unlike Morgan (1979) and Sykes *et al.* (1980), Flatt *et al.* (1989) observed significant IRGIP release by fructose. In addition, Flatt *et al.* (1989) demonstrated a delayed but large IRGIP response by 2-deoxyglucose, which is not transported by the glucose or fructose luminal transport system (Hopfer, 1987). Sykes *et al.* (1980), however, found no significant response to luminal perfusion of 2-deoxyglucose in the rat. Whether these observations indicate species differences or a mechanism other than the Na<sup>+</sup>-dependent glucose transporter for GIP release is not clear.

In hopes of better characterizing the mechanism by which GIP cells 'sense' glucose, Fushiki *et al.* (1992) studied IRGIP release into the portal vein of rats in response to duodenal infusion of glucose in the presence of inhibitors of some putative glucose sensors and carriers in the intestinal lumen. Gymnemic acid, the active principle of *Gymnema sylvestre* leaves, is a glucuronide of triterpene which can inhibit Na<sup>+</sup>-dependent active glucose transport in the small intestine (Yoshioka, 1986). Fushiki *et al.* (1992) found that like phloridzin, gymnemic acid markedly suppressed the increase in portal IRGIP in response to intraduodenal glucose infusion. In the presence of cytochalasin B, a competitive inhibitor of facilitative glucose transporters, there was no significant change in the portal IRGIP concentration after glucose administration, suggesting that a facilitative glucose transporter was not directly involved in the IRGIP release by the small intestinal endocrine cells (Fushiki *et al.*, 1992).

Fushiki *et al.* (1992) investigated the possibility that IRGIP release by glucose occurred by a mechanism similar to that by which glucose acts to release insulin from the pancreatic  $\beta$ -cell. Glucokinase has been demonstrated to be a key enzyme for glucose recognition by  $\beta$ -cells as well as liver cells (Matschinsky, 1990). Infusion of mannoheptulose, an inhibitor of glucokinase (Matschinsky, 1990), did not affect the portal vein plasma IRGIP concentration (Fushiki *et al.*, 1992). Glybenclamide, a potent insulin secretagogue acting via closure of the ATP-sensitive  $K^+$  channels (Trube *et al.*, 1986), also had no effect on portal vein plasma IRGIP concentration. These findings suggested that the mechanism underlying GIP release in response to luminal glucose in the small intestine was different from that underlying insulin release in response to plasma glucose in the pancreas.

A final putative mechanism for glucose 'sensing' by GIP cells was mediation by a neural pathway (Fushiki *et al.*, 1992). In 1978, Mei found a glucoreceptor existed in the duodenum and proximal jejunum, which transmitted via vagal neurons. During recording from nodose ganglia by means of extracellular glass microelectrodes, Mei (1978) noted that glucose and galactose preferentially stimulated the sensor, but fructose did not. Furthermore, this duodenal glucose sensor could be blocked by 1% procaine infusion. Fushiki *et al.* found that infusion of 10 g/l procaine and 10 g/l lidocaine in saline did not affect the glucose-induced portal vein plasma IRGIP concentration, suggesting that the procaine-sensitive vagal glucoreceptor was not involved in IRGIP release.

Overall, the present data suggest that glucose-mediated GIP release does not occur as a result of the direct actions of the duodenal procaine-sensitive vagal glucose sensor, a cytochalasin B sensitive facilitative glucose transporter, a mannoheptulose sensitive glucokinase, or the ATP-sensitive  $K^+$  channels. It appears that neither glucose metabolism, nor glucose transport out of the cell are necessary for GIP release, although this requires further verification. It seems glucose transport via a phloridzin sensitive  $Na^+$ -dependent transporter like SGLT1 is involved in glucose recognition leading to GIP

release but some data indicate a specificity different from that known for SGLT1. Furthermore, it is not known whether the GIP cells directly 'sense' glucose, or whether messages are transmitted by neighbouring glucose-transporting mucosal cells. Sykes *et al.* (1980) postulated that tight junctions between the two types of cells allowing changes in the flux of sodium ions generated in the mucosal cells by interaction between the stimulatory monosaccharides and their transport proteins might offer a possible explanation for their ability to stimulate release of IRGIP.

### 1.10.3 RESPONSE TO FATS

Triglycerides form the primary lipids of a normal diet. Their digestion occurs primarily in the duodenum and upper jejunum, where pancreatic lipases act on fat emulsified by bile. In the intestinal lumen, triglycerides are converted to free fatty acids and monoglycerides, form micelles, and then diffuse among the microvilli that form the brush border. The high lipid solubility of the fatty acids and monoglycerides promotes their diffusion into the enterocytes. The subsequent fate of the fatty acids depends on their size. Fatty acids containing less than 10-12 carbon atoms pass from the mucosal cells directly into the portal blood, where they are transported as free (unesterified) fatty acids. Larger fatty acids are reesterified with monoglycerides to re-form triglycerides, which accumulate in chylomicra and are ejected by exocytosis out of the cell, into the lymph. In order for GIP to fulfill the role of an enterogastrone, it is necessary to establish its release by some stage of this fat digestion process.

The first report of IRGIP release in response to fat was made in 1974 by Brown shortly after the development of a GIP radioimmunoassay (Kuzio *et al.*, 1974). Brown observed a peak in serum IRGIP levels in human volunteers approximately 2 h after ingestion of 100 ml corn oil suspension (Lipomul). A similar response was noted by Falko *et al.* (1975), and Cleator and Gourlay (1975), and these investigators also reported no increase in serum glucose or insulin concentrations. These studies showed quite

conclusively that endogenously released IRGIP was not insulinotropic in the absence of hyperglycemia. Pederson *et al.* (1975b) studied the IRGIP response to graded oral doses of fat and glucose in dogs, and found triglycerides produced a greater and more prolonged IRGIP response (5 h) than the glucose loads (2.5 h). It was suggested that the rate of gastric emptying might be a contributing factor to these observations. Pederson *et al.* (1975b) pointed out that the release of IRGIP by fat and the nature of this response fit well with an enterogastrone-like action, whereas the rapid response to oral glucose was more relevant to the potentiation of a rapidly rising insulin response.

In 1981, Ross and Shaffer found that the process of hydrolysis was necessary for GIP release. This came from the observation that there was no IRGIP release from patients with cystic fibrosis and defective fat lypolysis following ingestion of corn oil, but that it returned to levels similar to controls with coingestion of pancreatic enzymes. A similar conclusion was made by Ohneda *et al.* (1983) when it was observed that an IRGIP response to fat in pancreatectomized dogs occurred only during coingestion of pancreatic enzymes. Imamura *et al.* (1988) found a reduced IRGIP response to ingestion of a fat-enriched meal in patients with disturbed fat metabolism resulting from external biliary drainage. Interestingly duodenal infusion of bile alone has been demonstrated to increase IRGIP release (Burhol *et al.*, 1980b). Thus emptying of the gallbladder in response to the presence of fat in the duodenum may enhance the release of IRGIP in response to fat hydrolysis.

The suggestion that fatty acids were the product of triglyceride hydrolysis responsible for IRGIP release came from observations that monoglycerides had no effect on IRGIP levels (Ross and Shaffer, 1981; Ohneda *et al.*, 1984; Williams *et al.*, 1981). In 1976, O'Dorizio *et al.* reported only a modest IRGIP response to an intraduodenal infusion of medium chain triglycerides in dogs, when compared to the IRGIP release previously obtained from long chain fatty acids (Brown, 1974; Falko *et al.*, 1975). Other studies on the effects of fatty acid chain length on IRGIP release have since confirmed

that long chain fatty acids exert strong stimulation, while medium or short chain fatty acids do not (Ross and Shaffer, 1981; Ohneda *et al.*, 1984 Kwasowski *et al.*, 1985). In addition to fatty acid chain length, it also appears that the degree of saturation might affect their ability to stimulate IRGIP release. Lardinois *et al.* (1988) compared the IRGIP response to saturated, monosaturated and polyunsaturated fats in man, and found the latter yielded the smallest IRGIP secretion.

As short and medium chain fatty acids are absorbed faster than long chain, GIP release is not related only to the rate of cellular uptake of fatty acids. Kwasowski *et al.* (1985) suggested that the stimulus for fat-induced GIP release might be generated during the intracellular handling and metabolism of fatty acids. Short- and medium-chain fatty acids are transferred across the intestinal epithelium without esterification. However, long-chain fatty acids are conveyed to the smooth endoplasmic reticulum for esterification prior to incorporation into chylomicrons and eventual exocytosis into the intercellular compartment. The GIP-releasing action of fatty acids may be coupled therefore to the extent of esterification, an energy-consuming metabolic process confined to long-chain fatty acids. Previous studies on the mechanism of carbohydrate-stimulated GIP release have shown that only actively transported sugars stimulate IRGIP secretion (see section 1.10.2). Thus there may be a common link between metabolic and secretory events responsible for nutrient-regulation of GIP release from the intestinal GIP cells.

#### *1.10.4 GIP RESPONSE TO PROTEINS*

The majority of protein digestion occurs in the duodenum and jejunum, where pancreatic proteases such as trypsin, chymotrypsin, and carboxypeptidase act to convert dietary protein to small peptides. The brush border of the upper small intestine contains a number of peptidases, including aminopeptidases, dipeptidases and dipeptidyl aminopeptidases, which further reduce the peptides to oligopeptides and amino acids. The small peptides and amino acids are then transported across the brush border plasma

membrane into the epithelial cells. Transport of dipeptides and tripeptides across the brush border plasma membrane is a secondary active transport process powered by the electrochemical potential difference of  $\text{Na}^+$  across the membrane. Normally amino acids are transported into the enterocyte by way of certain specific amino acid transport systems, some dependent on  $\text{Na}^+$  and others not. Once in the enterocyte, transport systems of the basolateral membrane then transport amino acids out of the cell, again with some dependent on  $\text{Na}^+$ , and others not.

Brown (1974) reported that ingestion of a meat extract did not result in a significant release of IRGIP. A similar result was observed by Cleator and Gourlay (1975) with either beef steak or a meat extract, while a small but significant response to leucine was noted. Thomas *et al.* (1976) were able to demonstrate that intraduodenal administration of a mixture of amino acids elevated serum IRGIP concentrations (peaking at 30 min) in association with an increased insulin response. More specifically, they showed that a mixture containing arginine, histidine, isoleucine, lysine and threonine caused a marked rise in integrated IRGIP and insulin secretion, while a combination of methionine, phenylalanine, tryptophan and valine had only a minimal effect on release of these two hormones. It was suggested by Thomas *et al.* (1978) that the higher concentration of amino acids as compared to the previous studies was responsible for the observed IRGIP response, as concentrations of 10 or 20 mM in the small intestine demonstrated no effect. O'Dorisio *et al.* (1976) demonstrated a significant IRGIP response with a mixture of amino acids totaling ~38 mM.

In 1982, Schulz *et al.* examined the IRGIP response to amino acids in rats treated with a corticosteroid or alloxan. These compounds are thought to increase  $\text{Na}^+$   $\text{K}^+$  ATPase activity, and thus the  $\text{Na}^+$ -dependent active absorption process (Schulz *et al.*, 1982). A mixture of amino acids, including those shown by Thomas *et al.* (1976) to stimulate IRGIP release, were also found to be stimulatory by Schulz *et al.* (1982), and, furthermore, both groups of treated rats showed significantly higher serum IRGIP levels.

These results were attributed to an increased absorption of the amino acids owing to an increased Na<sup>+</sup> K<sup>+</sup> ATPase activity in the intestinal mucosa of corticosteroid- and alloxan-treated rats. These data therefore suggested that as with glucose or fat, the release of GIP after amino acid stimulation seemed to depend more on an active absorption than on their presence within the intestinal lumen.

In 1982, Wolfe and McGuigan measured the IRGIP response following a peptone meal in the dog. The increase in IRGIP release in response to the peptone meal in this study was maximal in peripheral venous serum at 15 min. This is similar to the response to glucose feeding and contrasts with the more prolonged response to fat ingestion. IRGIP release has not been demonstrated previously after feeding or infusion of a protein of peptone meal that is free of carbohydrate or fat. It was suggested that these differences might result from the sensitivity of the GIP radioimmunoassay used in this study which measured basal and peak IRGIP levels at  $34 \pm 10$  and  $323 \pm 95$  pg/ml, respectively; values lower than those previously reported. No significant increase in insulin was noted, consistent with the glucose-dependent action of GIP. Wolfe and McGuigan (1982) suggested that their demonstration of GIP release after a peptone meal accompanying peptone-stimulated gastrin release and gastric acid secretion was supporting of the enterogastrone role of GIP.

#### *1.10.5 AUTONOMIC NERVOUS SYSTEM CONTROL OF GIP RELEASE*

Conflicting data, both in human and animal studies, exist regarding the involvement of cholinergic and adrenergic factors in GIP secretion. Vagotomy has been demonstrated either to enhance (Thomford *et al.*, 1974; Imamura *et al.*, 1984; Yoshiya *et al.*, 1985), reduce (Lauritsen *et al.*, 1982; Imamura *et al.*, 1984), or to have no effect (Gayle and Ludewig, 1978; Yoshiya *et al.*, 1985) on the IRGIP response to nutrient ingestion. Sham feeding, which produced cephalic-vagal stimulation, had no effect on IRGIP release (Taylor and Feldman, 1982), as did direct vagal stimulation (Berthoud *et*

*al.*, 1982). Studies with atropine have demonstrated either reduced (Baumert *et al.*, 1978; Larrimer *et al.*, 1978) or no effect (Sirinek *et al.*, 1981; Nelson *et al.*, 1986) on the IRGIP response to nutrient ingestion. Finally, acetylcholine decreased the IRGIP response to intragastric glucose administration (Williams and Beisbroeck, 1980).

As with parasympathetic stimulation, controversy also exists on the effect of the sympathetic nervous system on GIP release. Epinephrine has been shown to inhibit (Williams and Biesbroeck, 1980) or have no effect (Sirinek *et al.*, 1977b) on glucose-stimulated IRGIP release. Frier *et al.* (1984) examined the influence of adrenergic denervation on the IRGIP response to feeding in man. There was no significant difference in meal-stimulated IRGIP release in sympathectomized subjects (with a complete transection of the spinal cord above the sympathetic outflow) compared to controls. Blockade of  $\alpha$ -adrenoreceptors has been shown to augment (Sirinek *et al.*, 1977b) or have no effect on nutrient stimulated IRGIP release (Flaten, 1981), while  $\beta$ -adrenoreceptor antagonists seem to inhibit nutrient stimulated IRGIP release (Sirinek *et al.*, 1977b; Flaten, 1981). Stimulation of  $\alpha$ -adrenoreceptors has been shown to reduce the IRGIP response to oral glucose (Salera *et al.*, 1982a), while  $\beta$ -adrenoreceptor stimulation has been shown to increase basal and glucose stimulated IRGIP release (Flaten *et al.*, 1982; Kogire *et al.*, 1990), or have no effect (Salera *et al.*, 1982a).

The multiple effects of the autonomic nervous system on the gastrointestinal tract probably contribute to the difficulty of establishing the role of its components on IRGIP release. Alterations in gastric emptying, intestinal secretion and absorption of fluids, motility, and blood flow by the autonomic nervous system are all likely to influence IRGIP release. It is therefore difficult to make inferences on the direct action of the sympathetic or parasympathetic nervous system on GIP cells.

### 1.10.6 OTHER MODULATORS OF GIP RELEASE

Somatostatin appears to be an effective inhibitor of GIP release. Pederson *et al.* (1975a) demonstrated that a rapid intravenous injection of somatostatin in dogs delayed the increase in serum concentrations of insulin, IRGIP and glucose following oral glucose. During a sustained somatostatin infusion, the IRGIP response to oral glucose was suppressed until cessation of the infusion, even though serum glucose levels were elevated to the same levels as controls. This suggested that inhibition of glucose-stimulated IRGIP release was not a result of the inhibition of glucose absorption. This hypothesis was confirmed by Creutzfeldt and Ebert (1977), who demonstrated that IRGIP and insulin responses to a test meal were completely suppressed during somatostatin infusion, whereas blood glucose levels were not different from controls. Kraenzlin *et al.* (1985b) demonstrated that a long acting somatostatin-analogue, SMS 201 995, also effectively suppressed the postprandial release of IRGIP.

Intravenous gastrin has been demonstrated to increase the IRGIP response to intraduodenal glucose in dogs (Sirinek *et al.*, 1977a). This augmentation could not have been caused by the stimulation of gastric H<sup>+</sup> secretion, since acidification of the duodenal glucose did not augment the IRGIP release. Jorde *et al.* (1981) further examined this putative effect of gastrin by examining the IRGIP response to a mixed liquid test meal in a group of achlorhydric patients with high serum gastrin levels compared to a normal group of human subjects. A significantly higher IRGIP response was observed in the achlorhydric group than in the control group. Studies on patients after total antero-duodeno-pancreatectomy that have undetectable levels of serum gastrin show a significant IRGIP response to a test meal, indicating that gastrin is not a prerequisite for GIP release (Creutzfeldt *et al.*, 1976). However, these patients have a very rapid gastric emptying, which may explain their elevated serum IRGIP levels.

Evidence for a relationship between GIP and gastrin has also been supplied by Morgan *et al.* (1985). These investigators observed a highly significant negative

correlation between circulating gastrin and IRGIP levels after control and guar gum meals. Addition of guar gum to an oral glucose load reduced the postprandial secretion of both IRGIP and insulin. Studies in dogs by Wolfe *et al.* (1983) using anti-GIP antibodies have shown that IRGIP can function as a physiological inhibitor of gastric acid secretion through its effect on gastrin release. Morgan *et al.* (1985) therefore suggested that the guar gum attenuated IRGIP response to the protein meal, leading to an attenuated somatostatin response and as a result unrestrained gastrin secretion. Exogenous GIP infusions have been shown to inhibit gastrin release (Villar *et al.*, 1976; Arnold *et al.*, 1978), the effect seemingly mediated via an increase in gastric somatostatin secretion (McIntosh *et al.*, 1979).

In addition to gastrin, other compounds have also been demonstrated to alter IRGIP release. Increases in IRGIP levels have been noted in response to intravenous administration of the neuropeptide gastrin-releasing peptide (McDonald *et al.*, 1981; Greely *et al.*, 1986a; 1986b), while the neuropeptide calcitonin gene-related peptide inhibited basal levels of IRGIP in man (Kraenzlin *et al.*, 1985a). A reduction in postprandial IRGIP release has also been noted in response to intravenous morphine (Champion *et al.*, 1982) and oral administration of the prostaglandins enprostil or rioprostil (Nicholl *et al.*, 1986; Demol and Wingender, 1989; Schwartz and Saito, 1989). It is not clear whether these actions result from direct interactions with GIP cells, or indirectly by slowing gastric emptying, reduced intestinal nutrient absorption, or by the action of another mediating peptide or neurotransmitter.

#### ***1.10.7 FEEDBACK INHIBITION OF GIP RELEASE***

In 1975, Brown *et al.* proposed a feedback inhibitory control mechanism of GIP release involving insulin. This hypothesis was based on their observation that an intravenous bolus injection of insulin during ingestion of fat significantly reduced the triglyceride-induced IRGIP increment. Support came from studies demonstrating

attenuated IRGIP responses to oral fat ingestion during simultaneous infusion of glucose, and, thereby, stimulation of endogenous insulin release (Cleator and Gourlay, 1975; Crockett et al., 1976; Ross and Dupré, 1978; Ebert *et al.*, 1979a). In 1978 Sirinek *et al.* suggested that insulin was also capable of attenuating the GIP response to oral glucose. However, Andersen *et al.* (1978) noted that during a euglycemic clamp with a continuous insulin infusion resulting in hyperinsulinemia, a significant IRGIP response to oral glucose was noted. Andersen *et al.* concluded that glucose-stimulated GIP release was not inhibited in the presence of marked hyperinsulinemia. This finding was supported by the observations that hyperinsulinemia induced by exogenous insulin (Service *et al.*, 1978), or endogenous insulin (Collier *et al.*, 1984) had no effect on carbohydrate-stimulated IRGIP secretion in normal subjects. Furthermore, in juvenile diabetics without insulin reserve, fat-, but not glucose-induced IRGIP secretion was reduced by exogenous insulin infusion (Creutzfeldt *et al.*, 1980). In a recent investigation with rats, however, Bryer-Ash *et al.* (1994) observed that the IRGIP response to oral glucose was suppressed by hyperinsulinemia, and this suppression was attenuated when hyperinsulinemia was accompanied by hyperglycemia. It is therefore possible that species differences may exist in the insulin feedback inhibition of GIP release by oral glucose.

Verdonk *et al.* (1980), using the glucose insulin clamp technique, questioned the effectiveness of insulin as an inhibitor of fat-stimulated IRGIP secretion. These authors used hypoglycemic, euglycemic, and hyperglycemic clamp conditions and found that insulin, under euglycemic conditions, did not exert an inhibitory effect on fat-stimulated IRGIP levels. Since in their study, the hypoglycemic clamp increased and the hyperglycemic clamp decreased the IRGIP response to oral fat, they suggested an effect of glycemia itself on IRGIP secretion in the presence of hyperinsulinemia. However, Stöckmann *et al.* (1984) postulated that the 2 h hyperinsulinemic period preceding ingestion of fat in the study by Verdonk *et al.* may have prevented the demonstration of

the insulin effect on fat-induced IRGIP release. Stöckmann *et al.* (1984) found that the response of IRGIP to oral fat was inhibited by 63% if insulin infusion was started at the time of fat ingestion, whereas no inhibition was seen if a 2 h hyperinsulinemic period preceded the fat load. It was therefore concluded that insulin does inhibit fat-induced IRGIP secretion in normal man, but prior hyperinsulinemia masks this insulin effect, probably by decreasing the sensitivity of the GIP cells to insulin.

Recently, Takahashi *et al.* (1991) compared the effect of insulin and glucose on fat-induced GIP and tGLP-I release in humans. The response of both hormones to fat ingestion was measured during continuous glucose infusion and during a hyperinsulinemic euglycemic glucose clamp. The release of GIP and tGLP-I was suppressed in the hyperglycemic, hyperinsulinemic state. However, while GIP was also suppressed in the normoglycemic hyperinsulinemic state, the release of tGLP-I was not. Takahashi *et al.* (1991) thus concluded, as previously reported, that insulin inhibited fat-induced GIP, but the secretion of tGLP-I was more likely inhibited by a direct action of glucose.

Dryburgh *et al.* (1980a; 1980b) investigated the possibility that C-peptide might inhibit the release of IRGIP. At a dose three-times that found in the fed rat, C-peptide totally abolished the IRGIP response of the perfused rat intestine to fat stimulation. It was also demonstrated that C-peptide released after stimulation of the pancreas by glucose and tolbutamide administration given intravenously in combination with insulin antiserum significantly inhibited fat-stimulated IRGIP release. However, this mechanism could not account for the reduced IRGIP response to fat during concomitant insulin infusion observed by Stöckmann *et al.* (1984) under their experimental conditions. C-peptide levels were not elevated, and were in fact decreased by about 50% by insulin infusion, consistent with the suggested feedback inhibition of insulin secretion by insulin (Service *et al.*, 1978; Elahi *et al.*, 1982).

## 1.11 GIP RECEPTORS

### 1.11.1 BIOLOGICALLY ACTIVE SITE(S)

It is possible, that the enterogastric and insulinotropic actions of GIP are mediated via two different receptor types, which interact with different regions of the polypeptide. Evidence for this hypothesis emerges from studies testing the biological activity of various regions of the GIP molecule. The cleavage of GIP by trypsin was first used during amino acid sequence determination of the polypeptide (Brown *et al.*, 1970). An unpurified mixture of tryptic fragments, including the major products 1-16, 19-30, and 34-42, had no enterogastrone activity, as tested in the denervated pouch of the fundus of the stomach in dogs. Brown and Pederson (1970b) reported that a purified cyanogen bromide produced C-terminal fragment with amino acids 15-42 was sufficient to inhibit acid secretion in the same model. Moroder *et al.* (1978) tested synthetic porcine GIP 1-38 and found it to be a "poor" inhibitor of gastric acid secretion. However, it was later found that there was an extra glutamine residue in this sequence at position 30, which perhaps contributed to that observation. Pederson *et al.* (1990) observed only a weak somatostatinotropic effect in a combined isolated perfused pancreas and stomach preparation of the rat with synthetic porcine GIP 1-30. Furthermore, Rossowski *et al.* (1992) found that unlike the parent peptide, porcine GIP 1-30 was not able to inhibit pentagastrin-stimulated gastric acid secretion in rats. These observations, when combined with that made by Brown and Pederson (1970b) indicate that the C-terminal portion of the polypeptide is likely the site of the acid inhibitory action of GIP.

The first GIP fragment tested for insulinotropic activity was the purified cyanogen bromide fragment 15-42 produced by Brown and Pederson (1970b). In 1976, these investigators tested this C-terminal peptide in the perfused rat pancreas and found that it was approximately 40% as potent as an insulinotropic agent as intact porcine GIP (1976b). Using the same bioassay, Maletti *et al.* (1986) established that an HPLC purified fragment of bovine GIP resulting from enterokinase digestion (amino acids 17-

42) was approximately 32% as insulinotropic as native bovine GIP. The N-terminal synthetic fragments bovine GIP 1-39 (Sandberg *et al.*, 1986), porcine GIP 1-38 (Moroder *et al.*, 1978), human GIP 1-31 (Carquist, 1987) and porcine GIP 1-30 (Pederson *et al.*, 1990; Gallwitz *et al.*, 1993) were shown to be equipotent with native GIP. In 1986, Schmidt *et al.* found that HPLC purified porcine GIP 19-42 derived by proteolytic cleavage of the natural peptide was not significantly insulinotropic in isolated rat pancreatic islets. This result, when considered with those by Maletti *et al.* (1986), and Pederson *et al.* (1990), suggests that the GIP region of 19-30 does not contain all the necessary amino acids for its insulinotropic activity, and 17-30 would be predicted to retain approximately 32% insulinotropic action. Blundell *et al.* (1976) pointed out that hydrophobic amino acid regions of the glucagon superfamily are probably important for receptor binding. The amino acid sequences 6-14 and 19-27 constitute the hydrophobic regions in GIP. It is also evident that the region 19-30 has so far been shown to be completely conserved in all species (Figure 1).

Interestingly, in 1981, Brown *et al.* reported that the purest preparations of GIP existing at that time, contained a minor peptide component contributing approximately 5% to the total peptide content as shown by isotachopheresis and HPLC. Concomitantly, Jörnvall *et al.* confirmed the heterogeneity in the porcine GIP preparation, and sequence analysis of the minor component suggested that it corresponded to GIP 3-42 (lacking residues 1 and 2). Jörnvall *et al.* (1981) reported 20% contamination by this fragment in GIP preparations, and suggested that it was formed by secondary processing or degradation, through susceptibility to attack by aminopeptidase, elastase, dipeptidyl aminopeptidase or related enzymes in the intestine. Again in 1981, Brown *et al.* compared the insulinotropic and somatostatinotropic actions of these two components in isolated perfused rat pancreas and stomach preparations. GIP 3-42 was found to lack significant actions on both IRSS and insulin release when compared with intact GIP 1-42. The lack of insulinotropic activity of GIP 3-42 was confirmed by Moody *et al.* (1981)

and Schmidt *et al.* (1986). In 1987, Schmidt *et al.* reported that this shorter form of GIP comprised 32% of available natural GIP, and also found 4% CCK to be present. Furthermore, they noted that GIP 3-42 did not exhibit antagonistic activity to GIP 1-42, even at a 10-fold molar excess. Carlquist *et al.* (1984) used *Staphylococcus aureus* V8 to cleave the N-terminal tripeptide from bovine GIP, resulting in GIP 4-42, and tested its insulinotropic activity in the perfused rat pancreas (Maletti *et al.*, 1986). In 3 out of 6 rats tested, a "small" stimulation of insulin release as compared to GIP 1-42 was noted (~10%). It is not clear why the biological activity of inactive GIP 3-42 appears to be regained with the further removal of N-terminal amino acids.

### 1.11.2 GIP BINDING STUDIES

An important step in confirming the actions of a peptide hormone on target tissues is the demonstration of specific binding sites. Attempts at studying the receptors that mediate the action of GIP on normal tissues have proven very difficult. Brown *et al.* (1989) suggested two reasons to explain the unsuccessful attempts. Firstly, it was noted that iodination of GIP resulted in a heterogeneous population of iodinated peptides, potentially altering the receptor binding and biological activity. Secondly, methods used to isolate  $\beta$ -cells or islets appeared to critically influence the GIP receptor. Indirect evidence for this was the observation that isolated pancreatic islets or islet cells only responded to pharmacological concentrations of GIP, unlike responses to other stimuli (Schäfer and Schatz, 1979; Schauder *et al.*, 1975; Schauder *et al.*, 1976). This implied that the GIP receptor was particularly susceptible to enzymatic damage during cell isolation. Verchere (1991) was able to demonstrate binding sites for GIP on cultured rat islets using HPLC purified, biologically active  $^{125}\text{I}$ -GIP. Significant displacement of the radioligand by GIP was observed at concentrations as low as 1 nM.

Tumor cells have been recognized as a potential source for large numbers of GIP receptors. Malletti *et al.* (1984) and Couvineau *et al.* (1984) were able to demonstrate

high affinity binding sites in membrane preparations from hamster  $\beta$ -cell tumors. Maletti *et al.* (1984) ensured that the label was homogenous by HPLC purification, and demonstrated biological activity in the perfused rat pancreas. Amiranoff *et al.* (1984; 1985) provided evidence for specific GIP binding sites in the pancreatic tumor cell line In III. Furthermore, GIP binding was correlated with an increase in cAMP levels and insulin release. Binding of  $^{125}\text{I}$ -GIP in these studies was found to be saturable and could not be displaced by peptides structurally related to GIP. Both high affinity (Amiranoff *et al.*:  $K_D = 7$  nM; 3000 binding sites/cell, Maletti *et al.*:  $K_D = 2$  nM; 219 binding sites/cell) and low affinity (Amiranoff *et al.*:  $K_D = 800$  nM; 150,000 binding sites/cell, Maletti *et al.*:  $K_D = 39$ ; 1250 binding sites/cell) binding sites for GIP were identified. By using a cross-linker to prevent dissociation of  $^{125}\text{I}$ -GIP bound to In III membranes, Amiranoff *et al.* (1986) identified a 59-kDa membrane protein that specifically bound GIP. Functional GIP receptors have also been demonstrated in human insulinoma plasma membranes (Maletti *et al.*, 1987), again with both high affinity ( $K_D = 0.2$  nM) and low affinity ( $K_D = 8.4$  nM) binding sites, and in the mouse derived  $\beta$ -cell line, BTC3 (Kieffer *et al.*, 1993). These receptors belong to a distinct family of G-protein coupled receptors referred to as the secretin-VIP receptor family (Christophe *et al.*, 1986; Rosselin, 1986).

Binding sites for GIP *in vivo* were reported by Whitcomb *et al.* (1984) using  $^{125}\text{I}$ -GIP in rats. Specific and displaceable binding of GIP to its receptors was demonstrated in the pancreas, glandular portion of the stomach, throughout the intestine and various muscle groups. No GIP receptors could be demonstrated in the liver, adrenal gland, spleen, kidney, submandibular gland, testis, epididymis, prostate or seminal vesicles. Recently, using a molecular approach, Usdin *et al.* (1993) cloned a novel receptor, which, when expressed in a cell line demonstrated activation only by GIP. Northern blots, reverse-transcription PCR and *in situ* hybridization demonstrated the receptor in tissues known to respond to GIP (pancreas, gut, adipose tissue) and in novel locations such as the heart, brain, and inner layers of the adrenal cortex, where physiological effects of GIP

have not been described. No receptor mRNA was found in kidney, spleen or liver. As neither GIP nor its effects have been described in the central nervous system, except at high concentrations (Ottlecz *et al.*, 1985) these findings might suggest the presence of a novel, homologous peptide in the brain. Interestingly, the related peptide tGLP-I has been observed and its release demonstrated in the rat brain (Shimazu *et al.*, 1987; Kreymann *et al.*, 1989) where it might act as a neurotransmitter.

As the results of biological assays with GIP fragments do not demonstrate interactions with the receptor as either an agonist or antagonist, the availability of specific receptor binding assays is important. Some fragments of GIP shown to be biologically active (GIP 1-31; 1-30; 17-42) were shown to competitively inhibit the binding of <sup>125</sup>I-GIP (Maletti *et al.*, 1987; Kieffer *et al.*, 1993; Maletti *et al.*, 1986; Gallwitz *et al.*, 1993). On the other hand, fragments not expected to yield insulintropic activity, such as GIP 19-30 (see section 1.11.1), did not displace binding of the tracer to insulinoma membranes (Maletti *et al.*, 1986). Likewise, the GIP fragment 1-27 was unable to displace GIP binding to RINm5F cells (Gallwitz *et al.*, 1993). Interestingly, the GIP fragment 4-42, which demonstrated only partial insulintropic action, was capable of competing equally with GIP 1-42 for tracer binding (Maletti *et al.*, 1986). In this regard, GIP 4-42 may act as a partial antagonist.

In 1991, Fehmann and Habener showed that the tGLP-I receptor in the glucose-responsive  $\beta$ -cell line HIT-T15 could rapidly and reversibly desensitize in response to supraphysiological concentrations of tGLP-I. Preperfusion with GIP had no effect on the tGLP-I response indicating the tGLP-I receptor on the HIT-T15 cells was distinct from that of GIP. Gallwitz *et al.* (1993) arrived at the same conclusion with the RINm5F cell line when it was observed that <sup>125</sup>I-GIP binding was not displaced by tGLP-I, and <sup>125</sup>I-tGLP-I binding was not altered by GIP. Fehman and Habener (1991a) also suggested that the GIP receptor could undergo homologous desensitization from their observation that prior exposure of HIT-T15 cells to GIP (100 nM) also reduced the insulin secretion

during stimulation with 10 nM GIP. This hypothesis was also supported by the demonstration that high concentrations of GIP inhibit both insulin secretion from the perfused pancreas (Szecowka *et al.*, 1982a) and cAMP generation in human (Malleti *et al.*, 1987) and rat (Gallwitz *et al.*, 1993) insulinoma cells, possibly by homologous desensitization. Furthermore, in rats subjected to total parenteral nutrition for a period of 6 days, there was a 30% increase in insulin release from the perfused pancreas in response to GIP (Pederson *et al.*, 1985). It was concluded that subjecting the pancreas to chronic low concentrations of GIP could induce an increase in the sensitivity or number of GIP receptors.

### 1.12 GIP METABOLISM

In 1975, Brown *et al.* determined the half-life of porcine IRGIP in the plasma of normal subjects to be approximately 21 min. Identical values were obtained using porcine GIP in man by Elahi *et al.* ( $20.4 \pm 2.37$  min; 1979) and Sarson *et al.* ( $20.3 \pm 1.2$  min; 1982), and the metabolic clearance rate was calculated as  $2.6 \pm 0.1$  ml/kg-min (Sarson *et al.*, 1982). While even higher values have been obtained for the half-life of tGLP-I in rat ( $39.5 \pm 15.5$  min; Oshima *et al.*, 1988) and man ( $45.9 \pm 8.8$  min; Oshima *et al.*, 1991), other related peptides are cleared from the plasma much quicker, with half-lives of 5 min or less (secretin: 2.5 min, Häcki *et al.*, 1977; vasoactive intestinal peptide: 1 min, Modlin *et al.*, 1978; glucagon: 5.5 min, Jaspan and Rubenstein, 1977). It therefore appears that exogenously administered GIP is cleared relatively slowly from human plasma. Wolfe and McGuigan (1982) measured the half-time of disappearance for porcine IRGIP in the dog at  $7.6 \pm 1.5$  min, thus indicating that some species differences might exist.

In 1979, Elahi *et al.* reported that the prevailing state of glycemia could influence the metabolic half-life of GIP. Using porcine GIP in human subjects, Elahi *et al.* (1979) found that under euglycemic conditions, the half-life was  $26.3 \pm 4.57$  min, during mild

hyperglycemia (8 mM),  $23.2 \pm 3.16$  min, and under moderate hyperglycemia (13 mM) dropped to  $13.1 \pm 2.59$  min. As the moderate hyperglycemia was also associated with hyperinsulinemia, Elahi and associates (1979) were unable to conclude which factor may have been responsible for the shortened IRGIP half-life. In order to differentiate between these two factors, Andersen *et al.* (1980) examined the half-life of exogenous porcine GIP in fasting dogs during hyperinsulinemia and hyperglycemia, alone and in combination. The half-life was significantly reduced by hyperglycemia alone or in combination, but there was no change with hyperinsulinemia alone, suggesting that hyperglycemia, but not hyperinsulinemia could significantly enhance the metabolic clearance rate of IRGIP. Recently, Sheu *et al.* (1987) also noted a greater metabolic clearance rate of porcine GIP in fed versus fasted rats. However, not all investigators have reported similar observations. Nauck *et al.* (1989) observed no difference in the half-life (18 min) or metabolic clearance rate ( $\sim 6$  ml/kg·min) of human GIP infused in normal subjects during basal (5 mM) or hyperglycemic (8 mM) clamps.

All pancreatic and gastrointestinal hormones must traverse the liver prior to reaching the general circulation. The liver is major clearance site for some hormones, such as insulin, of which approximately 50% of that secreted is removed with each transhepatic circulation (Ishida *et al.*, 1983; Stoll *et al.*, 1970). On the other hand, there appears to be no hepatic extraction of endogenous or exogenous GIP (Hanks *et al.*, 1984; Chap *et al.*, 1987). The kidney also inactivates or clears several polypeptide hormones, such as insulin, glucagon and gastrin from the circulation (Chamberlain and Stimmler, 1967; Sherwin *et al.*, 1976; Davidson *et al.*, 1973). O'Dorisio *et al.* (1977) found a renal arterial-venous IRGIP difference of 39% in dogs during intraduodenal perfusion of glucose, and found that uremic patients had higher than normal fasting and stimulated IRGIP concentrations. It therefore appears that the kidney is the major site of GIP clearance.

An important factor that must be considered when examining the metabolism of a hormone is the possibility that hormone measurements associated with immunoreactivity may not necessarily correspond to the biological activity of that hormone. There are numerous proteases in plasma that could potentially render a hormone biologically inactive, yet still immunoreactive in an RIA. Thus, while measurements of the half-life of IRGIP levels in plasma are useful, they must also be combined with studies examining the duration of biological activity of circulating GIP. The importance of this issue has recently been underscored by the observation that dipeptidyl peptidase IV hydrolyzes GIP in human serum to the biologically inactive fragment GIP 3-42 and thus may play an important role in the metabolism of GIP (Mentlein *et al.*, 1993). Present GIP antisera may be unable to distinguish this truncated N-terminal form, suggesting that GIP 3-42 likely contributes to IRGIP measurements in the circulation. Further discussion of this issue appears in Chapter 4 of this thesis.

### 1.13 GIP PATHOPHYSIOLOGY

The possibility exists that GIP may be involved in the etiology of pathophysiological states. In particular, in light of the fact that GIP is a major incretin candidate of the enteroinsular axis, the role of GIP in diseases associated with insulin deficiency and excess have been extensively investigated. The results of these studies, however, leave no clear answer to the function of GIP in these states. For instance, in non-insulin dependent diabetes mellitus (NIDDM), circulating IRGIP levels have been reported to be increased (Brown *et al.*, 1975; Mazzaferri *et al.*, 1985; Elahi *et al.*, 1984; Cox *et al.*, 1981; Lardinois *et al.*, 1985; Jones *et al.*, 1989b; Ross *et al.*, 1977), normal (Service *et al.*, 1984; Levitt *et al.*, 1980; Alam *et al.*, 1992), or decreased (Alam and Buchanan, 1980; Service *et al.*, 1984; Groop, 1989; Nauck *et al.*, 1993) following the oral administration of nutrients. Similarly, in obese subjects, some studies have demonstrated elevated fasting levels of IRGIP (Elahi *et al.*, 1984; Mazzaferri *et al.*, 1985; Salera *et al.*,

1982a), and others exaggerated (Creutzfeldt *et al.*, 1978; Elahi *et al.*, 1984; Mazzaferri *et al.*, 1985; Ebert *et al.*, 1979a; Jones *et al.*, 1989a; Fukase *et al.*, 1993), normal (Lauritsen *et al.*, 1980; Jorde *et al.*, 1983; Amland *et al.*, 1984; Ebert and Creutzfeldt, 1989) or blunted (Service *et al.*, 1984; Groop, 1989) levels after oral nutrient ingestion.

Among other defects in insulin secretion in NIDDM patients, there is a reduced or absent incretin effect: insulin release is no longer stimulated more by oral as compared to "isoglycemic" intravenous glucose (Perley and Kipnis, 1967; Nauck *et al.*, 1986; Tronier *et al.*, 1985). A reduced incretin effect could be caused by impaired secretion of relevant incretin hormones or by  $\beta$ -cell insensitivity to their insulinotropic action. While the data on GIP secretion are not conclusive, reports of the insulinotropic action of GIP in NIDDM patients seem to be in agreement. In streptozotocin diabetic rats, the insulinotropic effectiveness of GIP was significantly reduced (Suzuki *et al.*, 1990). In human diabetic subjects, there appears to be a significantly lower insulin response to intravenous GIP when compared to normal subjects (Jones *et al.*, 1987; Krarup *et al.*, 1987c; Nauck *et al.*, 1993; Meneilly *et al.*, 1993). Meneilly *et al.* (1993) found that the sulfonylurea glyburide significantly increased the  $\beta$ -cell response to GIP infusion in diabetic subjects, but the observed insulin release was still approximately 10-fold less than that of controls. Interestingly, Nauck *et al.* (1993) found no significant difference in the insulin response to tGLP-I in diabetic vs. normal subjects. Holz *et al.* (1993) postulated that since tGLP-I appeared able to compensate for a defect in the glucose signaling pathway that regulates insulin secretion from  $\beta$ -cells, making them "glucose competent", tGLP-I might be useful for the treatment of NIDDM.

The presence of a glucose threshold for the insulinotropic action of GIP is teleologically necessary to prevent inappropriate insulin release and hypoglycemia. Since untreated patients with NIDDM have plasma glucose levels above this threshold even in the fasting state, Jones *et al.* (1989) investigated whether GIP was insulinotropic during fasting in these individuals. It was found that the glucose threshold for the insulinotropic

action of GIP was not altered in the NIDDM patients, and thus there was a significant insulin response to intravenous GIP even during fasting. The resulting secretion of insulin was, however, insufficient to decrease plasma glucose levels. In normal subjects, persistently elevated GIP levels only have a short lived effect on insulin release, as plasma glucose levels are only above the necessary threshold for a relatively short time following meals. Jones *et al.* (1989b) reasoned that in untreated NIDDM subjects exhibiting an insulintropic effect to GIP in the hyperglycemic fasting state, persistently elevated GIP would be likely to exert an effect on insulin secretion throughout the day.

Obesity is often associated with glucose intolerance and hyperinsulinemia in both the fed and fasted state (Perley and Kipnis, 1967; Creutzfeldt *et al.*, 1978; Chan *et al.*, 1984). Unlike diabetics, however,  $\beta$ -cell sensitivity to GIP appears to be unaltered in obese humans. Intravenous infusion of porcine GIP during a hyperglycemic clamp produced a similar insulin response in both lean and obese volunteers (Amland *et al.*, 1985). Elahi *et al.* (1984) calculated  $\beta$ -cell sensitivity to GIP from the insulin and IRGIP responses to oral glucose and found it normal in obese individuals. The GIP sensitivity of  $\beta$ -cells from the hyperinsulinemic obese Zucker rat has also been measured. Chan *et al.* (1984) found that the insulin response to GIP was enhanced in the pancreas of obese animals and additionally, that the glucose threshold for the insulintropic action of GIP was well below fasting levels in these rats. Thus, as with NIDDM, there may be inappropriate GIP insulintropic activity occurring associated with obesity.

How might the insulintropic activity of GIP become uncontrolled? A defect in the feedback control of GIP secretion by insulin was proposed as the explanation for the exaggerated IRGIP release after food stimulation in obesity (Brown and Otte, 1978) and in NIDDM (Crockett *et al.*, 1976; Ross *et al.*, 1977). Hampton *et al.* (1983) studied the effects of pre-treatment with a high fat diet on the IRGIP and insulin responses to oral fat and glucose in rats. The pre-treatment resulted in increased IRGIP secretion in response to oral fat and abolition of the feedback inhibition of exogenous insulin on fat-stimulated

IRGIP release. It was also noted that some degree of insulin resistance was established. Willms *et al.* (1978), found that 5 days of caloric restriction abolished the exaggerated IRGIP response to fat or a mixed meal in obese subjects, suggesting that a previously high calorie intake might have been responsible for the excessive IRGIP production. Creutzfeldt *et al.* (1978) also reported an exaggerated IRGIP response in obese subjects given a high calorie mixed meal. This was not, however, observed in subjects after 5 days of dietary restriction. Hampton *et al.* (1983) suggested that this might be due to a decreased responsiveness of the GIP secreting cells to insulin due to a reduction in insulin receptor numbers. Investigation of insulin receptors on human monocytes has shown that insulin receptor number can be altered by dietary changes (Pedersen *et al.*, 1980) and that these changes can occur rapidly - within 24 h (Schluter *et al.*, 1980).

#### **1.14 THESIS INVESTIGATION**

In summary, the hormone GIP, has been extensively studied as a potential enterogastrone and an incretin involved in the enteroinsular axis. In addition, evidence has been presented that suggests GIP has other roles, such as direct actions on fat metabolism. These activities have been substantiated by the location of specific GIP receptors on the respective target tissues. Studies have revealed specific regions of the molecule to be important for receptor interactions, and activation of intracellular messenger systems have been associated with GIP binding. The endocrine cells that release GIP have been identified, and the gene coding for the prepropeptide sequenced. Numerous GIP assays have been established allowing for the measurement of GIP responses to the ingestion of nutrients or the intravenous administration of various secretagogues, and have enabled an estimation of the half-life of GIP immunoreactivity in plasma.

Observations from circulating IRGIP profiles do not allow direct assessment of the actions of GIP secretagogues and inhibitors because of the potential interactions

among paracrine, endocrine, neural and luminal influences at the level of the GIP cell. These confounding factors can only be overcome by studying GIP release at the cellular level. Such methods allow the investigation of many components potentially influencing GIP release, individually and in combination. Studies at the cellular level also allow investigation of the intracellular mechanisms by which agents act on the GIP cell. One objective of these thesis investigations was therefore to develop methods that would enable the study of GIP secretion at the cellular level in a controlled environment.

Observations of circulating IRGIP levels have also been used to evaluate the immunoreactive half-life of the hormone. It is not known, however, how accurately these measurements reflect the time of biological activity of circulating GIP. There is evidence to suggest that peptidases may convert GIP to a biologically inactive fragment, that is immunologically indistinguishable from the native form. Reports of circulating IRGIP levels may therefore have little meaning in terms of the actual physiological potency of plasma GIP. A second aim of the thesis investigations was consequently to carefully examine the metabolic processing of GIP and to determine a physiological time frame during which this process occurs. A better understanding of GIP secretion and metabolism is critically important to resolving the potential role this hormone may have in both normal and pathophysiological situations.

## **CHAPTER 2**

### **GIP RELEASE FROM ISOLATED CANINE AND PORCINE ENDOCRINE CELLS**

#### **2.1 INTRODUCTION**

A major factor impeding study of isolated GIP cells is the diffuse nature of the distribution of endocrine cells throughout the small intestine. Separation techniques to produce enriched preparations of gastric endocrine cells were developed by Soll and colleagues in 1984. These techniques were used to study the release of somatostatin and have been modified to permit the study of the local regulation of peptide secretion from purified gastrin (Giraud *et al.*, 1987; Campos *et al.*, 1990), cholecystokinin (Barber *et al.*, 1986b; Koop and Buchan, 1992), neurotensin (Barber *et al.*, 1986a), peptide YY (Aponte *et al.*, 1988), motilin (Poitras *et al.*, 1993) and enteroglucagon cells (Buchan *et al.*, 1987). The aim of these studies was to modify these techniques to allow for the isolation, enrichment, and culture of intestinal endocrine cells in order to study the local regulation of GIP secretion.

#### **2.2 MATERIALS AND METHODS**

##### **2.2.1 ANIMAL TISSUES**

Initial studies were performed with adult mongrel dogs (n = 10), but due to unavailability of dogs, the model was later adapted for use with pigs (n = 10). Fasted male and female dogs or pigs were sedated with phentanol triperidol (0.1 ml/kg) administered with atropine (0.05 mg/kg), anesthetized with intravenous sodium pentobarbital (30 mg/kg) and prepared for abdominal surgery. The animals were then bled from the abdominal vena cava, and the upper small bowel was removed. The intestine was immediately cut open and washed in ice-cold Hanks' balanced-salt solution (HBSS; Gibco Laboratories, Burlington, Ont.) ~pH 7.4 containing 0.1% bovine serum albumin (BSA fraction V; Sigma Chemical, St. Louis, MO).

### 2.2.2 ISOLATION OF MUCOSAL CELLS

The canine or porcine mucosa was bluntly dissected from the intestine, weighed and chopped finely with scalpels. Aliquots (10 g) of canine mucosa were initially digested in 50 ml basal medium Eagle (BME; Gibco) ~pH 7.4, with a mixture of enzymes consisting of 75 U/ml type I collagenase (Sigma), 75 U/ml type XI collagenase (Sigma), 0.9 U/ml type IX protease (Sigma) and 1 U/ml trypsin (Worthington Biochemical Corp, Freehold NJ) for 1 h in a shaking water bath at 37°C. The total volume was then doubled with HBSS-BSA and allowed to settle ~10 min. The supernate containing detached cells was discarded, and the remaining tissue was further digested in the enzyme mixture for two 45 min periods, with each step followed by the addition of 300 µl of 0.5 M ethylenediaminetetraacetic acid (EDTA; BDH, Toronto, Ont.) for 15 min. A similar procedure was followed for the porcine mucosa, but as it was much thinner, the digestions were performed only with 75 U/ml type I collagenase (Sigma). The cell suspension resulting from digest 3 or 4 of the canine and porcine mucosa was filtered through Nitex mesh (200 µm, B & SH Thompson, Scarborough, Ont.) and washed and centrifuged at 200 x g (Baxter Biofuge) twice with HBSS-BSA supplemented with 0.01% dithiothreitol (DTT; Sigma) and 0.001% deoxyribonuclease (DNase; Sigma). The cells were then filtered a second time through fine Nitex mesh (62 µm, B & SH Thompson), counted, and diluted in HBSS-BSA-DTT-DNase to  $6 \times 10^6$  cells/ml for elutriation.

### 2.2.3 ENRICHMENT OF MUCOSAL ENDOCRINE CELLS

Counter-flow centrifugal elutriation of cells was first reported by Lindahl in 1948. This procedure separates cells on the basis of their sedimentation coefficients. The cell suspension is pumped into a rotating chamber, and cells are held where their sedimentation rate is balanced by the flow of fluid through the separation chamber. Different fractions of homogeneous cells are then 'eluted' by either increasing the flow rate through the chamber or decreasing the centrifugal speed. The appropriate flow rates

and centrifugation speeds were determined empirically. Batches consisting of  $1.5 \times 10^8$  canine or porcine dispersed cells were introduced into the Beckman elutriator (model J2-21M/E; Beckman, Los Angeles, CA) via a pump (Cole Parmer) connected to a sterile source of HBSS-BSA. The enzyme dispersed canine cells were loaded into the elutriator chamber at a rotor speed of 2500 rpm with a flow rate of 25 ml/min and washed for 2 min. A 100 ml fraction (F1) was collected after changing the flow rate to 30 ml/min. A second 100 ml fraction (F2) was obtained at a rotor speed of 2100 rpm and a flow rate of 55 ml/min. The elutriation procedure for the dispersed porcine mucosal cells was slightly different. Cells were loaded at a rotor speed of 3000 rpm and a flow rate of 25 ml/min. The F1 and F2 fractions were eluted at 2800 rpm, 30 ml/min and 2200 rpm, 55 ml/min respectively.

Samples of cells ( $5 \times 10^6$ , in triplicate) from the pre-eluted cells, F1 and F2 were extracted in 1 ml of 2 M acetic acid in 1.5 ml Eppendorf microcentrifuge tubes. The tubes were boiled for 10 min, centrifuged for 5 min at 7000 x g (Baxter Biofuge) at 4°C and the supernate stored at -20°C for subsequent radioimmunoassay (RIA). Prior to being assayed, these extracts were neutralized with 10 M NaOH. Results indicated that the majority of canine and porcine IRGIP cells eluted in F2, and consequently these cells were cultured for immunocytochemical and hormone release studies.

#### *2.2.4 ENDOCRINE ENRICHED CELL CULTURE*

Canine and porcine cells from F2 were concentrated by centrifuging at 200 x g for 10 min, and then resuspended in sterile culture medium. The canine culture medium consisted of 47.5% Dulbecco's modified Eagle medium (DMEM, Terry Fox Laboratory, Vancouver, BC) and 47.5% Ham's F-12K (Terry Fox Lab), containing 5.5 mM glucose, supplemented with 5% fetal calf serum (FCS, Gibco), 2 ng/ml nerve growth factor (Collaborative Research, Bedford, MA), 8 µg/ml insulin (Sigma), 1 µg/ml hydrocortisone (Sigma), 50 µg/ml gentamycin sulfate (Sigma), 0.25 µg/ml amphotericin B (Fungizone,

Gibco), 50 U/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco), and 20 µM cytosine β-D-arabinofuranoside (Gibco). The porcine culture medium was identical with the exception of 10% heat inactivated porcine serum substituted for the 5% FCS. Plates for culturing the cells (12-well Costar, Cambridge, MA) were coated with collagen to promote cell adherence. To prepare the collagen coated plates, rat tail collagen was dissolved in 0.017 M acetic acid (3.5 mg collagen/ml) and then added to each well and left for 45 min. The solution was then aspirated, and the plates allowed to air dry prior to storage at 4°C. Cells from F2 were seeded at  $5 \times 10^6 \cdot \text{ml}^{-1} \cdot \text{well}^{-1}$  in the collagen coated culture plates and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 40 h.

### *2.2.5 IMMUNOCYTOCHEMISTRY*

Both cultured cells and intact intestine were prepared for immunocytochemical analysis. Sections of intestine (1 cm<sup>2</sup>) were fixed in Bouin's solution (75 ml saturated picric acid, 25 ml 40% formaldehyde, 5 ml acetic acid) for 1 h, and then washed and stored in 70% ethanol prior to processing. The tissue was dehydrated through a graded series of alcohol and xylene using a histomatic tissue processor (model 166; Fisher). The samples were then embedded in paraffin wax using a histo-center II-N (Thermolyne). Sections 5 µm thick were cut using a microtome (1130/Biocut; Reichert-Jung) and dried on gelatin coated slides. Prior to staining, wax was removed by soaking the sections for 10 min in xylene followed by clearing in petroleum ether for 2 min. Cultured cells were washed 2 x in phosphate-buffered saline (8.0 g/l NaCl, 0.2 g/l KH<sub>2</sub>PO<sub>4</sub>, 1.15 g/l Na<sub>2</sub>HPO<sub>4</sub>) plus 0.1% sodium azide (NaN<sub>3</sub>; PBS-azide). The cells were then fixed in Bouin's solution for 10 min and then washed 3 x and stored in PBS-azide.

Tissue sections and cultured cells were immunostained with primary antibodies for all hormones listed in Table 1 using the avidin-biotin peroxidase method. To prevent nonspecific staining caused by the presence of endogenous peroxidase activity, samples

were immersed in PBS-azide containing 0.3% H<sub>2</sub>O<sub>2</sub> (BDH Inc. Toronto, Ont.) for 30 min at room temperature and then washed in PBS-azide. The fixed cells and 5 µm sections of jejunum were incubated with the antibodies diluted in PBS-azide containing 10% swine serum as listed in Table 1 for a period of 48 h at 4°C. After washing 3 x in PBS-azide, the biotinylated secondary antibodies (Table 1) were applied and incubated for 1 h at room temperature. The secondary antibodies were then localized with a premixed (10 min) avidin-biotin peroxidase solution (Vectastain ABC Kit; Vector Laboratories, Burlingame CA) for 1 h. The resulting complex was identified with pH 7.6 tris buffer solution of diaminobenzidine (BDH; 125 µg/100 ml) containing 40 mg NH<sub>4</sub>Cl, 200 mg D-glucose, and 0.3 mg glucose oxidase (Sigma). Staining was monitored with a Zeiss Axiophot microscope, and the reaction was terminated after the appearance of positive cells (15-45 min) by washing in PBS-azide. In order to quantify staining, a minimum of 10 groups of approximately 100 cells were observed for positive cells, which were then expressed as a % of the total number counted.

The cultures and intact mucosa were also stained for mucin containing cells using a combined alcian blue-periodic-acid Schiff technique. Fixed cells were treated with 1% alcian blue (Fisher, Fair Lawn, NJ) in 3% acetic acid for 5 min and then washed in distilled water. The cells were treated with 1% aqueous periodic acid solution for 5 min and again washed in distilled water. Finally, they were washed in Schiff's reagent (0.5% basic fuchsin, Fisher; 0.5% sodium metabisulphite, Fisher; 0.5% hydrochloric acid, and 0.5% decolorizing charcoal, Fisher) for 15 min followed by 10 min of running water. Neutral mucins stained red and acid mucins blue (pink and purple respectively in figures).

#### *2.2.6 RELEASE EXPERIMENTS*

On the experimental day, the culture medium was removed by pipet and the porcine or canine cells washed with 1 ml release medium, consisting of DMEM (Sigma) supplemented with 25 mM NaHCO<sub>3</sub>, 25 mM HEPES, 5 mM glucose, 1% FCS, and 2%

aprotinin (Trasylol, 500 kallikrein-inhibiting U/ml, Miles Pharmaceuticals, Rexdale, Ont.). This was followed by addition of 1 ml release medium containing the appropriate glucose concentration to each well. Stock solutions of other secretagogues were added as 25  $\mu$ l of a 40 x concentration to 975  $\mu$ l of release medium in 5 mM glucose, in triplicate. Cells were incubated for 2 h, after which the release medium was removed and stored in 1.5 ml Eppendorf microcentrifuge tubes on ice. The release medium was centrifuged for 5 min at 7000 x g (Baxter Biofuge) at 4°C and the supernate stored at -20°C for subsequent GIP and somatostatin RIA. Adherent cells in control wells (2 wells per plate) were extracted in 2 M acetic acid for determination of total cell peptide content per well. After centrifugation to remove particulate matter, the supernate was stored at -20°C. Prior to being assayed, these extracts were neutralized with 10 M NaOH. Peptide secretory values from each well were expressed as percentage of total cell peptide content (%TCC) as measured from extracts.

### *2.2.7 SECRETAGOGUES*

The somatostatin antibody (SOMA 10; Regulatory Peptide Group, Vancouver, BC, Canada) was diluted in release medium to 10  $\mu$ g/ml/well. Porcine gastrin releasing peptide (GRP, Peninsula Laboratories Inc, Belmont CA) was diluted to a concentration of  $4 \times 10^{-6}$  M with release medium and kept on ice. This solution was serially diluted to form other stock solutions of  $4 \times 10^{-7}$ ,  $4 \times 10^{-8}$ , and  $4 \times 10^{-9}$  M. Forskolin (Sigma) was prepared as a stock solution of  $4 \times 10^{-3}$  M in ethanol and serially diluted in DMEM release medium to concentrations of  $4 \times 10^{-4}$ ,  $4 \times 10^{-5}$ , and  $4 \times 10^{-6}$  M. A23187 (Sigma) was prepared as a stock solution of  $4 \times 10^{-4}$  M in dimethylsulfoxide (DMSO) and serially diluted in DMEM release medium to concentrations of  $2 \times 10^{-4}$  and  $4 \times 10^{-5}$  M. The effect of the solvents ethanol and DMSO on IRGIP or IRSS release was tested by adding identically diluted solvents to medium in corresponding control wells. Peptide values

resulting in these controls were subtracted from the appropriate values obtained with forskolin or A23187.

### 2.2.8 RADIOIMMUNOASSAY FOR GIP

The RIA used to measure immunoreactive GIP (IRGIP) was originally described by Kuzio *et al.* (1974) and modified by Morgan *et al.* (1978). The  $^{125}\text{I}$ -GIP used for the RIA was prepared by a slight modification of the chloramine-T method as was performed by Greenwood *et al.* (1963). The column for label purification was prepared prior to the iodination with Sephadex G-15 (Pharmacia, Uppsala, Sweden). The G-15 was first swollen in 0.2 M acetic acid with the 'fines' repeatedly decanted, and then degassed under water vacuum for ~2 h. A 1:1 'slurry' of gel and acetic acid was then poured into a 0.5 x 20 cm plastic pipet with a glass wool plug and allowed to settle at an eluent (0.2 M acetic acid plus 5.0% BSA; RIA Grade) flow rate of ~250  $\mu\text{l}/\text{min}$  for 4-5 h. The column buffer was supplemented with 2% aprotinin for ~1 h before the iodination.

The iodination reaction was performed in a siliconized test tube containing 5  $\mu\text{g}$  of porcine GIP (Regulatory Peptide Group) dissolved in 100  $\mu\text{l}$  40 mM pH 7.5 phosphate buffer (10 x stock prepared by titrating 0.4 M  $\text{Na}_2\text{HPO}_4$  with 0.4 M  $\text{NaH}_2\text{PO}_4$  to pH 7.5). In a fume hood behind lead, 0.5 mCi  $\text{Na}^{125}\text{I}$  (NEN Research Products, Wilmington, DE) and 10  $\mu\text{l}$  chloramine-T (Fisher, Fair Lawn, NJ; 4 mg/ml phosphate buffer) were added while the reaction vessel was agitated. The reaction was stopped after 15 sec by the addition of 20  $\mu\text{l}$  sodium metabisulfite (Fisher; 12.6 mg/ml phosphate buffer). Separation of the free iodine from the  $^{125}\text{I}$ -labeled hormone was achieved using gel filtration on the Sephadex G-15 column previously poured. Fractions of 400  $\mu\text{l}$  were collected at a flow rate of 250  $\mu\text{l}/\text{min}$  and 10  $\mu\text{l}$  aliquots were counted (LKB Wallace 1277 Gammamaster). A typical column profile obtained with these counts is shown in Figure 23.

Fractions comprising the  $^{125}\text{I}$ -GIP peak were assayed for 'damage' - or the ability of the label to bind to charcoal. The 10  $\mu\text{l}$  fractions were diluted to ~5000 cpm/100  $\mu\text{l}$  of assay

buffer (pH 6.5, 40 mM phosphate buffer containing 5% CEP and 2% aprotinin). Then, 100  $\mu$ l aliquots of the diluted fractions were added in triplicate to tubes containing 900  $\mu$ l assay buffer. Dextran-coated charcoal was prepared by dissolving 2.5 mg/ml dextran (T-70, Pharmacia) in 40 mM phosphate buffer (pH 6.5), adding 2.5 mg/ml activated charcoal (Norit Decolorizing Carbon), 0.75% aprotinin, plus 5% CEP, and stirring for 2h at 4°C. Each tube of diluted label received 200  $\mu$ l of the dextran-coated charcoal and was vortexed and centrifuged (30 min, 1000 x g). Both the supernatant and the charcoal pellet were counted and the percent of total radioactivity bound (%B) to charcoal (i.e.  $^{125}$ I-GIP) was calculated. The fraction from the  $^{125}$ I-GIP peak with the highest radioactivity and the first 1-2 fractions on the descending portion of the peak usually had the greatest adsorption to charcoal (usually >95%). These fractions were also previously shown to have the greatest immunoreactivity in the RIA, possibly due to decreased substitutions of iodine into the GIP molecule (Kuzio *et al.*, 1974). Therefore, these fractions were pooled and diluted to  $2.5 \times 10^6$  cpm/100  $\mu$ l in a 1:1 mixture of column buffer and acid ethanol (750 ml/l 95% ethanol plus 15 ml/l concentrated HCl), and stored at -20°C until used in the RIA.

The rabbit anti-porcine GIP serum used for the RIA (RK343F also referred to as LMR34) was generously supplied by Dr LM Morgan (University of Surrey, UK). Negligible cross-reactivity of this antibody was demonstrated with cholecystokinin, insulin, pancreatic polypeptide, glucagon, secretin, and vasoactive intestinal peptide (Morgan *et al.*, 1978). This antisera was stored as 100  $\mu$ l aliquots of a 1:40 dilution in assay buffer at -20°C, and diluted to 1:6000 for use in the RIA (final dilution in RIA of  $1:3 \times 10^5$ ).

Stock standards for the RIA were prepared from 100  $\mu$ g porcine GIP (Regulatory Peptide Group) dissolved in 0.2 M acetic acid plus 0.5% BSA (Pentex) and 2% aprotinin to a concentration of 100  $\mu$ g/ml. Aliquots of 1  $\mu$ g (10  $\mu$ l) were then lyophilized and stored at -20°C. When needed, an aliquot was dissolved in 100  $\mu$ l acetic acid (100 mM) and 100  $\mu$ l assay buffer to achieve a concentration of 5  $\mu$ g/ml. This was then further diluted to 20 ng/ml

by diluting 40  $\mu$ l up to 10 ml in assay buffer, and then serially diluted in assay buffer to provide a range of standards down to 78 pg/ml.

On the first day of the assay, 200  $\mu$ l assay buffer was added to all sample tubes followed by either 100  $\mu$ l of porcine GIP standard (7.8 - 2000 pg/100  $\mu$ l) or 100  $\mu$ l of unknown sample, and 100  $\mu$ l of antibody (LMR34). For the non-specific binding (NSB) and zero-binding ( $B_0$ ) tubes, 100  $\mu$ l of buffer was used to replace the antibody or standard, respectively. All tubes were vortexed and incubated 24 h at 4°C. On day two, 100  $\mu$ l of porcine GIP tracer (~5000 cpm/100  $\mu$ l) was added to each tube and vortexed. On day three, 500  $\mu$ l of polyethylene glycol (250 g/l H<sub>2</sub>O PEG 8000; Fisher) was added to all but the total counts and the tubes were vortexed and centrifuged at 1000 x g for 45 min, after which the supernatant was decanted and the pellets were allowed to dry overnight. The specific radioactivity in the bound fraction (B) was then determined by counting the pellets for 2 min on a gamma counter (LKB Wallace 1277 Gammamaster) and subtracting the NSB. Sample values were computed from a spline - smooth plot of B expressed as a percentage of the  $B_0$  against concentration using an IBM 386 microprocessor with RiaCalc (Pharmacia).

Controls for the GIP RIA were prepared by dissolving 1  $\mu$ g porcine GIP (Regulatory Peptide Group) in 500 ml of 40 mM phosphate buffer (pH 6.5) containing 0.5% BSA (Pentex) and 2% aprotinin (200 pg/100  $\mu$ l). Aliquots of 1 ml were stored in Eppendorf tubes at -20°C until use in the RIA. Inter- and intra-assay variation calculated from these controls was  $10 \pm 2\%$  and  $5 \pm 1\%$ , respectively. Control studies were also performed to examine the effect of incubation of GIP at 37°C in release medium, with and without isolated cells. No significant difference was noted in standard curves prepared from porcine GIP standards under normal assay conditions or after 2 h incubation with cells.

### 2.2.9 RADIOIMMUNOASSAY FOR SOMATOSTATIN

The RIA used to measure immunoreactive somatostatin (IRSS) was as described by McIntosh *et al.* (1978; 1987). Synthetic Tyr<sup>1</sup>-somatostatin was iodinated by the chloramine-T method, and the labeled peptide was separated from free <sup>125</sup>I and 'damaged' material by adding 1 ml hormone free plasma followed by 20 mg microfine silica (QUSO G32; Philadelphia Quartz Co., USA). After centrifugation the pellet was washed twice with 1 ml distilled water, and the label eluted with 1 ml acetic acid/acetone/water (0.1/3.9/4). The label was then diluted in 0.1 M acetic acid containing 0.5% BSA (Pentex, Miles Labs) to 1 x 10<sup>6</sup> cpm/10 µl and lyophilized and stored at -20°C. On the day of the RIA, an aliquot of label was dissolved in 1 ml 2 mM ammonium acetate buffer (pH 4.6) and applied to a column (0.9 x 10 cm) of CM-cellulose (CM 52; Whatman) equilibrated in the same buffer. The column was then washed with ~50 ml buffer pumped at a flow rate of 1 ml/min and the label was eluted with 200 mM ammonium acetate (pH 4.6). The peak fraction (2 min/fraction) was then neutralized with 2 M NaOH, and diluted in assay buffer (50 mM barbital buffer pH 7.4 containing 0.01% merthiolate, 1% aprotinin, and 0.5% BSA) to ~3500 cpm/100 µl.

A somatostatin monoclonal antibody (SOMA 03; Regulatory Peptide Group) was used in the assay, prepared from a stock of 1:100 (in 0.9% NaCl containing 0.1% NaN<sub>3</sub> and 0.5% BSA) stored at 7°C. Antibody was diluted to 1:1 x 10<sup>6</sup> in assay buffer the day of the RIA for a final concentration of 1:4 x 10<sup>6</sup>. This antibody was shown not to cross-react with GIP, gastrin or motilin and is directed towards the central region of the somatostatin-14 molecule, recognizing somatostatin-14 and -28 equally (McIntosh *et al.*, 1987).

Assay standards were prepared by dissolving synthetic cyclic somatostatin-14 (Penninsula) in 100 mM acetic acid containing 0.5% BSA (Pentex) to obtain a concentration of 100 µg/ml. Aliquots of 5 µg were then lyophilized and stored at -20°C. On the day of the assay, an aliquot was reconstituted in 100 µl cold distilled water

followed by 400  $\mu\text{l}$  assay buffer to obtain a concentration of 10  $\mu\text{g}/\text{ml}$ . This was then serially diluted (1:10, 1:10, 1:10, 1:20) to a concentration of 500  $\text{pg}/\text{ml}$ , and then sequentially 1:2 to 3.9  $\text{pg}/\text{ml}$  for a total of 8 standards for the RIA.

The same general procedure was used for the RIA as in the GIP assay, with the following differences. All the assay constituents were added to the tubes the first day in the following order, 100  $\mu\text{l}$  standard or sample, 100-300  $\mu\text{l}$  assay buffer, 100  $\mu\text{l}$  antibody and 100  $\mu\text{l}$  label for a total volume of 400  $\mu\text{l}/\text{tube}$ . The assays were then vortexed and incubated for ~72 h at 4°C. The bound peptide was then separated from the unbound by adding 1 ml dextran-coated charcoal (plus CEP) to each tube (except total counts), allowing the assays to sit ~15 min, centrifuging at 1000 x g for 30 min, and then decanting the supernatant. After drying, the pellet was counted for 3 min on a gamma counter, and sample values were computed from a spline - smooth plot of standard B expressed as a percentage of the  $B_0$  against concentration.

Samples containing known amounts of somatostatin for controls could not be kept for long periods of time because of loss of immunoreactivity. Interassay variation was therefore computed from standards of 10 standard curves at  $11 \pm 2\%$ . Intraassay variation as computed from standards (20 duplicates) placed randomly throughout the assay was  $5 \pm 1\%$ .

### *2.2.10 EXPRESSION OF RESULTS*

Release data were calculated as mean  $\pm$  SEM for a percentage of the total cell content (%TCC) to reduce variation between animals. Statistical significance was determined using the Student's t test and was set at the 5% level. Support for using this method as opposed to an analysis of variance test followed by one of the *a posteriori* tests was provided by Sinclair (1988).

## 2.3 RESULTS

### 2.3.1 MUCOSA DIGESTION

The digestion protocol described for both canine and porcine mucosal cells was empirically determined to give the greatest yield of IRGIP cells. Other methods attempted, such as tissue homogenization, were not successful. The duration of time between bleeding the animal and retrieving the tissue into cold Hank's (period of tissue anoxia) was determined to be a critical factor for the cell survivability rate. Porcine bowel supplied by a slaughter house which was anoxic for 5-10 min usually did not yield significant numbers of viable endocrine cells. In addition, these animals were usually not fasted prior to slaughter, and the presence of pancreatic and intestinal enzymes in the intestinal lumen undoubtedly contributed to tissue degradation.

While the enzymatic digestion used in dispersing the canine mucosal cells undoubtedly resulted in cell damage, collagenase on its own was not sufficient. With just type I collagenase, 8-10 digests were required to disperse the mucosa and this repetitive process led to excessive cell death. In contrast, the porcine mucosa was much thinner and digestion with type I collagenase was found sufficient to disperse the cells after 3-4 digests. The enzyme mixture used for canine mucosa completely digested the porcine tissue in 1-2 digests, but the cells did not recover from this process. In both the canine and porcine experiments, extracts of cells were kept from each digestion for assay of IRGIP content. It was usually noted that the later digests (3 and 4) had the greatest concentration of IRGIP. Early digests were observed to contain greater numbers of red blood cells, cellular debris, and non-viable cells. Viability of cells from later digests was ~98%, as assessed by trypan blue exclusion.

### 2.3.2 ENDOCRINE CELL ENRICHMENT AND CULTURE

The elutriation procedure yielded two main fractions, designated F1 and F2. The F1 fraction contained most of the bacteria, red blood cells, and debris that was present in

the digested cells. The F2 fractions for both porcine and canine cells were collected under similar elutriation conditions, and made up a population of cells of similar size. The IRGIP and IRSS content of  $5 \times 10^6$  canine cells from the eluted fractions was compared to that of the same number of cells from a pre-elutriation (PE) sample (Figure 3A). While there was no significant difference between the PE and F1 fractions, the F2 fractions contained approximately 3 times the IRGIP and IRSS concentrations as the PE fractions ( $p < 0.005$ ). The canine F2 fraction ( $5 \times 10^6$  cells) contained  $38 \pm 6$  ng IRGIP and  $7.0 \pm 0.6$  ng IRSS. A similar enrichment of IRGIP was observed in the elutriated porcine cells (Figure 3B), with the F2 fraction ( $15.4 \pm 2.5$  ng) containing ~3 times the PE fraction ( $5.0 \pm 0.8$  ng). Although IRSS was not consistently measured in the porcine extracts, preliminary results suggest that while there was enrichment, considerably less IRSS was present when compared to the same number of eluted canine cells (F1: 52 pg, F2: 321 pg;  $n = 2$ ).

The culture conditions were found to be an important factor in the success of the peptide secretion experiments. Collagen coated plates provided a suitable substrate for endocrine cells to attach to. These cells did not adhere to non-coated plates, and other substrates, such as poly-l-lysine, laminin, and fibronectin produced various degrees of success. As only 20 - 25% of the cells in each well adhered, the maximum number of cells deemed possible to culture ( $5 \times 10^6$ ) were added to each well. This was important in order to attain sufficient numbers of IRGIP cells to produce measurable levels of IRGIP. Attempts at culturing the dispersed and enriched cells in large flasks and then transferring to Eppendorfs for release experiments were also not successful. After 40 h culture, 98% of adherent cells were viable as assessed by trypan blue exclusion. The viability decreased rapidly as cells were cultured longer. Extracts of canine cells contained  $11.5 \pm 2.5$  ng IRGIP/well and  $1.4 \pm 0.2$  ng IRSS /well, while porcine cells contained  $6.6 \pm 0.5$  ng IRGIP/well.

### 2.3.3 IMMUNOCYTOCHEMISTRY OF INTACT MUCOSA AND CULTURED CELLS

Figure 4 shows canine jejunum immunostained for IRGIP using the peroxidase method. The IRGIP cells, shown by arrows are sparsely distributed, and account for < 0.1% of the cells of the intact mucosa. A similar observation was made for IRSS distribution (data not shown). Figures 5 and 6 show the isolated canine mucosal cells after enrichment and 40 h culture, immunostained using the peroxidase method for GIP and SS, respectively. At the time of plating, the F2 fraction contained mainly single, spherical cells, but after culture, endocrine cells appeared to flatten and adopt a more ovoid or triangular cell appearance (see Figure 5 & 6). During the culture period, adhered clusters of up to 200 cells formed, including the endocrine cells, and these cells were observed to form connections with neighbouring cells. IRGIP cells accounted for approximately 10% of the adherent canine cell population, while IRSS cells accounted for 5% on average. A similar proportion of cultured porcine cells stained positive for IRGIP (8%), while IRSS cells added up to ~1% of the adherent population (data not shown). A large portion of cells in the intact mucosa were mucin containing (Figure 7), and accounted for ~80% of the adherent canine (Figure 8) and porcine cell population. Less than 1% of the canine or porcine cultured cells stained for motilin, secretin, gastrin/CCK, glicentin, or neurotensin (data not shown).

### 2.3.4 IRGIP SECRETION IN RESPONSE TO $K^+$

The effect of depolarization of the isolated canine and porcine endocrine cells was investigated using  $K^+$ . Figure 9 shows the IRGIP secretory response to increasing concentrations of  $K^+$  from 10 to 55 mM over a 2 h period from canine and porcine cells. Basal release of IRGIP in 5 mM glucose, 5 mM  $K^+$  was  $2.7 \pm 0.4$  %TCC ( $310 \pm 45$  pg/ml) for canine cells and  $4.6 \pm 0.4$  %TCC ( $304 \pm 23$  pg/ml) for porcine cells. Potassium concentrations ranging from 20 to 55 mM significantly stimulated IRGIP release from canine cells in a concentration-dependent fashion when compared to basal

( $p < 0.05$ ). A similar response was observed in the porcine cells, however, a significant increase in IRGIP release was also observed at 10 mM  $K^+$  ( $p < 0.05$ ). Stimulation at 55 mM  $K^+$  was  $18.8 \pm 2.1$  %TCC ( $2.2 \pm 0.2$  ng/well), or approximately 7 times basal release ( $p < 0.001$ ) for canine cells and approximately 4 times basal release ( $p < 0.001$ ) for porcine cells ( $19.9 \pm 2.0$  %TCC or  $1.3 \pm 0.1$  ng/well). Basal release of IRSS from canine cells was  $2.0 \pm 0.2$  %TCC ( $28 \pm 3$  pg/ml) and increased significantly by 55 mM  $K^+$  to  $4.1 \pm 0.3$  %TCC ( $57 \pm 4$  pg/well;  $p < 0.05$ ,  $n = 3$ , data not shown).

### *2.3.5 IRGIP SECRETION IN RESPONSE TO GLUCOSE*

Figures 10 and 11 show the effects of graded glucose concentrations from 5 to 20 mM on IRGIP secretion from canine and porcine cells, respectively. In the canine cells, the release of IRGIP was enhanced significantly ( $p < 0.05$ ) over basal by glucose levels of 15 and 20 mM, while the addition of 10 mM glucose had no significant effect. In contrast, IRGIP release from porcine cells was significant at glucose concentrations  $\geq 10$  mM ( $p < 0.05$ ). With the addition of 10  $\mu$ g/well somatostatin antibody SOMA 10 to the canine cells, IRGIP release in the presence of 5 mM glucose significantly increased to  $6.1 \pm 0.7$  %TCC ( $p < 0.05$ ), comparable to that induced by 20 mM glucose alone (Figure 10). Glucose had a minimal effect on IRSS release from canine cells, yielding a significant increase only at 20 mM glucose (from  $2.0 \pm 0.2$  %TCC to  $2.8 \pm 0.3$  %TCC;  $p < 0.05$ ,  $n = 4$ , data not shown).

### *2.3.6 IRGIP SECRETION IN RESPONSE TO A23187*

The involvement of  $Ca^{2+}$  in the release of IRGIP was investigated using the  $Ca^{2+}$  ionophore A23187. Figure 12 shows the effect of A23187 at concentrations of 1, 5, and 10  $\mu$ M on IRGIP release from canine epithelial cells in the presence of 5 mM glucose. While the addition of 5  $\mu$ M A23187 produced only a small but significant ( $p < 0.05$ ) increase over basal IRGIP release, the addition of 10  $\mu$ M A23187 resulted in an IRGIP

output approximately 4.1 times basal (to  $11.2 \pm 1.8$  %TCC;  $p < 0.01$ ). IRSS release was increased significantly by  $10 \mu\text{M}$  A23187, from  $2.0 \pm 0.2$  %TCC to  $3.9 \pm 0.6$  %TCC ( $p < 0.05$ ,  $n = 4$ , data not shown).

### *2.3.7 IRGIP SECRETION IN RESPONSE TO GRP*

To investigate the potential for peptidergic control of IRGIP secretion, the effect of graded concentrations of porcine GRP on IRGIP release from canine epithelial cells in the presence of  $5 \text{ mM}$  glucose was examined (Figure 13). GRP significantly ( $p < 0.05$ ) stimulated IRGIP release in a concentration-dependent fashion with significant release occurring at a concentrations of  $1 \text{ nM}$  and greater. At  $100 \text{ nM}$  GRP, IRGIP release was  $7.2 \pm 1.0$  %TCC, approximately 2.7 times basal ( $p < 0.01$ ). This concentration of GRP also significantly increased IRSS release from  $2.0 \pm 0.2$  %TCC to  $2.9 \pm 0.4$  %TCC ( $p < 0.05$ ,  $n = 4$ , data not shown).

### *2.3.8 IRGIP SECRETION IN RESPONSE TO FORSKOLIN*

A possible role for adenylate cyclase in IRGIP secretion was investigated using forskolin. Figure 14 shows IRGIP release from canine epithelial cells in response to forskolin concentrations ranging from  $0.1$  to  $100 \mu\text{M}$ . Forskolin at a concentration of  $1 \mu\text{M}$  significantly ( $p < 0.05$ ) increased IRGIP release over basal. The maximum effect observed was achieved with the highest forskolin concentration tested ( $100 \mu\text{M}$ ) where IRGIP release was approximately 4.9 times basal (to  $13.1 \pm 1.8$  %TCC;  $p < 0.01$ ). Concentrations of forskolin from  $1$ - $100 \mu\text{M}$  also significantly increased IRSS release, with  $100 \mu\text{M}$  yielding  $3.3 \pm 0.5$  %TCC compared to  $2.0 \pm 0.2$  %TCC in the absence of forskolin ( $p < 0.05$ ,  $n = 4$ , data not shown).

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Peptide	Dilution	Source	Species
Gastrin/CCK	1:100	Walsh	Mouse
Glucagon	1:100	Gregor	Mouse
Secretin	1:500	Polak	Rabbit
Somatostatin	1:1000	RPG	Mouse
Insulin	1:1000	RPG	Guinea Pig
Glicentin	1:1000	Moody	Rabbit
Neurotensin	1:2000	RPG	Mouse
PP	1:2000	Buchanan	Rabbit
GIP	1:5000	RPG	Mouse
Motilin	1:5000	RPG	Mouse
Rabbit IgG	1:300	Vector	Goat
Mouse IgG	1:300	Vector	Goat
Guinea Pig IgG	1:300	Vector	Goat

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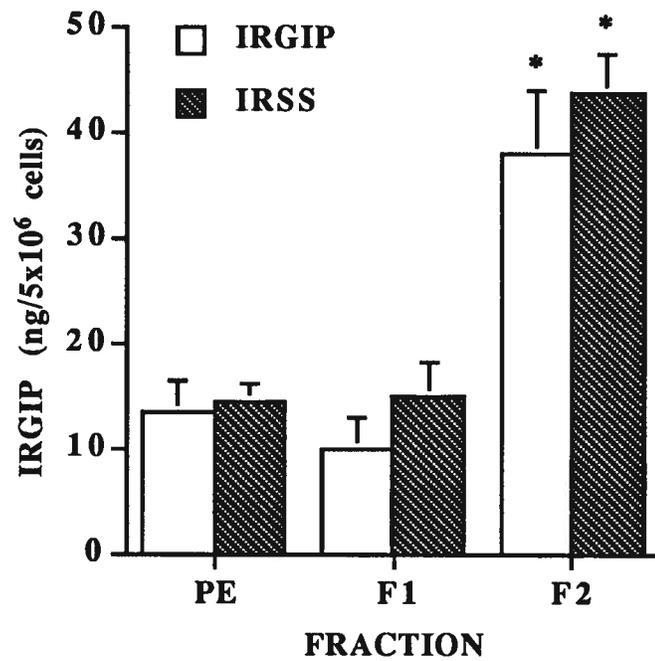


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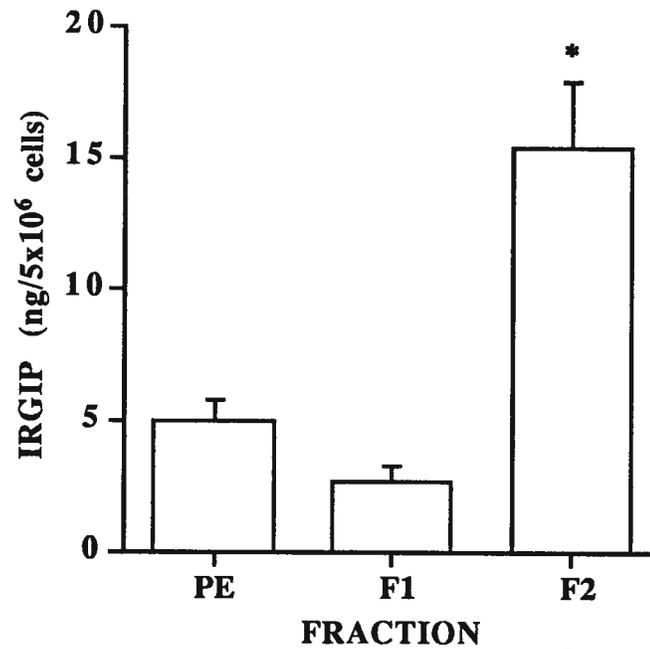
**TABLE 1:** Antisera used in this thesis.

CCK - cholecystokinin; GIP - gastric inhibitory polypeptide; PP - pancreatic polypeptide  
 RPG - Regulatory Peptide Group; Dr. J. Walsh - Cure, UCLA; Dr. J. Polak -  
 Hammersmith Hospital, RPMS; Dr. A. Moody - Novo Nordisk, Copenhagen; Dr. K.  
 Buchanan - University of Belfast; Vector Laboratories Inc. - Burlingame CA.

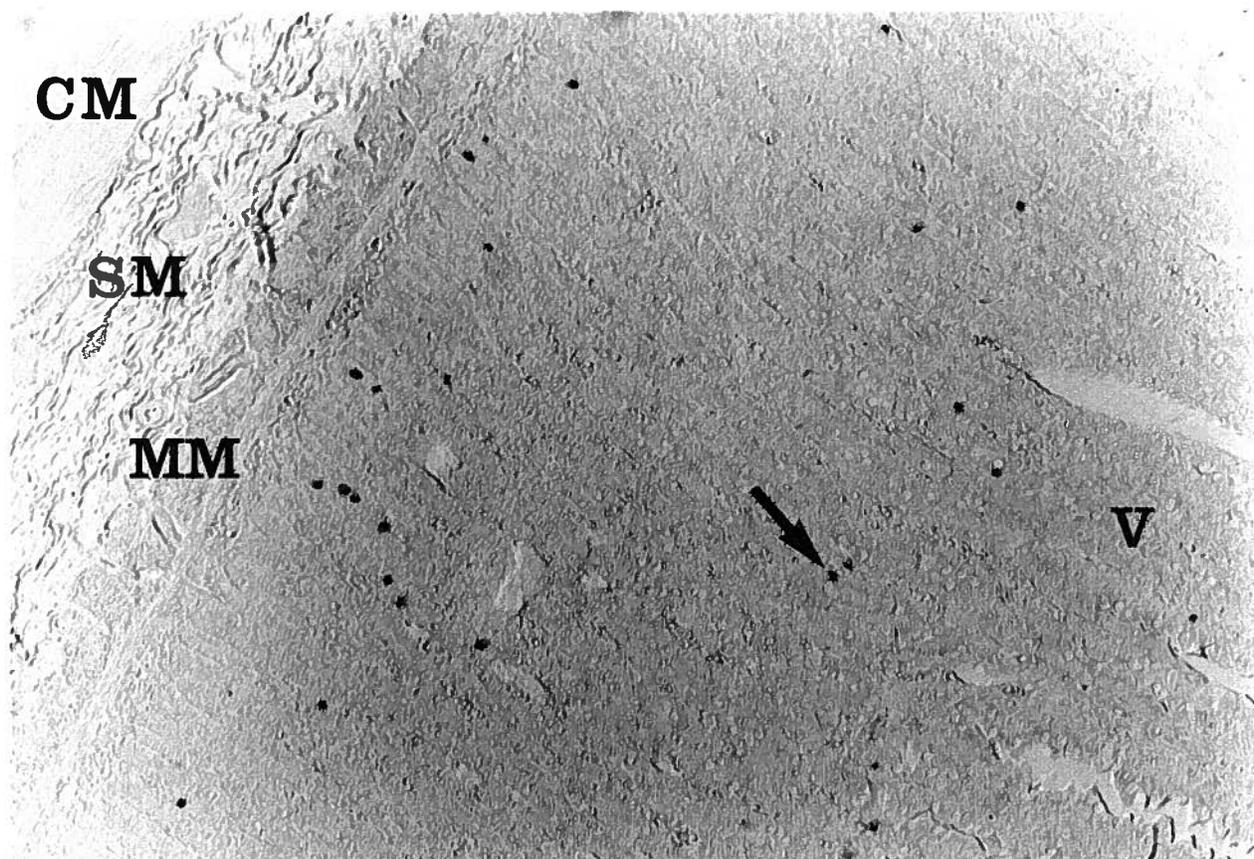
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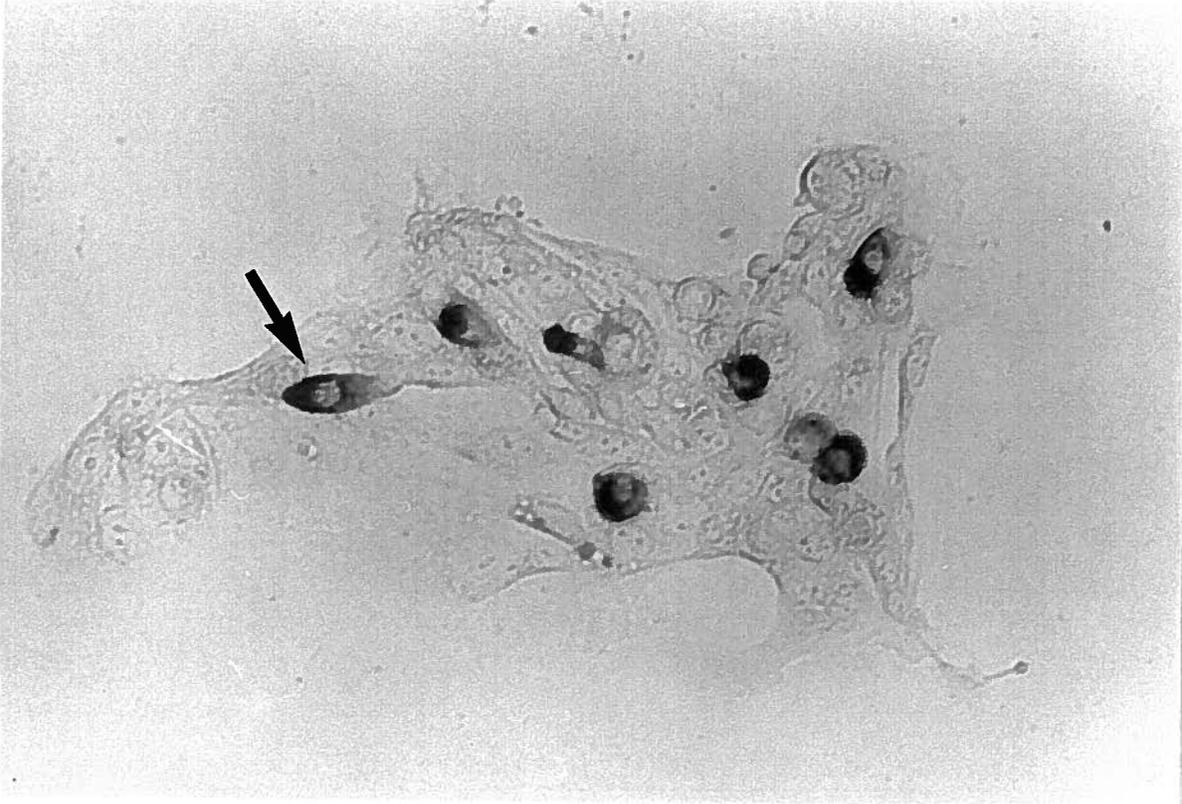
B



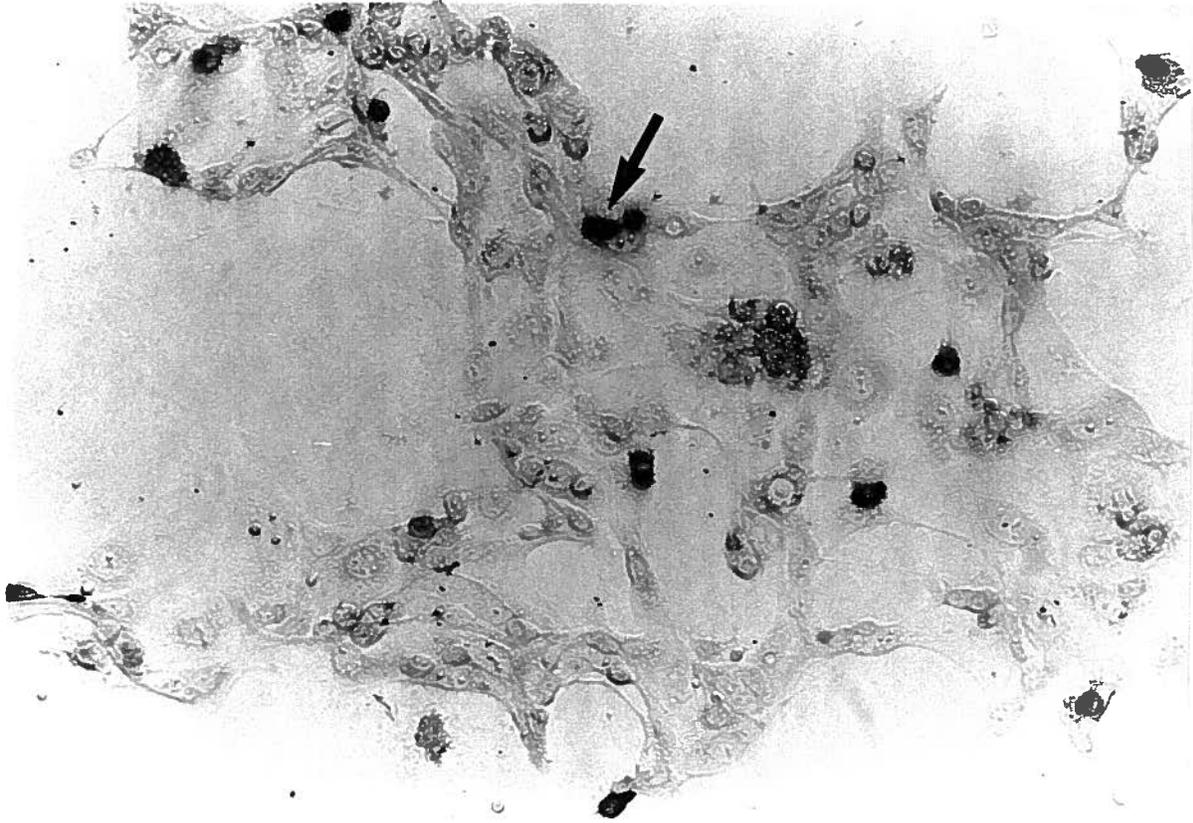
**FIGURE 3:** A) IRGIP and IRSS content (ng/5 x 10<sup>6</sup> cells) from the canine single cell suspension prior to elutriation (PE) and the two fractions resulting from elutriation (F1 & F2). For IRGIP n = 9, for IRSS n = 4. B) IRGIP content from isolated porcine cell fractions. n = 10. \* represents significance to a level of p<0.05 when compared to PE.



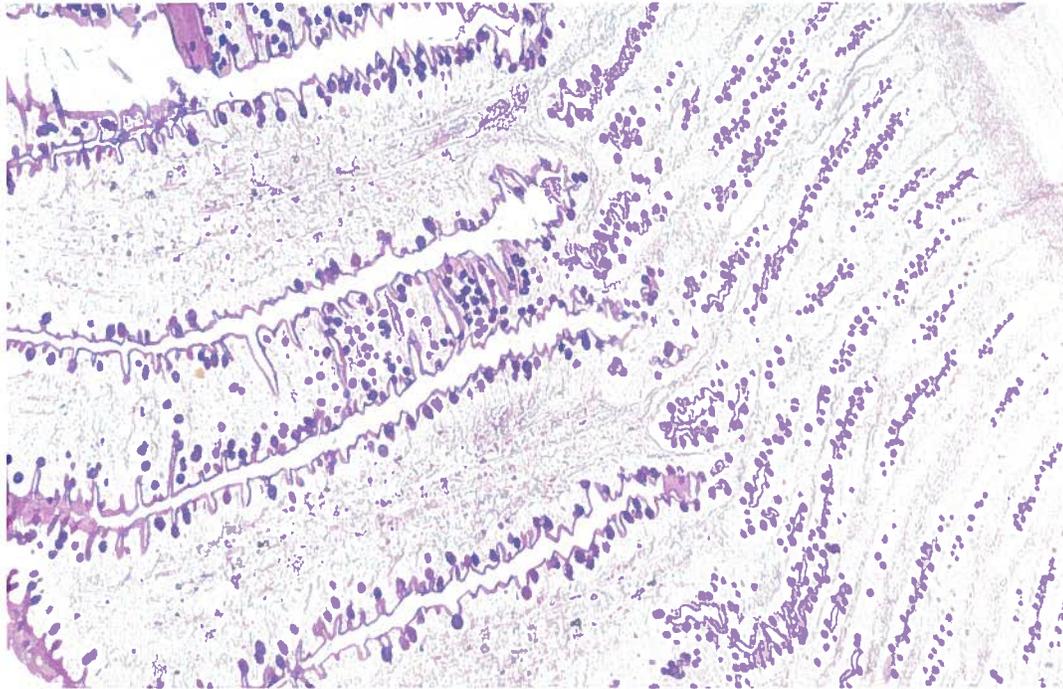
**FIGURE 4:** Canine jejunum immunostained for GIP using the peroxidase method. Magnification is 50 x. CM = circular muscle, MM = muscularis mucosae, SM = submucosa, V = villus.



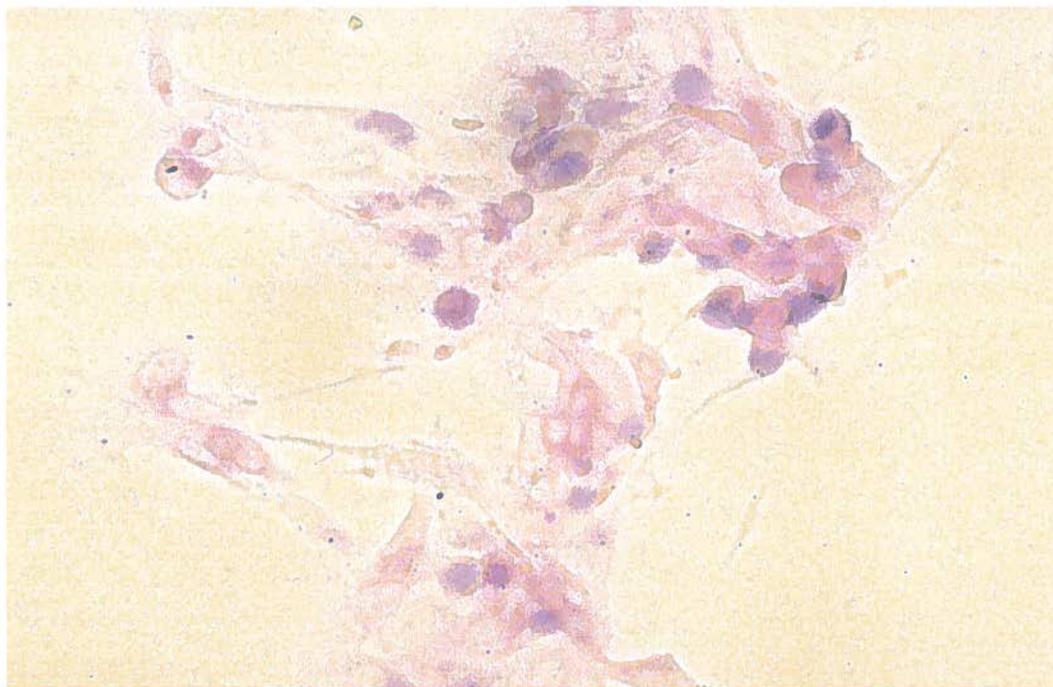
**FIGURE 5:** Isolated canine epithelial cells after 40 h culture, immunostained for GIP. Magnification is 400 x. Arrow indicates a positive cell.



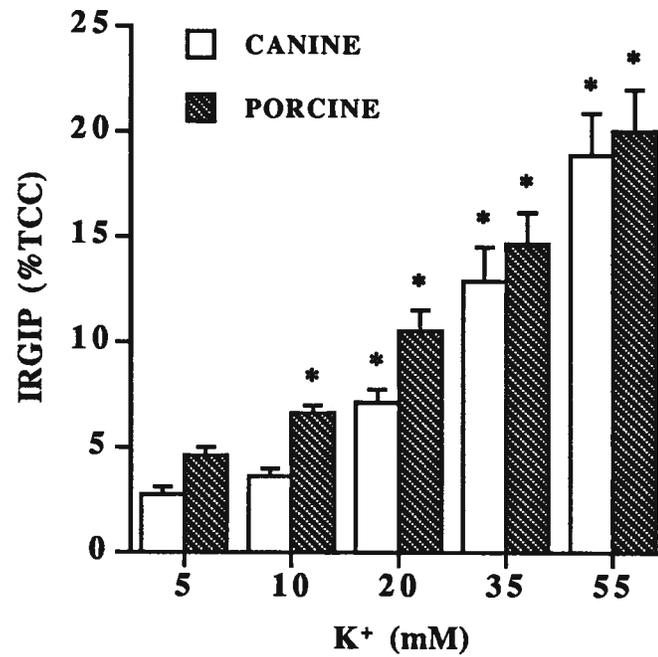
**FIGURE 6:** Isolated canine epithelial cells after 40 h culture, immunostained for somatostatin. Magnification is 200 x. Arrow indicates a positive cell.



**FIGURE 7:** Canine jejunum stained for neutral (red) and acid (blue) mucins using a combined alcian blue-periodic-acid Schiff technique. Magnification is 50 x.

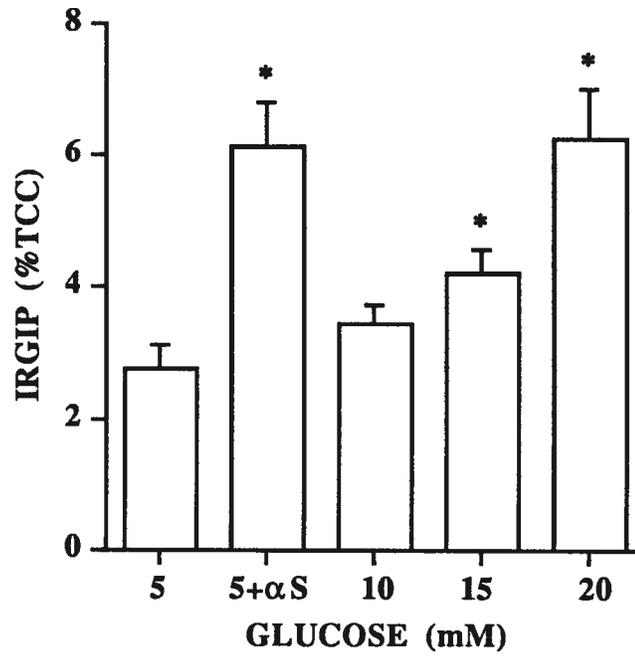


**FIGURE 8:** Isolated canine epithelial cells after 40 h culture, stained for neutral (red) and acid (blue) mucins using a combined alcian blue-periodic-acid Schiff technique. Magnification is 400 x.

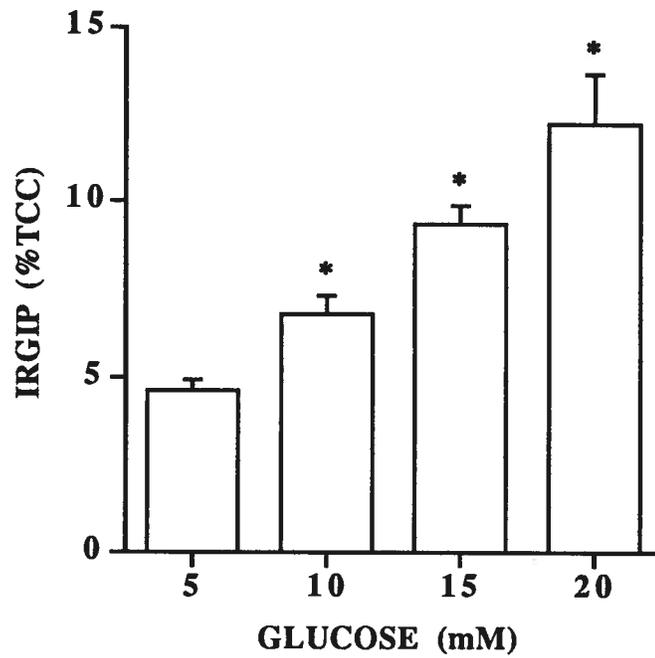


**FIGURE 9:** Effect of K<sup>+</sup> concentrations of 10 to 55 mM on basal IRGIP release (5 mM K<sup>+</sup>) from isolated canine and porcine endocrine cells in the presence of 5 mM glucose. In this and subsequent figures, levels are expressed as mean  $\pm$  SEM %TCC. IRGIP responses were compared to basal release (5 mM glucose, 5 mM K<sup>+</sup>).

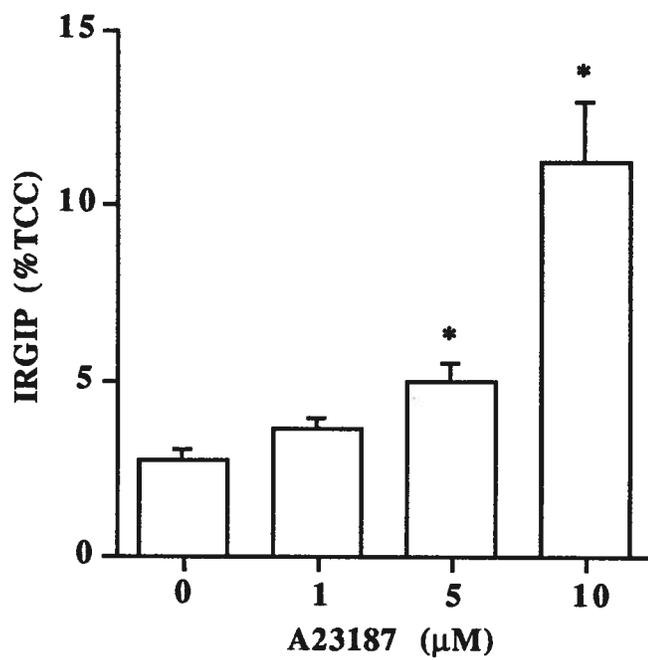
\*  $p < 0.05$ , canine  $n = 5$ , porcine  $n = 8$ .



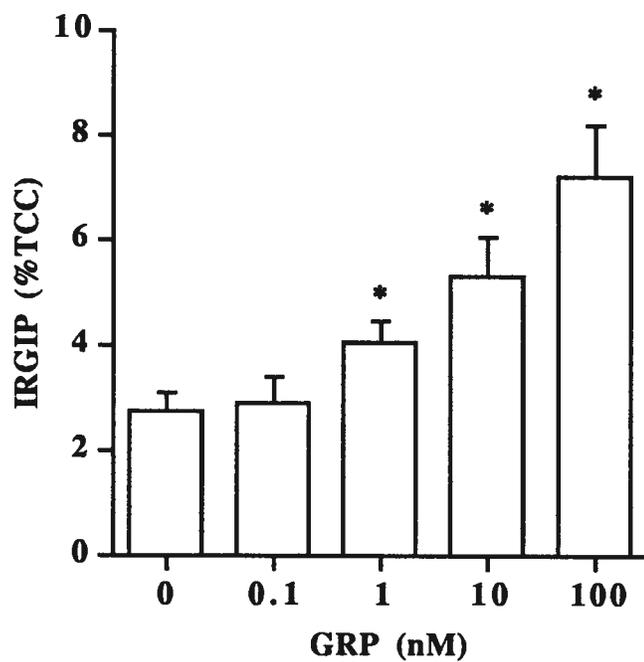
**FIGURE 10:** Concentration-response relationship between glucose (5 to 20 mM) and IRGIP secretion from isolated canine epithelial cells. 5+αS indicates 5 mM glucose with 10 μg/ml anti-somatostatin monoclonal antibody (n = 4). IRGIP responses were compared to basal. \*p<0.05, n = 6.



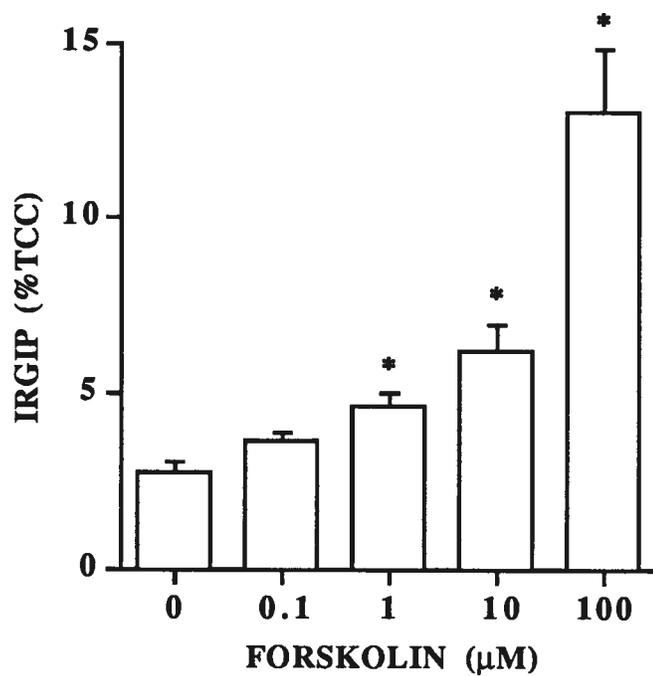
**FIGURE 11:** Concentration-response relationship between glucose (5 to 20 mM) and IRGIP secretion from isolated porcine epithelial cells. IRGIP responses were compared to basal. \* $p < 0.05$ ,  $n = 7$ .



**FIGURE 12:** Effect of 1, 5, and 10  $\mu\text{M}$  A23187 on basal release of IRGIP in the presence of 5 mM glucose. IRGIP responses were compared to basal. \* $p < 0.05$ ,  $n = 10$ .



**FIGURE 13:** Effect of graded concentrations of GRP (0 to 100 nM) on IRGIP release from cultured canine epithelial cells. IRGIP responses were compared to basal. \* $p < 0.05$ ,  $n = 4$ .



**FIGURE 14:** Effect of 0.1 to 100 µM forskolin in the presence of 5 mM glucose on IRGIP release from isolated canine epithelial cells. IRGIP responses were compared to basal. \* $p < 0.05$ ,  $n = 4$ .

## 2.4 DISCUSSION

Although it is known that IRGIP is released into the circulation in response to a meal, the mechanisms by which the components of a meal in the lumen of the small intestine act to cause GIP secretion are unknown. A greater understanding of these mechanisms will only become available with *in vitro* experiments to explore the direct effect of various substances on the GIP cell. Studying the release of IRGIP at the cellular level requires enrichment of the otherwise diffusely located endocrine cells or the use of tumor-derived cell lines which express IRGIP. The objective of these experiments was to develop a model for the isolation, enrichment and culture of canine and porcine intestinal endocrine cells.

Dispersion of canine and porcine duodenal and jejunal mucosa by sequential enzyme digestion progressively increased the IRGIP content of the resulting cell suspension. This cell suspension was further enriched for endocrine cells by elutriation followed by culture. The culture technique has often been found useful after counterflow elutriation to facilitate further enrichment in the studied cell content by providing selective attachment of the endocrine cells to the culture dishes (Aponte *et al.*, 1988; Barber *et al.*, 1986; Buchan *et al.*, 1987; Giraud *et al.*, 1987; Koop and Buchan, 1992; Soll *et al.*, 1984). The overall enrichment of cultured endocrine cells was as much as 100-fold, with IRGIP and IRSS cells accounting for 10% and 5% respectively of the adherent canine cells, plus 8% and 1% respectively of the attached porcine cell population. The majority of the remaining cells were mucin and goblet cells, staining positive for neutral and/or acidic mucins.

The fact that 1 to 2 days was required for cells to adhere and adopt a more normal appearance indicates that these cells likely sustained damage as a result of the chemical and mechanical forces of the digestion procedure. Using similar methods, Barber *et al.* (1986) documented the beneficial effects of short term culture of isolated canine ileal mucosal cells. Freshly isolated neurotensin containing cells incubated at 4°C for 60 min

released ~12% of their neurotensin content, while after 48 h culture, basal release was ~1%. The culture period was thus important for the isolated cells to functionally stabilize. In the present investigations, basal release of IRGIP was  $2.7 \pm 0.4$  %TCC (canine) and  $4.6 \pm 0.35$  %TCC (porcine), values similar to those previously reported from other comparably studied isolated cells.

Membrane potential difference plays an important role in the control of many cellular processes, including endocrine secretion (Barber *et al.*, 1986; Matthews and Sakamoto, 1975; Taraskevich and Douglas, 1984). To assess the ability of the cells to release peptide in response to a depolarizing stimulus, cells were exposed to a series of  $K^+$  concentrations. Basal IRGIP release in the presence of 5 mM  $K^+$  was significantly increased by  $K^+$  concentrations of 20 mM and greater for canine cells and from 10 mM for porcine cells. Release in response to 55 mM  $K^+$  was approximately 7 times and 4 times that of the basal canine and porcine cell release, respectively. This same concentration of  $K^+$  doubled IRSS release compared to basal in the canine cells.  $K^+$  likely produces these effects by depolarizing the cells, thus opening  $Ca^{2+}$  channels and increasing the intracellular  $Ca^{2+}$  concentration, leading to peptide release. IRGIP release by  $K^+$  indicated these cells were electrically active and that depolarization resulted in exocytosis.

Glucose is thought to be one of the most important physiological stimuli for GIP secretion *in vivo*. The isolated porcine and canine endocrine cells responded to graded glucose by releasing IRGIP in a concentration-dependent fashion. There was, however, no significant increase in IRGIP release from the canine endocrine cells until a concentration of 15 mM glucose was reached in the release medium. Normal physiological diets result in luminal concentrations of glucose in dogs of < 20 mM (Ferraris *et al.*, 1990). Therefore, one would expect canine GIP cells to be sensitive to lower concentrations of glucose than was observed in this study. A probable explanation for the apparent lack of sensitivity of the isolated canine endocrine cells to glucose is that

the GIP cells may have been tonically inhibited. Unlike the intact intestinal mucosa, the isolated epithelial cells in this model are in a non-vascular system. Metabolic products and other secreted peptides that would normally be removed by the circulation remain in the medium. Immunocytochemistry of the cultured canine intestinal mucosal cells revealed that cultures contained 5% IRSS cells, and IRSS levels during basal conditions were  $28 \pm 3$  pg/ml. IRSS release was increased modestly by 20 mM glucose to  $39 \pm 1$  pg/ml. Although IRSS cells may not appear adjacent to IRGIP cells in the intact gut (Buchan *et al.*, 1982), somatostatin has been previously demonstrated to inhibit IRGIP release in man (Salera *et al.*, 1982b), dog (Pederson *et al.*, 1975a) and rat (Ho *et al.*, 1987). It is possible, therefore, that endogenous somatostatin present in the medium inhibited IRGIP release. Support for this is the observation that the addition of somatostatin immunoneutralizing antibody (Seal *et al.*, 1987) to basal medium significantly increased IRGIP release. The significant IRGIP release from porcine endocrine cells at a lower glucose concentration (10 mM) might reflect the fewer IRSS cells present, and resultant lower levels of endogenous IRSS.

Considering that glucose transport is a requirement for GIP release (see section 1.10.2), the sensitivity of the isolated cells to glucose may also be a reflection of the activity of luminal glucose transporters. It is possible that these transporters were damaged by the digestive process, and were not functioning normally after 40 h culture. Alternatively, these transporters which exist in a glucose-free environment (during the interdigestive period), may be down regulated by 40 h culture in 5.5 mM glucose. It is also possible that the GIP cells may not possess luminal glucose transporters and thus require messages from neighbouring 'glucose-sensitive' cells in order to release GIP in response to luminal glucose. The degree of IRGIP response to glucose may therefore be related to the degree of reaggregation of the cells upon the seeding on collagen coated plates in order that the appropriate connections are made. Thus other factors could

potentially contribute to the weak IRGIP response to glucose observed from the isolated cells.

The  $\text{Ca}^{2+}$  ionophore A23187 increases cytosolic  $\text{Ca}^{2+}$  concentrations. Concentrations of 5  $\mu\text{M}$  and greater were able to significantly increase IRGIP release from the cultured canine epithelial cells. At 10  $\mu\text{M}$  A23187, release of IRGIP was approximately 4.1 times that of basal. These data suggest that signal transduction mechanisms in the IRGIP cell involve an increase in intracellular  $\text{Ca}^{2+}$  concentration. IRSS levels were also increased to twice that of basal by 10  $\mu\text{M}$  A23187. Somatostatin secretion from isolated canine fundic mucosal cells (Chan and Soll, 1988) or human antral cells (Buchan *et al.*, 1990) has been reported to be stimulated by treatment with A23187. In contrast, cultured fetal rat intestinal cells did not respond to modulation of  $\text{Ca}^{2+}$  fluxes (Brubaker *et al.*, 1990). It is not known if the action of A23187 on IRSS secretion observed in the present study was direct, or whether IRGIP release in response to A23187 stimulated IRSS release. GIP has been shown previously to stimulate gastric IRSS release (McIntosh *et al.*, 1981).

Gastrin releasing peptide (GRP) has been located in neurons and nerve fibres of canine duodenum and jejunum (Vigna *et al.*, 1987). In rats and dogs, GRP infusion has been demonstrated to cause IRGIP release (Greely *et al.*, 1986a, 1986b; McDonald *et al.*, 1981) although it was not known whether this was a direct effect on GIP cells. GRP is thought to act through  $\text{IP}_3$  to release  $\text{Ca}^{2+}$  from intracellular stores (Gallacher *et al.*, 1990). It was therefore of interest to determine the effects of GRP on IRGIP secretion in the isolated epithelial cell model. Concentrations of GRP from 1 to 100 nM produced a significant increase in IRGIP release over basal in a concentration-dependent fashion. This provides evidence that GRP may be involved *in vivo* in the control of GIP release, possibly by an  $\text{IP}_3/\text{Ca}^{2+}$  mediated pathway. It is, however, notable that other isolated endocrine cell preparations have yielded significant responses to GRP at concentrations as low as 0.01 fM (Campos *et al.*, 1990). It is possible, therefore, that IRGIP release in

response to GRP observed in the present study was mediated by a different subtype of the GRP receptor, or via a related receptor, such as that for neuromedin B. The addition of 100 nM GRP also resulted in a modest increase in IRSS release (to  $2.9 \pm 0.4$  %TCC). GRP infusion in dogs has previously been demonstrated to increase plasma IRSS (Schusdziarra *et al.*, 1980).

To determine if activation of cAMP-dependent intracellular pathways would result in IRGIP secretion from the cultured canine epithelial cells, forskolin, an activator of adenylate cyclase was tested. Forskolin at concentrations of 1 to 100  $\mu$ M produced significant increases in IRGIP release when compared to basal. The effect of 100  $\mu$ M forskolin was disproportionately higher than at lower concentrations tested, and may be reflective of the lack of specificity of forskolin for adenylate cyclase at this dose. High concentrations of forskolin may also affect a variety of ion channel functions, independent of cAMP systems (Leidenheimer *et al.*, 1990; Ticku and Mehta, 1990; Watanabe *et al.*, 1987). With these limitations in mind, it appears that canine GIP cells are responsive to activation of adenylate cyclase. Whether the effects on stimulation of  $Ca^{2+}$  and cAMP systems yield secretory responses that are additive or potentiating is a subject for further investigation. Forskolin concentrations of 1 to 100  $\mu$ M also significantly increased the release of IRSS ( $p < 0.05$ ). Somatostatin release in response to dibutyryl cAMP or forskolin has been reported in isolated canine fundic somatostatin cells (Soll *et al.*, 1984) in human antral somatostatin cell cultures (Buchan *et al.*, 1990), and in fetal rat intestinal cells in culture (Brubaker *et al.*, 1990).

In conclusion, a method has been established to enrich duodenal/jejunal endocrine cells, yielding large adhered cell clusters in culture of approximately 10% IRGIP in the canine and 8% IRGIP in the porcine preparation. Both depolarization by  $K^+$  and nutrient stimulation with glucose yielded an increase in IRGIP release from the cultured canine and porcine cells. In addition, pharmacological investigation of potential signal transduction systems involved in stimulus-secretion coupling in canine GIP cells

suggested the participation of both cAMP and Ca<sup>2+</sup> - dependent pathways. Evidence was also provided for a direct role of GRP on receptor-dependent stimulation of IRGIP release. The use of a somatostatin immunoneutralizing antibody indicated that basal IRGIP release was tonically inhibited by exogenous IRSS. The fewer IRSS cells in the isolated porcine cells may, therefore, have contributed to the greater basal release and sensitivity to lower glucose or K<sup>+</sup> concentrations. IRSS secretion was significantly stimulated by high levels of all the above agents used to examine IRGIP release, but it is unclear if these are direct actions or a result of endogenously released peptides such as GIP. This model may prove useful for further elucidating the cellular mechanisms controlling the release of the intestinal endocrine hormones GIP and somatostatin. Furthermore, it may be possible to adapt these methods to isolate epithelial cells from the rat, where models for disease states potentially characterized by abnormal GIP release, such as obesity, exist. Such studies at the cellular level may be the only way to resolve possible aberrant GIP release, and the mechanisms responsible.

## CHAPTER 3

### **GIP RELEASE FROM A TUMOR-DERIVED CELL LINE (STC 6-14)**

#### **3.1 INTRODUCTION**

The rationale for studying GIP release at the cellular level was presented in chapter 2. By using cell preparations, the culture environment can be controlled and progressive changes in intracellular and intercellular events can be directly monitored (Hassall *et al.*, 1989). A major factor impeding such studies is the diffuse distribution of the entero-endocrine cells (including IRGIP cells) throughout the small intestine. One strategy employed has been the development of methods for the isolation enrichment and culture of gut endocrine cells, and the adaptation of these methods to specifically examine GIP release (chapter 2). While this model has proven useful for studying the mechanisms controlling IRGIP release, endocrine cell yield remains a critical problem.

Recently, hormone secreting cell lines have been obtained from neuroendocrine tumors of transgenic mice expressing oncogenes in neuroendocrine cells. The general strategy involves the construction of a hybrid gene composed of the regulatory region from a hormone gene linked to the coding region of an oncogene. This hybrid gene is then transferred into a mouse germ line via microinjection of fertilized eggs and the resulting transgenic mice are able to express the oncogene in the cell types that normally express the hormone regulatory sequences (Hanahan, 1988). Recently, two lineages of transgenic mice were crossed to produce double transgenics. One of these lines carried a hybrid gene construct linking the rat insulin promoter, which drives expression in pancreatic  $\beta$ -cells, to the SV40 early region encoding the potent oncogene large Tag (RIP1Tag2; Hanahan, 1985). The other line carried a polyoma small T antigen gene linked to the rat insulin promoter (RIP2PyST1; Seth *et al.*, 1991). Progeny of these mice followed two distinct tumorigenesis pathways, either resulting in the development of encapsulated  $\beta$ -cell tumors of the pancreas, or unexpected neuroendocrine cell tumors of

the small intestine (Grant *et al.*, 1991). These tumors were excised, minced and injected as small clumps into mice to yield cell lines (Grant *et al.*, 1991). The tumor cells did not stain with periodic acid-Schiff/alcian blue, indicating a lack of mucus secretion, but retained strong positivity for the Grimelius' silver method and chromogranin A, revealing their endocrine nature (Rindi *et al.*, 1990). It appeared that a well differentiated endocrine cell producing secretin was the original target of transgene expression in the gut of RIP1Tag2/RIP2PyST1 mice, as T-antigen-positive mucosal neuroendocrine cells only expressed secretin (and not the other tested hormones). A high proportion of cells stained for secretin, and thus the name Secretin producing Tumor Cells (or S-Type Cell; abbreviated STC) was given to the cell line. Other cells stained for the different areas of the proglucagon molecule (GLPI, GLPII, glicentin and glucagon) and scattered rare cells reacted to neurotenin and pancreatic polypeptide antisera, while no immunoreactivity was detected for insulin in the STC-1 cells (Rindi *et al.*, 1990). Experiments reported in this chapter explored the use of this tumor cell line to study GIP release at the cellular level.

## **3.2 MATERIALS AND METHODS**

### ***3.2.1 CULTURE AND PRODUCTION OF STC 6-14 CELL LINE***

The STC-1 cell line was kindly supplied by Dr. D. J. Drucker (Banting and Best Research Group, Department of Medicine, Toronto Ont). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Terry Fox Laboratory, Vancouver, BC) supplemented with 25 mM glucose, 2.5% fetal calf serum (FCS; Sigma Chemical, St. Louis, MO), 12.5% horse serum (HS; Gibco Laboratories, Burlington, Ont.), l-glutamine (Sigma, 0.15 mg/ml), penicillin (Gibco, 50 units/ml), and streptomycin (Gibco, 50 µg/ml) at 37°C in 5% CO<sub>2</sub> in air in 250 ml Falcon tissue culture flasks (Becton Dickinson Canada Inc., Toronto, Ont.). The cells were subcultured as required or when approximately 80% confluency was reached, by harvesting with trypsin-EDTA (Sigma).

Some cells were cultured on collagen coated 12-well plates until ~80% confluency was achieved and fixed in Bouins for immunocytochemical analysis, as described in section 2.2.5. STC-1 cells were also routinely frozen in media containing DMSO and stored in liquid nitrogen.

Data from immunocytochemical studies indicated that a small number of STC-1 cells were immunoreactive for GIP. In an attempt to increase the proportion of cells expressing GIP, the STC-1 cells were sub-cloned. Cells were plated at an average of 1 cell/well in 96 well Falcon culture plates (10 plates; Becton Dickinson Canada Inc.) and cultured as above to produce clones. Supernatant from each well containing clones was stored at -20°C for later IRGIP determination using the GIP RIA described in section 2.2.8. Cells from RIA-positive wells were transferred to progressively larger wells to increase cell numbers, and also immunostained to verify the presence of IRGIP containing cells (see section 2.2.5 for methods). Sub-clones were produced with varying proportions of IRGIP expressing cells. However, those clones with a majority of IRGIP cells appeared slow growing and difficult to culture. A stable clone, STC 6-14, was therefore chosen as the cell line to use for IRGIP release studies. The STC 6-14 cells were transferred to culture flasks and maintained as above to establish sufficient cell numbers for experiments to be conducted. Cells were routinely frozen in media containing DMSO and stored in liquid nitrogen. The cells were subcultured as required or when approximately 80% were confluent, by harvesting with trypsin-EDTA. Cells from passages 26-39 were used in this study.

### *3.2.2 RELEASE EXPERIMENTS*

For IRGIP secretory studies,  $5 \times 10^5$  cells in 1 ml of culture medium were plated in each well of collagen coated Costar 12-well plates and cultured for 4 days. On the experimental day, the medium was removed by pipet, and changed to 5 mM glucose DMEM (prepared as described above) and incubated 4-5 h. The culture medium was

then removed by pipet and the cells washed with 1 ml release medium consisting of DMEM supplemented with 5 mM glucose, 1% FCS, and 2% aprotinin (Trasylol, 200 kallikrein-inhibiting U/ml, Miles Pharmaceuticals, Rexdale, Ont.). This was followed by addition of 1 ml of release medium containing the appropriate stimulus to each well. Each stimulus was tested in triplicate, for  $n = 1$ . The cells were incubated for 2 h, after which the release medium was removed and stored in 1.5 ml Eppendorf microcentrifuge tubes on ice. Samples were centrifuged for 5 min at 7000 x g (Baxter Biofuge) at 4°C and the supernatant stored at -20°C for subsequent peptide determination. Adherent cells in control wells (2 wells per plate) were extracted in 2 N acetic acid for determination of total cell peptide content per well. After boiling 10 min and centrifugation to remove particulate matter, the supernatant was stored at -20°C for subsequent IRGIP or immunoreactive SS (IRSS) assay. IRGIP and IRSS secretory values from each well were expressed as percentage of total cell content (%TCC) of IRGIP or IRSS as measured from extracts. Control plates that were not used for the secretion experiments were fixed with Bouins for 10 min and then stored in phosphate buffered saline (PBS) to be subsequently examined for peptides by immunocytochemistry.

### *3.2.3 SECRETAGOGUES*

Porcine GIP (pGIP; Regulatory Peptide Group, Vancouver, BC) was diluted to a concentration of 4  $\mu$ M with glucose-free DMEM release medium and kept on ice. This solution was serially diluted to 400, 40, and 4 nM. Final concentrations of pGIP per well were obtained by adding 25  $\mu$ l of the different pGIP solutions to 1 ml medium in each well. An appropriate volume of a solution of 400 nM SS (SS-14; Peninsula, Belmont CA) was added to release media to give a final concentration of 10 nM/well before the media were added to the appropriate wells. The SS antibody (SOMA-10; Regulatory Peptide Group) was diluted in release medium to 250  $\mu$ g/ml/well. Synthetic porcine GIP used for HPLC standard was obtained from Peninsula.

### *3.2.4 PEPTIDE QUANTIFICATION*

Peptides (IRGIP & IRSS) were quantified by RIA as described in sections 2.2.8 and 2.2.9. Acetic acid extracts were adjusted to pH ~7 with 10 M NaOH prior to assaying.

### *3.2.5 IMMUNOCYTOCHEMISTRY*

The STC 6-14 cells were immunostained with primary antibodies for all hormones listed in Table 1 using the avidin-biotin peroxidase method as previously described in section 2.3.3.

### *3.2.6 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)*

Reverse-phase HPLC was used to characterize the IRGIP extracted from STC 6-14 cells, and in the culture medium. A pellet of  $1.5 \times 10^7$  cells was extracted with 5 ml 2 N acetic acid, followed by boiling 10 min and centrifugation (7000 x g, 10 min). The supernatant was then dried using a SpeedVac (Savant Instruments Inc., Farmingdale, NY) and stored at -20°C. Medium that had cultured STC 6-14 cells for 4 days was also processed for HPLC analysis. Samples of 20 ml were adjusted to pH ~2 with acetic acid. SepPak cartridges (C<sub>18</sub>; Waters) were prepared by priming with 5 ml acetonitrile plus 0.1% trifluoroacetic acid (CH<sub>3</sub>CN + 0.1% TFA), followed by 10 ml H<sub>2</sub>O (plus 0.1% TFA). The media samples were then applied to the cartridge and washed with 50 ml H<sub>2</sub>O plus TFA, followed by 20 ml 10% CH<sub>3</sub>CN plus TFA. It was then eluted with 2 ml 60% CH<sub>3</sub>CN plus TFA, and the products were dried by Speed Vac and stored at -20°C. An aliquot of purified culture medium or the cell extract (10 µg/µl) was dissolved in distilled filtered water and 75 µl of this solution was loaded onto a µBondpak C<sub>18</sub> column (4.6 x 250 mm; Waters). The HPLC equipment (Waters) consisted of a model 512 WISP autoinjector, two model 510 pumps, a model 441 absorbance detector, and a NEC computer and printer for signal processing using the Waters Maxima 820 program. The loaded material was eluted using a

gradient that had previously been empirically determined to resolve the components of natural porcine GIP. Samples were loaded at 28% CH<sub>3</sub>CN plus 0.1% TFA, and after 1 min, were eluted by a CH<sub>3</sub>CN gradient (28 - 33% in water) containing 0.1 % TFA, run over 10 min at a flow rate of 1 ml/min. The column was then maintained a further 3 min at 33% CH<sub>3</sub>CN followed by a 4 min gradient up to 70% CH<sub>3</sub>CN. After 2 min at 70% CH<sub>3</sub>CN, it was then re-equilibrated to 28% CH<sub>3</sub>CN over 2 min. The run was complete after a 10 min period of equilibration, and was followed by 100 µl H<sub>2</sub>O injected and subjected to the same gradient to ensure no residual GIP remained on the column and was collected in the next sample run. Fractions were collected every 0.5 min and were dried using a SpeedVac prior to being stored at -20°C. Samples were then dissolved in 500 µl assay buffer prior to being assayed for IRGIP.

### *3.2.7 EXPRESSION OF RESULTS*

Release data are presented as mean  $\pm$  SEM for a percentage of the total cell content (%TCC). Statistical significance was determined using the Student's *t* test and was set at the 5% level.

## **3.3 RESULTS**

### *3.3.1 CHARACTERIZATION OF STC 6-14 CELLS*

Sub-cloning of the STC-1 cell line resulted in a stable cell line, designated STC 6-14, that consisted of approximately 30% IRGIP cells (Figure 15). Roughly the same proportion of cells exhibited SS immunoreactivity, and <2% immunostained for either glucagon, gastrin, secretin, glicentin, pancreatic polypeptide, or neurotensin. No immunoreactivity for insulin was observed. In order to examine IRGIP expressed in STC 6-14 cells, HPLC analysis of cell extracts was employed. The narrow gradient was chosen to resolve the components of natural porcine GIP in a fashion similar to that which had been previously reported (see section 1.11.1). Three major components of

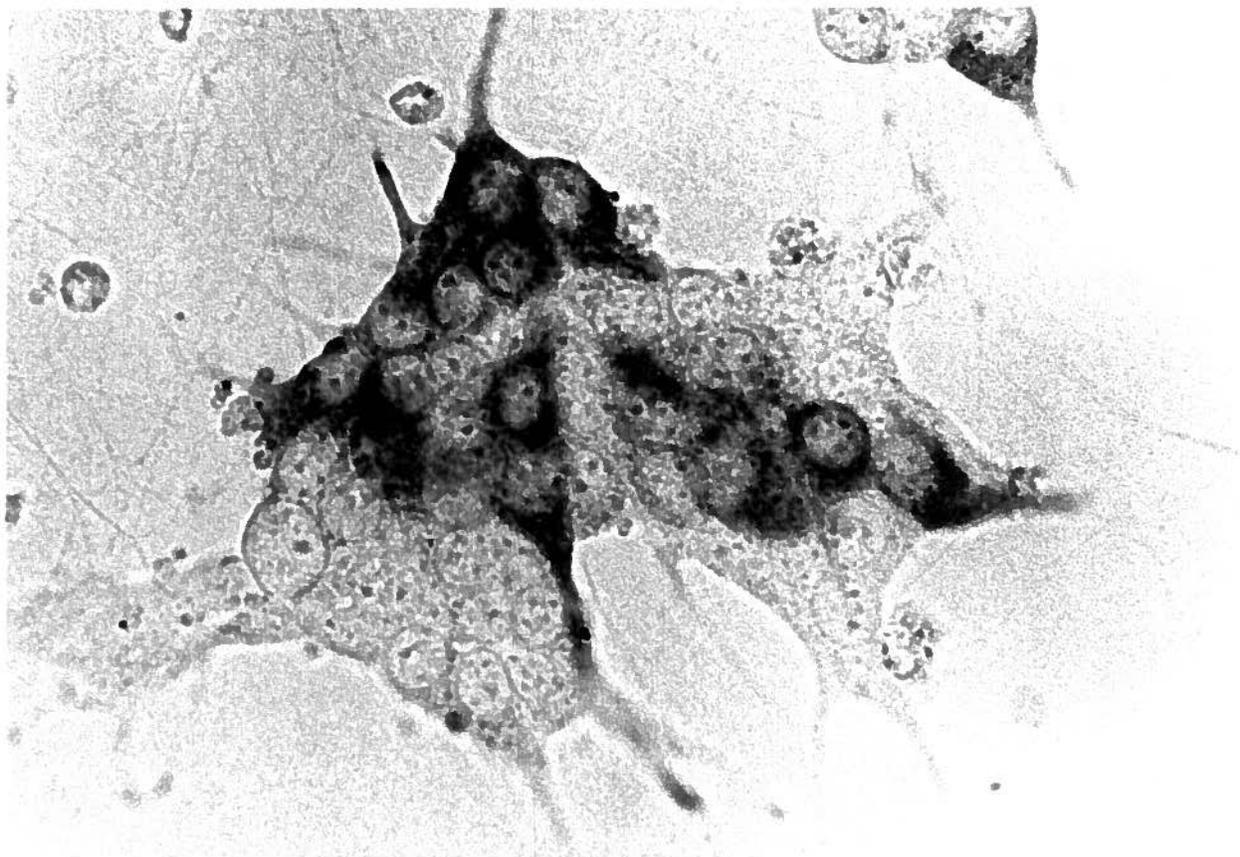
natural porcine GIP were resolved, with retention times of 11.37, 12.47 and 13.12 min respectively, while synthetic porcine GIP eluted as a mono-component at 13.01 min (Figure 16). For following runs, fractions were collected and assayed for IRGIP, as samples were below the absorbance detection limits of the HPLC system. Analysis of a natural porcine GIP standard in this fashion yielded 3 IRGIP peaks (a, b, c), the second of which (b) eluted in the same fraction as synthetic porcine GIP (Figure 17A). On a separate elution, RIA analysis of collected fractions revealed 1 major IRGIP peak for the STC 6-14 cell extract, but 3 major IRGIP peaks (i, ii, iii) for the STC 6-14 culture medium (Figure 17B). While the IRGIP in the cell extract was present in the same fractions as synthetic porcine GIP 1-42, the 3 IRGIP peaks from cell medium were in different fractions. The largest IRGIP fraction of the cell medium (peak i) eluted at the same fraction as peak a of natural porcine GIP.

### *3.3.2 IRGIP SECRETION IN RESPONSE TO GLUCOSE*

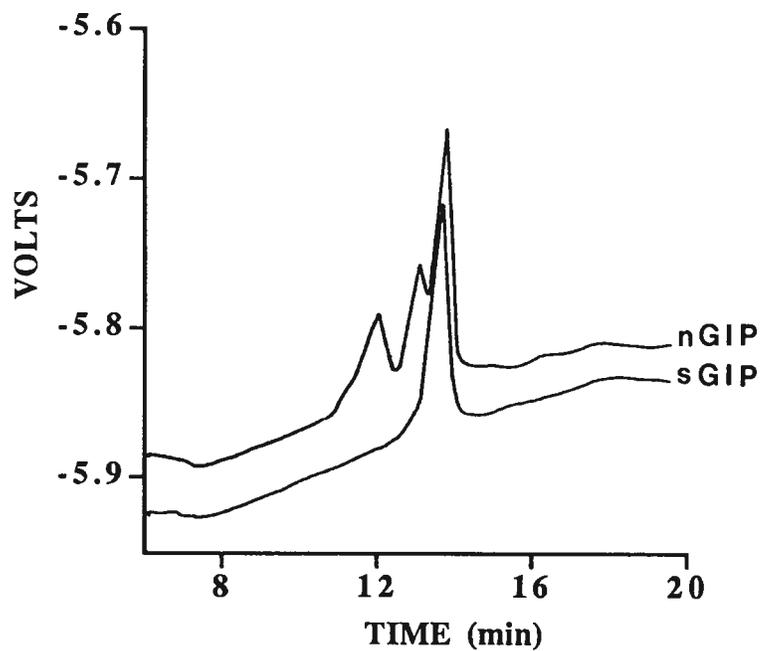
The effects of SS and SS monoclonal antibody (SOMA-10) on glucose stimulated IRGIP release are shown in Figure 18. After 4 days culture, each well of cells on average contained  $33.3 \pm 1.4$  ng of IRGIP, and basal release in the presence of 5 mM glucose was  $733 \pm 58$   $\text{pg}\cdot\text{ml}^{-1}\cdot\text{2h}^{-1}$  ( $2.20 \pm 0.17$  %TCC). Incubation of the cells at glucose concentrations of 10 mM and higher resulted in significantly greater IRGIP release than at 5 mM glucose, to a maximum as tested of  $4.20 \pm 0.42$  %TCC at 20 mM. In the presence of 10 nM SS however, glucose had no significant effect on IRGIP release. The addition of SOMA-10 to the graded glucose concentrations resulted in significantly greater IRGIP responses at all but 20 mM glucose. At 5 mM glucose, IRGIP release was increased by the addition of SOMA-10 to a level comparable to that obtained for 15 mM glucose alone.

### *3.3.3 IRSS SECRETION IN RESPONSE TO GLUCOSE AND GIP*

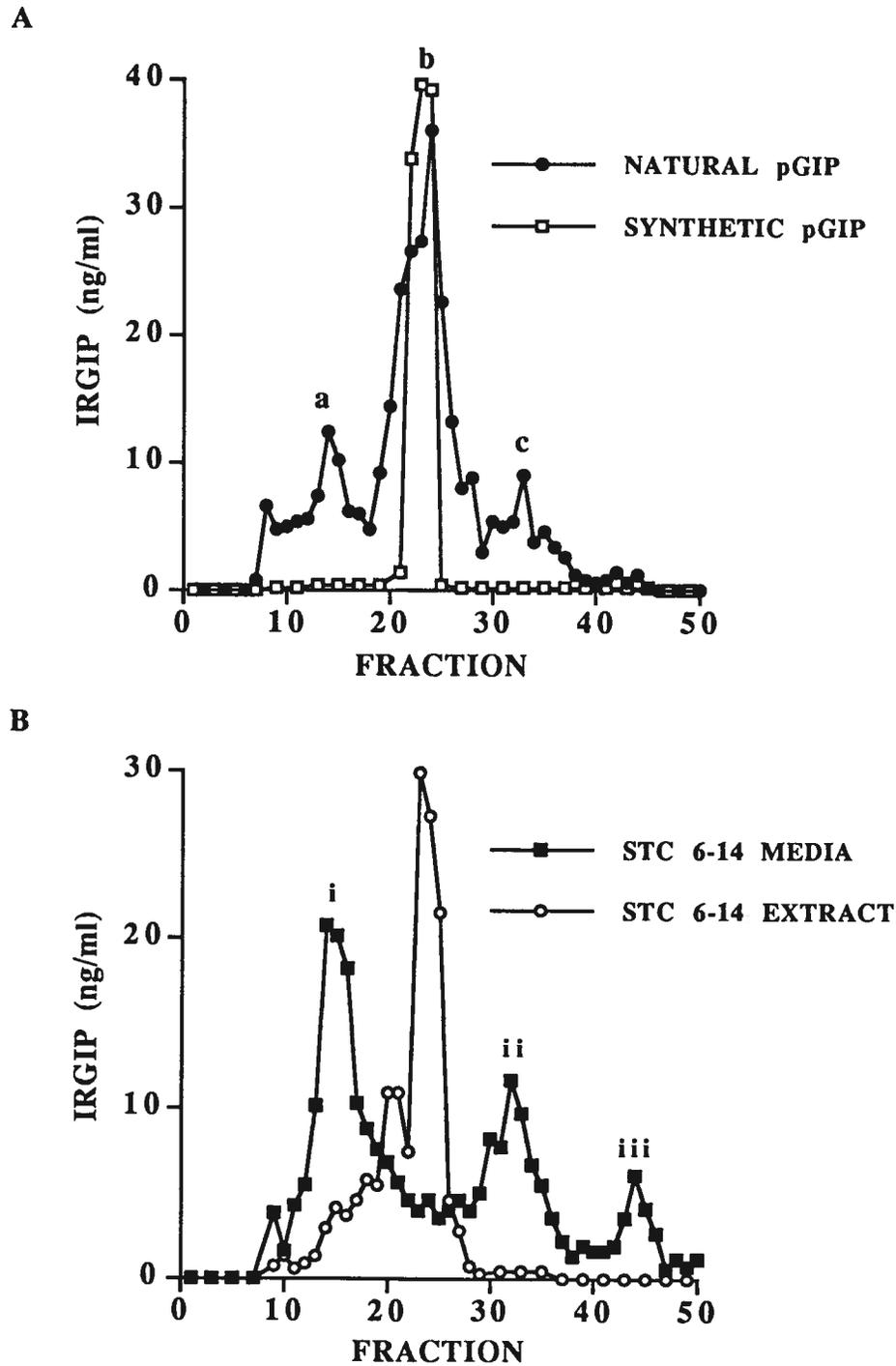
The release of IRSS from STC 6-14 cells in response to 4 concentrations of glucose is shown in Figure 19. The average content of IRSS in each well after 4 days culture was  $18.4 \pm 1.5$  ng. Basal release of IRSS was  $2.05 \pm 0.19$  %TCC, and increased significantly in response to 15 and 20 mM glucose. The addition of natural porcine GIP at concentrations of 1 nM and greater to release medium containing 5 mM glucose also significantly increased IRSS release in a concentration dependent fashion (Figure 20). At the highest pGIP concentration tested (100 nM) IRSS release was doubled ( $4.10 \pm 0.44$  %TCC).



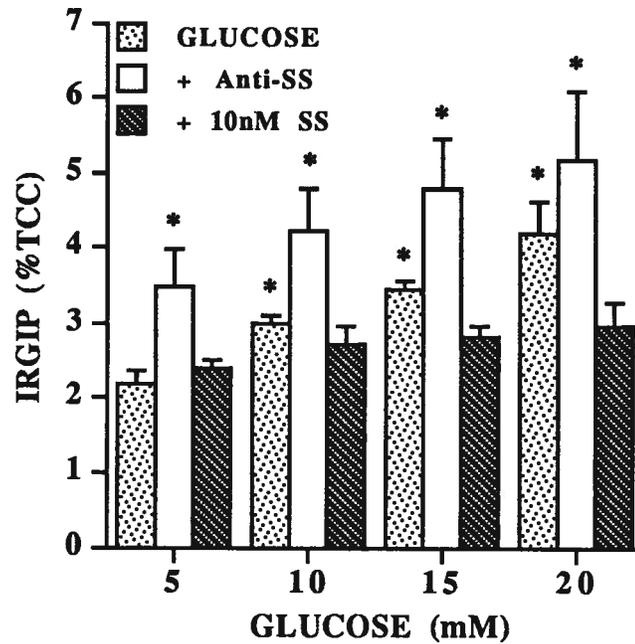
**FIGURE 15:** Cultured STC 6-14 cells immunostained for GIP (black), x 400.



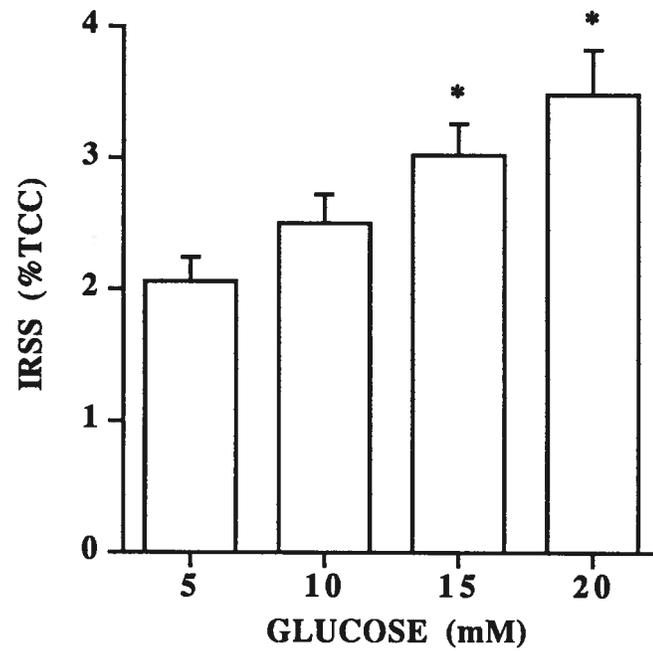
**FIGURE 16:** HPLC elution profile of natural porcine GIP (5  $\mu\text{g}$ ) and synthetic porcine GIP (3  $\mu\text{g}$ ) from a  $\text{C}_{18}$  column with an acetonitrile gradient of 28-33% over 10 min, at a flow rate of 1 ml/min.



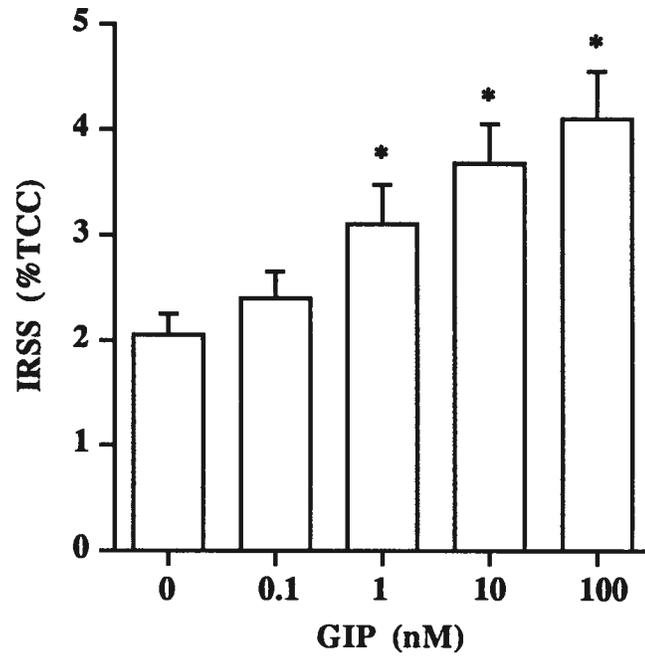
**FIGURE 17:** Immunoreactive GIP (IRGIP) content of HPLC fractions eluted from a  $C_{18}$  column with an acetonitrile gradient of 28-33% over 10 min. **A)** Overlay of elution profiles for natural porcine GIP and synthetic porcine GIP. **B)** Overlay of elution profiles for STC 6-14 cell extract and culture medium.



**FIGURE 18:** Effect of 250  $\mu\text{g/ml}$  anti-somatostatin antibody (SOMA-10) or 10 nM somatostatin (SS) on the dose-response relationship between glucose concentrations (5 to 20 mM) and IRGIP secretion from STC 6-14 cells. For glucose alone,  $n = 10$ ; for glucose + anti-SS,  $n = 7$ ; for glucose + 10 nM SS,  $n = 4$ . In this and subsequent figures, IRGIP levels are expressed as mean  $\pm$  SEM percent total cell content (TCC). IRGIP responses were compared to 5 mM glucose alone. \*  $p < 0.05$ .



**FIGURE 19:** Concentration-response relationship between glucose concentrations (5 to 20 mM) and immunoreactive somatostatin (IRSS) secretion from STC 6-14 cells.  $n = 9$ . IRSS responses were compared to 5 mM glucose. \*  $p < 0.05$ .



**FIGURE 20:** Effect of graded concentrations of porcine GIP (0-100 nM) on immunoreactive somatostatin (IRSS) release from STC 6-14 cells in the presence of 5 mM glucose.  $n = 7$ . IRSS responses were compared to 5 mM glucose in the absence of GIP. \*  $p < 0.05$ .

### 3.4 DISCUSSION

Cell lines producing gut peptides are able to proliferate in culture and can provide large numbers of viable tumor cells free of contamination by other cell types. They are therefore valuable models for studying the synthesis, storage and release of gut peptides in definable environments. The STC-1 cell line was derived from an intestinal endocrine tumor that developed in mice carrying transgenes consisting of the rat insulin promoter linked to the potent viral oncogene SV40 T antigen and to the polyoma virus small T antigen (Rindi *et al.*, 1990). This cell line has been used previously to study the release of cholecystokinin (CCK) and secretin (Chang *et al.*, 1992; Mangel *et al.*, 1993). In these studies, it was concluded that the STC-1 cells may serve as a useful model system to investigate the secretory mechanisms for these peptides. In the present study, these primarily secretin-secreting tumor cells were sub-cloned to enrich the population of IRGIP secreting cells, resulting in the STC 6-14 clone.

Immunohistochemical analysis of the STC 6-14 cells revealed that IRGIP and IRSS were the main endocrine cell types comprising the cell line, and that small numbers of glucagon, gastrin, secretin, glicentin, pancreatic polypeptide, and neurotensin were also present. The fact that the STC cell lines secrete a number of peptides must be considered. Heterogeneity of hormone expression might be related to the known plasticity of endocrine cells when transformed (Tischler, 1983). Rindi *et al.* (1990) noted that the spectrum of hormones expressed apparently increased with tumor progression, suggesting that the proliferating intestinal neuroendocrine cell is able to switch to multiple alternative differentiated states. This quality has been ascribed to stem cells of the intestinal epithelium (Cheng and Leblond, 1974a; 1974b; Pictet *et al.*, 1976). Rindi *et al.* (1990) also noted it was surprising that in RIP1Tag2/RIP2PyST1 the regulatory sequences of rat insulin II gene induced transformation in the secretin cell, an epithelial endocrine cell anatomically and functionally quite distinct from the insulin producing  $\beta$ -cell. This might be explained by the fact that the pancreas and upper gut have a common

embryologic origin (Pictet and Rutter, 1972; Falkmer *et al.*, 1984). Whether more than one peptide is secreted from one cell was not determined, but is the subject of further investigation.

CCK and secretin released from the STC-1 cell line have been previously shown to have the same retention times on HPLC as the naturally occurring peptides (Chang *et al.*, 1992). HPLC was also employed in the current investigations to characterize GIP expressed by the STC 6-14 cells. The gradient derived for the elution of GIP by HPLC was capable of resolving three main components of natural porcine GIP, similar to that which had been previously reported (Brown *et al.*, 1981; Jörnvall *et al.*, 1981; Schmidt *et al.*, 1987). The main component had a retention time comparable to that of synthetic porcine GIP, and was therefore assigned GIP 1-42. The other two peaks were identified by comparisons to previous HPLC profiles of natural porcine GIP (Brown *et al.*, 1981; Jörnvall *et al.*, 1981; Schmidt *et al.*, 1987). These reports identified the two preceding peaks as CCK and GIP 3-42, respectively. As the content of GIP in the STC 6-14 culture medium and extracts loaded on the HPLC (using the same gradient conditions) was below the absorbance detection limit, eluted fractions of subsequent runs were collected, examined for GIP immunoreactivity, and compared to identically treated GIP standards run the same day. Three main IRGIP components were resolved from natural porcine GIP. The largest component (b) was found in the same fraction as synthetic porcine GIP and was assigned GIP 1-42. Peak a of the natural GIP preparation eluted prior to peak b and was hypothesized to be GIP 3-42 based on the previous reported elution profiles of this GIP preparation. The difference in elution profiles between the absorbance (Figure 16) and the GIP immunoreactivity (Figure 17A) may have occurred as a result of replacing the C<sub>18</sub> column between these runs. IRGIP in the eluted STC 6-14 extract was found in the same fractions as synthetic porcine GIP and was assigned GIP 1-42. However, IRGIP in eluted STC 6-14 culture medium was found in three fractions, the largest of which (peak i) eluted in the same fractions as peak a of the natural GIP,

believed to be GIP 3-42. The other IRGIP peaks of the STC 6-14 culture medium could represent putative forms of GIP that were previously suggested to exist (see section 1.9.3), or could be other IRGIP fragments. Thus while IRGIP contained in the STC 6-14 cells does appear to have identical HPLC characteristics to synthetic GIP, IRGIP purified from the cell culture medium appears to have been degraded, possibly largely to GIP 3-42. This phenomenon is further investigated in chapter 4 of this thesis.

Glucose is thought to be one of the main physiological regulators of GIP secretion *in vivo*. Concentrations of glucose from 10 to 20 mM significantly increased IRGIP release from STC 6-14 cells, however, there was only an approximately 2-fold increase in IRGIP release at 20 mM when compared to basal. This increase in IRGIP secretion is significantly smaller than that noted in the isolated canine and porcine endocrine cells (Figures 10 & 11). It is possible that these tumor cells have glucose regulatory mechanisms that differ from those of normal endocrine cells. Such a case has previously been observed with other tumor-derived endocrine cell lines (Brant *et al.*, 1992; Nagamatsu and Steiner, 1992; Visher *et al.*, 1987). In view of the high proportion of somatostatin-secreting cells present in the STC 6-14 cell cultures, it is likely that endogenous somatostatin contributes to the blunted IRGIP response observed following addition of glucose. Studies of glucose-stimulated IRGIP release in the presence of the somatostatin monoclonal antibody tend to support this hypothesis. This antibody significantly increased glucose-stimulated IRGIP release at glucose concentrations less than 20 mM, presumably by immunoneutralizing IRSS. Furthermore, the addition of exogenous SS was able to completely suppress glucose-stimulated IRGIP release, at all glucose concentrations tested. SS has been previously demonstrated to inhibit IRGIP release in man (Salera *et al.*, 1982), dog (Pederson *et al.*, 1975) and rat (Ho *et al.*, 1987). The cellular mechanisms responsible for the inhibitory effect of SS on IRGIP secretion are presently not understood, probably because of the prior lack of suitable *in vitro* models.

Concomitant determination of IRSS revealed that glucose concentrations of 15 and 20 mM significantly increased IRSS release when compared to basal. Previous *in vivo* studies have also demonstrated the release of gut IRSS in response to luminal glucose (Schusdziarra *et al.*, 1978). It is not clear in the present case whether this secretion is a direct result of glucose, or whether glucose-stimulated IRGIP is acting as a secretagogue of IRSS. In order to examine if IRGIP could stimulate release of IRSS from the STC 6-14 cells, pGIP was added to the basal release medium. At concentrations of 1 nM and greater, pGIP significantly increased IRSS release. Verification that endogenously released IRGIP stimulates IRSS release requires the development of antibodies capable of neutralizing the action of IRGIP. GIP has previously been demonstrated to stimulate secretion of IRSS from the perfused rat stomach (McIntosh *et al.*, 1981).

In conclusion, the tumor-derived STC 6-14 cell line represents a readily available source of large numbers of IRGIP and IRSS cells for intensive study of cellular mechanisms of hormone release. IRGIP expressed by these cells has the same HPLC retention time as porcine GIP 1-42. Glucose-stimulated IRGIP release is augmented by the addition of a somatostatin antibody, possibly by immunoneutralization of endogenous IRSS. The addition of exogenous SS suppressed glucose-stimulated IRGIP release. IRSS release from STC 6-14 cells is enhanced by the addition of glucose, but it is unclear if this increased release is secondary to glucose-stimulated IRGIP release. The addition of exogenous pGIP stimulated IRSS release in a concentration dependent fashion. The STC 6-14 cell line may thus be a useful model to further study the interactions and release of GIP and SS.

## CHAPTER 4

### METABOLISM OF GIP

#### 4.1 INTRODUCTION

Studies in chapter 3 indicated that while IRGIP extracted from STC 6-14 cells eluted with the same retention time on HPLC as native porcine GIP, GIP secreted from these cells had a significantly different HPLC profile. Furthermore, it appeared that the major constituent of IRGIP in the STC 6-14 culture medium eluted identically with GIP 3-42 that was a component of preparations of porcine GIP produced by the method of Brown *et al.* (1970). This observation suggested that the conversion of GIP 1-42 to GIP 3-42 might be occurring in the medium. As this culture medium contained a total of 15% serum, it was also hypothesized that the N-terminal Tyr-Ala of GIP could be the substrate for an enzyme present in serum. The physiological implication of this process was evident from the observation that GIP 3-42 separated from the natural preparations by HPLC was biologically inactive (see section 1.11.1).

Jörnvall *et al.* (1981) suggested that the GIP fragment which constituted >20% of purified GIP was formed by secondary processing or degradation, through susceptibility to attack by aminopeptidase, elastase, dipeptidyl aminopeptidase or related enzymes in the intestine. As GIP belongs to a superfamily of peptides sharing sequence homology (see section 1.2.4), it could be predicted that such an enzyme would act on other hormones with similar N-terminal amino acid sequences. In 1986, Frohman *et al.* observed that growth hormone-releasing hormone (GRF 1-44) was cleaved to 3-44 by an enzyme in plasma. Furthermore, this enzyme product, like the GIP fragment, was biologically inactive. As a member of the glucagon superfamily, N-terminal amino acids (1 and 2) of GRF are identical to GIP (Tyr-Ala). In 1989, Frohman *et al.* identified the plasma enzyme responsible as dipeptidyl peptidase IV (DPP IV). They clearly

demonstrated that this enzyme was present in human plasma and quickly inactivated GRF *in vivo*.

The objective of studies in this chapter was to determine if DPP IV was also responsible for the putative production of GIP 3-42 found in the STC 6-14 growth medium, and more importantly, whether this enzyme might play an important role in GIP metabolism *in vivo*. Furthermore, in light of a potential role for GIP in some disease states (see section 1.13), it was also of interest to determine if levels of DPP IV were altered in the obese Zucker rat, and NIDDM subjects. It was hypothesized that elevations of plasma DPP IV leading to rapid incretin degradation, might manifest as hyperglycemia due to a reduction in the biological potency of the enteroinsular axis.

## 4.2 MATERIALS AND METHODS

### 4.2.1 INCUBATION OF GIP WITH SERUM

In order to test the hypothesis that the serum component of the STC 6-14 culture medium was responsible for the degradation of GIP, synthetic porcine GIP (5 µg/ml; Peninsula) was incubated at 37°C in 40 mM phosphate buffer (pH 7.0), with and without serum (12.5% HS, 2.5% FCS). At time intervals of 1, 10, 30 min; and 1, 3, 6, and 24 h, 100 µl aliquots were transferred to Eppendorf tubes, immediately flash frozen by submersion in ethanol and dry ice and then stored at -70°C. Sample volumes of 50 µl were then analyzed by reverse-phase HPLC as previously described in section 3.2.6. These studies indicated a time-dependent breakdown of GIP 1-42 to the product believed to be GIP 3-42, in the presence of serum. Similar experiments were performed with and without the addition of 0.1 mM diprotin A (Sigma). Diprotin A is a bacterial tripeptide (Ile-Pro-Ile) that has been demonstrated to competitively inhibit DPP IV (Umezawa *et al.*, 1984) and was shown previously by Frohman *et al.* (1989) to greatly reduce the conversion of GRF 1-44 to GRF 3-44.

#### 4.2.2 DIPEPTIDYL PEPTIDASE IV-NEGATIVE RATS

Recently, studies have demonstrated that Fischer-344 rats from the Japanese Charles River Inc. (DPP IV-negative) specifically lack DPP IV, whereas Fischer-344 rats from sources in the United States (DPP IV-positive) possess normal DPP IV activity (Tiruppathi *et al.*, 1990; Watanabe *et al.*, 1987). It was felt that these animals would serve as ideal models with the DPP IV-negatives serving as controls, to investigate a possible role for DPP IV in the metabolism of GIP *in vivo*. Two breeding pairs of these rats were generously supplied by Dr. F. H. Liebach (Medical College of Georgia, Augusta, Georgia) and a colony was established at the department of Physiology at UBC.

#### 4.2.3 DIPEPTIDYL PEPTIDASE IV ASSAY

In order to verify the lack of DPP IV in the DPP IV-negative rats, a DPP IV assay was established, using a slightly modified method from that which was previously described (Matumura, 1985). Blood was collected from the tail of five DPP IV-negative and five DPP IV-positive rats, and the serum separated. The substrate for the enzyme was a solution of 1.4 mM Gly-Pro-*p*-nitroanilide (Sigma) in 114 mM Tris buffer pH 8.0 (700 mg Tris HCl + 844 mg Tris Base to 100 ml distilled H<sub>2</sub>O). Test tubes containing 1 ml of the Tris buffer were incubated at 37°C, followed by the addition of 100 µl test serum. The absorbance at 410 nm was then recorded immediately and at 5 min intervals, using a U.V. spectrophotometer (Canlab Pye Unicam SP8-100) to monitor the appearance of the yellow product of enzyme activity, *p*-nitroaniline. A standard curve was prepared by reading the absorbance of *p*-nitroaniline (Sigma) solutions of 0.005 to 1 mM in Tris buffer (Figure 21A). Absorbance of serum samples with substrate (time = 0) was subtracted from all further timed readings. The resulting absorbance values were then determined from the standard curve, and the resulting conversion rate to *p*-nitroaniline was plotted.

#### 4.2.4 HPLC PURIFICATION OF <sup>125</sup>I-GIP

In order to perform *in vivo* studies to confirm the action of DPP IV on GIP, it was desirable to develop a highly sensitive assay system to monitor the conversion of GIP 1-42 to GIP 3-42. Since GIP 1-42 can be separated from GIP 3-42 by HPLC (see section 3.3.1), it was hypothesized that HPLC could also resolve <sup>125</sup>I-GIP 1-42 from <sup>125</sup>I-GIP 3-42. The use of radioactive tracer would enable the use of physiological levels of GIP *in vivo*, and thus allow for a highly sensitive, accurate estimation of the conversion of GIP 1-42 to inactive GIP 3-42. Tracer was therefore prepared, using the methods outlined in section 2.2.8, with the exception that synthetic porcine GIP (Peninsula) was used instead of the natural purified form (known to contain a high proportion of GIP 3-42; see section 1.11.1). Immediately after iodination, the peak fraction of <sup>125</sup>I-GIP was purified on an HPLC system that was used exclusively for purifying radioiodinated peptides.

Water (distilled and treated through a Waters MilliQ H<sub>2</sub>O filtration system) and acetonitrile (CH<sub>3</sub>CN) were prepared by adding 0.1% trifluoroacetic acid (TFA) to each, and degassing and filtering through either 0.22 μm (H<sub>2</sub>O) or 0.45 μm (CH<sub>3</sub>CN) filters (Waters). These solvents were delivered to a μBondapak C18 column (Waters) using two 110B Solvent Delivery Module pumps (Beckman Instruments Inc, San Ramon, CA) controlled by a programmable 421A Controller (Beckman). The <sup>125</sup>I-GIP was injected using a 100 μl needle syringe (Hamilton Co., Reno, NV) into a 210A Injector Port (Beckman) with 100 μl capacity. The tracer was loaded at a CH<sub>3</sub>CN concentration of 32%, and eluted by increasing the CH<sub>3</sub>CN content to 38% over 10 min, and maintained for a further 5 min. The column was then washed by increasing the CH<sub>3</sub>CN concentration to 60% over 5 min, and re-equilibrated at 32% for 10 min prior to injection of another sample. This program had previously been empirically determined by Verchere (1991) to provide separation of the tracer components. Radioactivity of the column eluant was measured with a 170 Radioisotope Detector (Beckman) and charted on a Recordall Series 5000 recorder (Fisher). Eluant fractions were collected every 0.5

min into 13 x 10 mm siliconized glass test tubes containing 10  $\mu$ l BSA (5%; RIA Grade). Aliquots (10  $\mu$ l) of these fractions were counted to ensure recovery and separation, and peak fractions were pooled, frozen at  $-70^{\circ}\text{C}$ , lyophilized, and stored at  $-20^{\circ}\text{C}$ .

The specific activity of the  $^{125}\text{I}$ -GIP was estimated by two methods. First, the total radioactivity (cpm) eluted from the G-15 column for the first peak ( $^{125}\text{I}$ -GIP) was determined as the sum of the radioactivity in each fraction of the peak ( $= x \mu\text{Ci}$ ). Assuming that all 5  $\mu\text{g}$  GIP was iodinated, the specific activity (S.A.) was then estimated as:  $\text{S.A. } (\mu\text{Ci}/\mu\text{g}) = x \mu\text{Ci}/5 \mu\text{g}$ . Specific activity of the purified tracer was estimated from self-displacement curves, using increasing concentrations of  $^{125}\text{I}$ -GIP in a GIP RIA. A displacement curve was formed beginning with  $\sim 5000$  cpm, and then doubling amounts up to 160,000 cpm. A standard curve was also prepared similar to the normal GIP RIA (see section 2.2.8), with the exception that the label was added at the same time as the standards. In this fashion, both displacement curves allowed equal time for labeled and unlabeled peptide to compete for binding to the antibody. The ratio of bound to total tracer (%B) was then calculated for each concentration of  $^{125}\text{I}$ -GIP and the results plotted as %B vs. total counts added, on the same axes as the standard curve. Assuming that the antiserum used bound  $^{125}\text{I}$ -GIP and unlabeled GIP equally well, both the GIP standard concentration and  $^{125}\text{I}$ -GIP radioactivity were determined for several values of %B in the mid-range of the curve and a plot of GIP mass (pg) vs radioactivity (cpm) was produced. The S.A. of the label was then estimated as the inverse slope of this plot.

#### *4.2.5 INCUBATION OF $^{125}\text{I}$ -GIP WITH SERUM*

The hypothesis that  $^{125}\text{I}$ -GIP could be metabolized by serum and the components resolved by HPLC was tested. Purified label (peak 2) was diluted to 500,000 cpm/50  $\mu$ l in 40 mM phosphate buffer (pH 7.0) and 50  $\mu$ l aliquots were added to siliconized test tubes containing 400  $\mu$ l of the same buffer. After gentle mixing, 50  $\mu$ l of rat serum was added to each tube, and incubated at  $37^{\circ}\text{C}$ . Some samples were also incubated at  $4^{\circ}\text{C}$  to

examine the effect of temperature. Similar serum/label mixtures were prepared with buffer containing either 0.1 mM diprotin A, 2% aprotinin, 50 U/ml bacitracin (Upjohn), or all three combined as a 'cocktail'. After incubation times ranging from 10 min to 48 h, 100  $\mu$ l aliquots were removed and analyzed by HPLC for breakdown of  $^{125}$ I-GIP 1-42.

#### 4.2.6 *IN VIVO* EXPERIMENTS WITH $^{125}$ I-GIP

Experiments were next carried out to measure the degradation of  $^{125}$ I-GIP 1-42 *in vivo*. Age matched Fischer 344 rats and DPP IV-negative rats (~350 g) were fasted overnight and anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg). The right jugular vein was cannulated with PE 90 tubing filled with heparinized saline and attached to a 3-way stopcock. The abdomen was then exposed by a midline incision and the abdominal aorta cannulated with heparinized saline-filled PE160 tubing attached to a 3-way stopcock. At time 0,  $\sim 5 \times 10^6$  cpm purified GIP label (peak 2;  $\sim 22.5$  ng GIP) in a volume of 100  $\mu$ l 40 mM phosphate buffer (pH 7.0) was injected into the jugular vein, followed by 200-300  $\mu$ l of heparinized saline. At intervals of 2, 5, 10, and 20 min, 2 ml blood samples were collected from the dorsal aorta and transferred immediately to Eppendorf tubes on ice containing diprotin A and aprotinin (final concentration 0.1 mM and 2%, respectively). Samples were then centrifuged at 7,000  $\times$  g for 5 min at 4°C and the serum removed and stored on ice.

A C<sub>18</sub> SepPak cartridge (Waters) was used to purify the  $^{125}$ I-GIP from the serum. The SepPak was first primed with 5 ml CH<sub>3</sub>CN (plus 0.1% TFA), followed by 10 ml H<sub>2</sub>O (plus 0.1% TFA). The serum sample was then applied to the cartridge, and washed with 10 ml H<sub>2</sub>O plus TFA followed by 10 ml of 20% CH<sub>3</sub>CN plus TFA. The label was then eluted into a siliconized test tube containing 10  $\mu$ l BSA (5%; RIA Grade) with 2 ml 40% CH<sub>3</sub>CN plus TFA, lyophilized and stored at -20°C. Each step of the procedure was monitored with a portable gamma detector to ensure maximal recovery of the tracer.

Lyophilized samples were then dissolved in 100  $\mu$ l of distilled H<sub>2</sub>O and analyzed by HPLC as described in section 4.2.4.

Control experiments were performed in order to ensure that during the sample collection procedure and sample treatment steps, no further degradation of the label occurred. Approximately  $6 \times 10^5$  cpm was divided into two aliquots of 1 ml serum containing diprotin A (0.1 mM) and aprotinin (2%). One aliquot was maintained on ice for 1 h, while the other was incubated at 37°C for 1 h. Both samples were then purified using the SepPak, and lyophilized as the rest of the samples. These controls were then analyzed by HPLC for any degradation of the <sup>125</sup>I-GIP.

#### *4.2.7 DIPEPTIDYL PEPTIDASE IV IN ZUCKER RATS AND NIDDM PATIENTS*

Serum samples were collected from fed Wistar rats (4) plus fed lean and fat Zucker rats (4 of each) and stored at -20°C. In collaboration with Dr. G. S. Meneilly (Department of Medicine, UBC) samples were also collected from 12 fasted non-obese NIDDM patients receiving treatment with oral hypoglycemic agents, and 12 fasted age matched controls with normal glucose tolerance, and stored at -20°C. All samples were then assayed for DPP IV by the method described in section 4.2.3.

### **4.3 RESULTS**

#### *4.3.1 INCUBATION OF GIP WITH SERUM*

Incubation of GIP with serum yielded a time-dependent degradation, resulting in products that eluted by HPLC identically to that observed for STC 6-14 cell culture medium (Figure 17 chapter 3). This process did not occur in the absence of serum, and was considerably reduced by the addition of 0.1 mM diprotin A (data not shown).

#### 4.3.2 DIPEPTIDYL PEPTIDASE IV ASSAY

The assay used to measure serum levels of DPP IV relied on the ability of this enzyme to convert Gly-Pro-*p*-nitroanilide to the colored product, *p*-nitroaniline. The development of the product was monitored with a spectrophotometer at 410 nm and was converted to mM *p*-nitroaniline produced using a standard curve (Figure 21A). Under the assay conditions used, serum from Fischer rats was able to produce *p*-nitroaniline at a rate of  $\sim 1.66 \mu\text{M}/\text{min}$ , while production with serum from DPP IV-negative rats occurred at a much slower rate of  $\sim 0.17 \mu\text{M}/\text{min}$  (Figure 21B). There was no significant difference in DPP IV activity in the same serum volume from Fischer, Wistar, lean Zucker, or fat Zucker rats (data not shown). Similarly, there was no significant difference in the DPP IV activity in serum from NIDDM patients tested when compared to controls. Absorbance values at 45 min were corrected for the absorbance of the serum + substrate at time 0, and then converted to mM *p*-nitroaniline produced using the standard curve. The average concentration of *p*-nitroaniline after 45 min for control subjects was  $0.134 \pm 0.004 \text{ mM}$  ( $\sim 2.97 \mu\text{M}/\text{min}$ ), and for diabetic subjects was  $0.128 \pm 0.004 \text{ mM}$  ( $\sim 2.84 \mu\text{M}/\text{min}$ ).

#### 4.3.3 LABEL SPECIFIC ACTIVITY

The specific activity of the  $^{125}\text{I}$ -GIP eluted from the G-15 column during iodination (Figure 23) was estimated at  $\sim 35 \mu\text{Ci}/\mu\text{g}$ . This material was then purified by reverse-phase HPLC, which revealed a heterogeneous population of iodinated peptides, consisting of 4 major components, the largest being peak 2 (Figure 25A). The specific activity of this fraction was estimated from self displacement curves to be  $\sim 100 \mu\text{Ci}/\mu\text{g}$  (Figure 24), representing a significant enrichment compared to the unpurified tracer.

#### 4.3.4 INCUBATION OF PURIFIED $^{125}\text{I}$ -GIP WITH WISTAR RAT SERUM

The stability of HPLC purified  $^{125}\text{I}$ -GIP 1-42 was investigated by incubation at 37°C for up to 24 h in the absence of serum. At intervals of ~6 h, labeled GIP was removed and analyzed by HPLC. Even after 24 h, a single component was observed (Figure 25B). This  $^{125}\text{I}$ -GIP 1-42 eluted slightly later (~21-22 min) than when collected as peak 2 (~19 min; Figure 25A), perhaps attributable to the use of a new C<sub>18</sub> column. A single component was also observed after 10 min incubation with 10% Wistar rat serum (Figure 26A), but after 1 h,  $^{125}\text{I}$ -GIP had resolved into two distinct major components. One component eluted in the original position (~21-22 min) and thus consisted of  $^{125}\text{I}$ -GIP 1-42 (peak 1), while the new component eluted earlier (19-20 min). This separation is similar to that observed in the identification of GIP 3-42 in natural GIP preparations (see Figure 16) and therefore this peak 2 was assigned  $^{125}\text{I}$ -GIP 3-42. With time, as aliquots of incubated  $^{125}\text{I}$ -GIP and serum were analyzed by HPLC, a progression from  $^{125}\text{I}$ -GIP 1-42 to putative  $^{125}\text{I}$ -GIP 3-42 was observed (Figures 27 & 28). Furthermore, other  $^{125}\text{I}$ -GIP fragments with different elution times appeared at a significantly slower rate, the majority eluting at ~3 min (peak 3; Figures 27 & 28).

The effect of temperature on the metabolism of  $^{125}\text{I}$ -GIP 1-42 was investigated by performing the same experiments at 4°C. Both peaks 1 & 2 could be observed, indicating that the conversion of  $^{125}\text{I}$ -GIP 1-42 to putative  $^{125}\text{I}$ -GIP 3-42 was still occurring (Figures 29 & 30). However, the rate of this reaction (as determined by the changes of the areas of the two peaks with time) was considerably slower. Interestingly, at this temperature, an absence of other peaks including peak 3 was noted, indicating no further degradation of the tracer had occurred (Figures 29 & 30)

The effect of enzyme inhibitors on the degradation of  $^{125}\text{I}$ -GIP was also investigated. Diprotin A, a competitive inhibitor of DPP IV, was able to significantly reduce the degradation of  $^{125}\text{I}$ -GIP 1-42 to putative  $^{125}\text{I}$ -GIP 3-42. This was evident from the significantly reduced rate of appearance of peak 2 and reduction in peak 1 when 0.1

mM diprotin A was added to the serum and  $^{125}\text{I}$ -GIP (Figures 31 & 32). However, this inhibitor did not alter the degradation of  $^{125}\text{I}$ -GIP to other fragments, with considerable radioactivity eluting in peak 3 as incubation time progressed (Figures 31 & 32). In contrast, the protease inhibitor aprotinin had no significant effect on the conversion of  $^{125}\text{I}$ -GIP 1-42 to putative  $^{125}\text{I}$ -GIP 3-42, as evident from the reduction of peak 1 and production of peak 2 with time by Wistar rat serum in the presence of 2% aprotinin (Figures 33 & 34). However, this inhibitor did reduce the rate of production of peak 3 (Figures 33 & 34). The antibiotic bacitracin (50 U/ml) significantly reduced the conversion of  $^{125}\text{I}$ -GIP 1-42 to putative  $^{125}\text{I}$ -GIP 3-42 by serum (peak 1 to peak 2; Figure 35), but not to the same degree as diprotin A. Bacitracin, however, greatly reduced the further fragmentation of  $^{125}\text{I}$ -GIP as indicated by the near absence of other peaks, including peak 3 (Figure 35). When used in combination, these inhibitors effectively reduced the degradation of  $^{125}\text{I}$ -GIP (Figure 36). After 20 h incubation of  $^{125}\text{I}$ -GIP with Wistar rat serum and the inhibitor 'cocktail' at 37°C, ~35% existed as putative  $^{125}\text{I}$ -GIP 3-42 (peak 2) and the remainder as  $^{125}\text{I}$ -GIP 1-42 (peak 1).

#### *4.3.5 INCUBATION OF PURIFIED $^{125}\text{I}$ -GIP WITH DPP IV-NEGATIVE RAT SERUM*

The effect of DPP IV-negative serum on the degradation of  $^{125}\text{I}$ -GIP as analyzed by HPLC is shown in Figure 37. For a period of up to 5 h, no peak 2 could be resolved, suggesting that no  $^{125}\text{I}$ -GIP 3-42 was produced. However, as with Wistar rat serum, other peaks representing other  $^{125}\text{I}$ -GIP fragments were observed, including peak 3 (Figure 37). The addition of 2% aprotinin significantly reduced the rate of production of other  $^{125}\text{I}$ -GIP fragments by serum from DPP IV-negative rats, as evident from the reduction in the size of other peaks, including peak 3 (Figures 38 & 39).

#### 4.3.6 INCUBATION OF PURIFIED $^{125}\text{I}$ -GIP WITH CEP

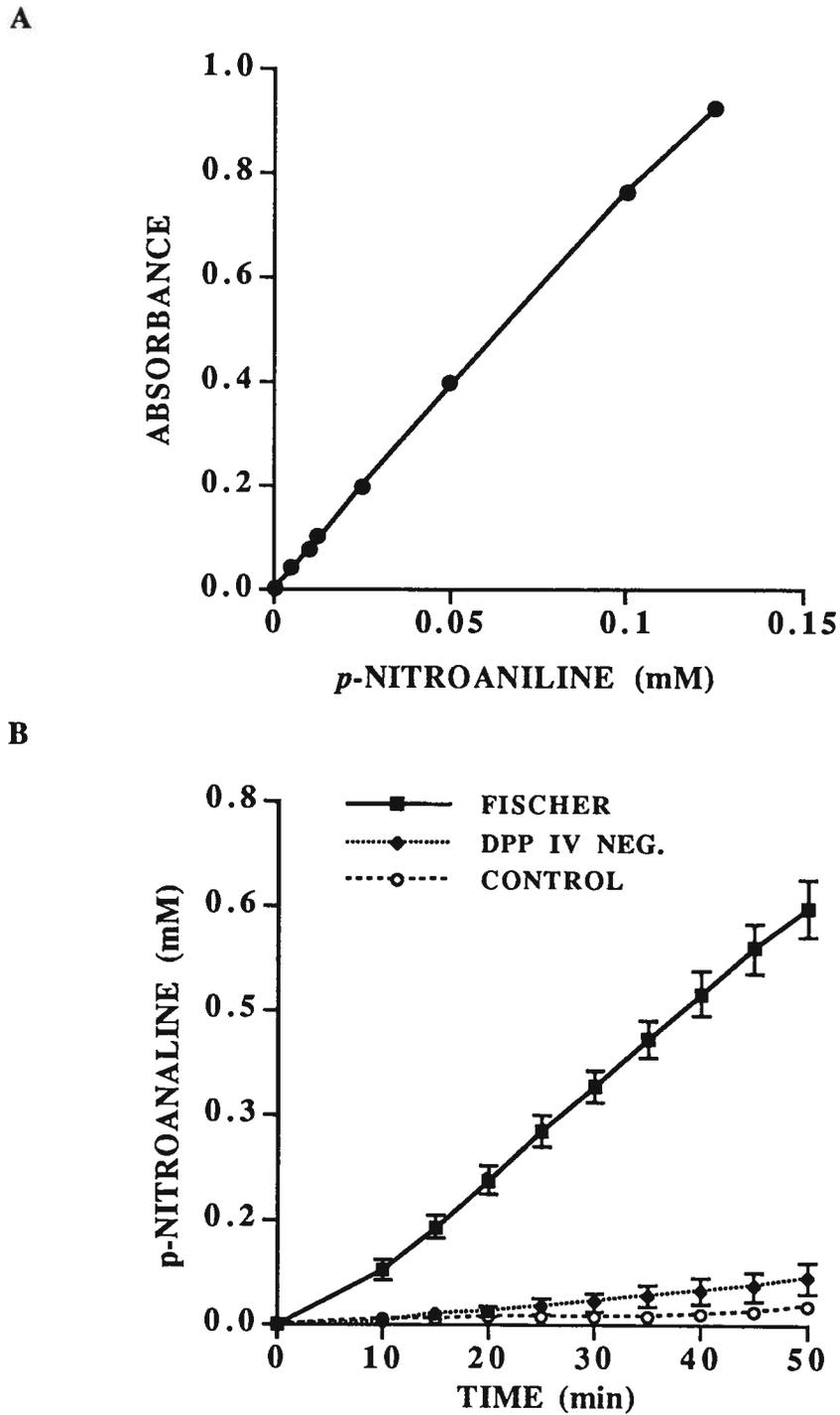
The buffer used for the GIP RIA contains 5% charcoal extracted human plasma (CEP; see section 2.2.8). In order to determine if this plasma contains enzymes capable of degrading GIP, CEP was diluted and incubated at 37°C with  $^{125}\text{I}$ -GIP and was analyzed by HPLC. After 3 h incubation, ~48%  $^{125}\text{I}$ -GIP was found in peak 2, the putative  $^{125}\text{I}$ -GIP 3-42 (Figure 40). Furthermore, by 7 h, further significant degradation to other  $^{125}\text{I}$ -GIP fragments had occurred, including a significant proportion in peak 3 (Figure 40). These observations would suggest that the addition of this plasma to the GIP assay buffer would promote the degradation of the GIP tracer, and possibly the standards and samples.

#### 4.3.7 THE ANALYSIS OF $^{125}\text{I}$ -GIP DEGRADATION IN VIVO

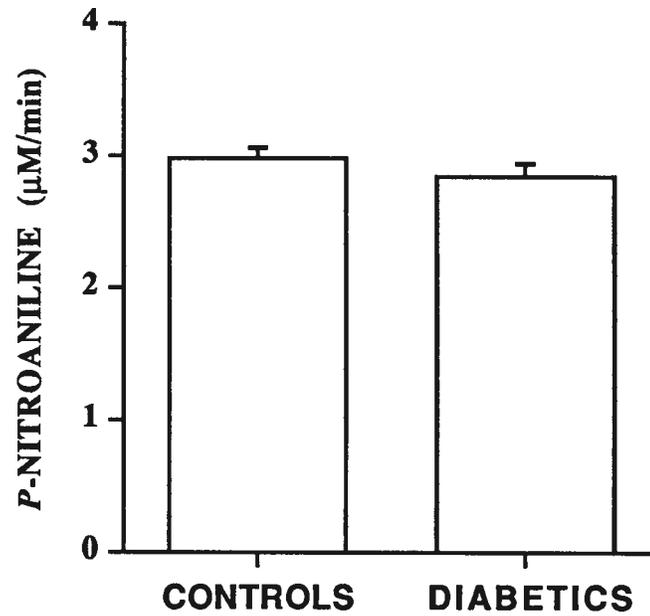
Methods were developed to investigate the degradation of GIP *in vivo*. It was expected that  $^{125}\text{I}$ -GIP 1-42 would be rapidly degraded to  $^{125}\text{I}$ -GIP 3-42 in the circulation, and rapid delivery of the tracer and expeditious blood sampling were deemed necessary. Label was therefore delivered by injection through a jugular vein cannula and plasma samples were collected directly from the aorta. It was also imperative that any further degradation subsequent to sample collection be prevented. Based on *in vitro* results it was determined that in order to keep degradation to a minimum, samples should be collected into tubes in ice, containing diprotin A and aprotinin (final concentrations 0.1 mM and 2%, respectively). The collected samples were then purified by SepPak as described earlier (section 4.2.6). Recovery of tracer from plasma collection to lyophilization was approximately 80%. In order to verify that there was minimal label degradation through all steps of the procedure,  $^{125}\text{I}$ -GIP was added to serum containing the inhibitors, and was treated identically to the experimental samples. However, prior to being purified by SepPak, one aliquot was stored on ice for 1 h, and another was incubated at 37°C for 1 h. The results of the HPLC analysis are shown in Figure 41.

Neither condition resulted in measurable degradation of the label, indicating that experimental samples were treated appropriately.

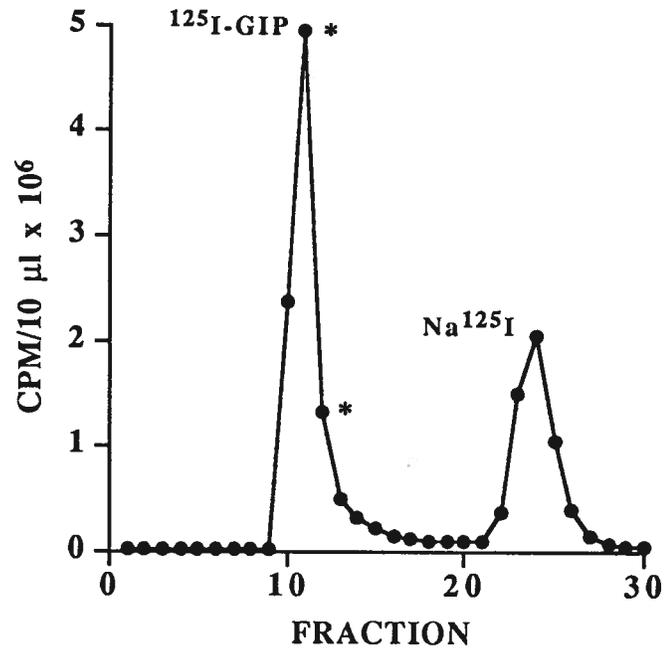
The degradation of label occurred very quickly *in vivo*. By 10 min, only ~9% of the  $^{125}\text{I}$ -GIP was represented by peak 1 ( $^{125}\text{I}$ -GIP 1-42; Figure 42 & 43). The remainder of tracer appeared to be  $^{125}\text{I}$ -GIP 3-42 (peak 2) with no other degradation products discernible. In contrast, there was no degradation of  $^{125}\text{I}$ -GIP infused into DPP IV-negative rats by 10 min (Figure 44). In order to summarize some of the data on  $^{125}\text{I}$ -GIP breakdown in serum, the relative proportions of putative  $^{125}\text{I}$ -GIP 3-42 and  $^{125}\text{I}$ -GIP 1-42 were computed by measuring the area of peak 1 and peak 2. By repeating this for various time intervals, plots of the rate of conversion to putative  $^{125}\text{I}$ -GIP 3-42 were produced (Figure 45). Figure 45A indicates that the time required to yield 50% putative  $^{125}\text{I}$ -GIP 3-42 was ~2.2 h in 10% normal Wistar rat serum. This time was significantly reduced by the addition of bacitracin to ~5.6 h, and to 16.1 h by storage at 4°C. The addition of diprotin A lengthened the time required to acquire 50% putative  $^{125}\text{I}$ -GIP 3-42 to ~29 h. Similar computations were done with the *in vivo* data from one Wistar rat. Only ~1.5 min was required for 50% of the  $^{125}\text{I}$ -GIP to be converted to putative  $^{125}\text{I}$ -GIP 3-42, a rate approximately 88-times faster than was observed with the 10% rat serum *in vitro*. No degradation of  $^{125}\text{I}$ -GIP 1-42 to putative  $^{125}\text{I}$ -GIP 3-42 was observed in DPP IV-negative rats, *in vitro* or *in vivo*.



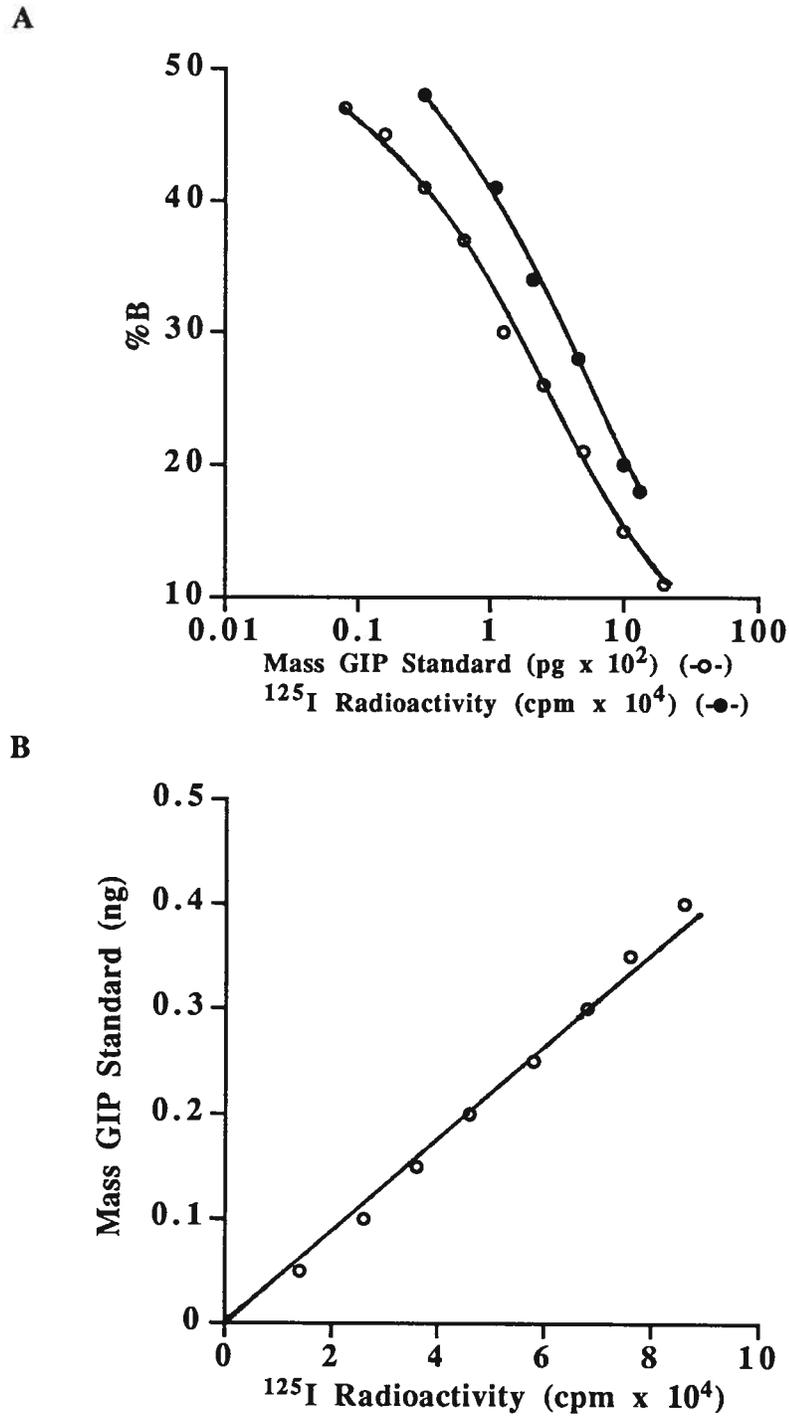
**FIGURE 21:** Determination of relative DPP IV levels in Fischer (DPP IV-positive) and DPP IV-negative rats from rate of production of *p*-nitroaniline. **A)** Standard curve of absorbance for increasing concentrations of *p*-nitroaniline (mM);  $n = 10$ . **B)** Plot of *p*-nitroaniline concentration vs. time (min) determined from absorbance values converted to mM *p*-nitroaniline from curve A. Control = substrate in the absence of serum;  $n = 8$ .



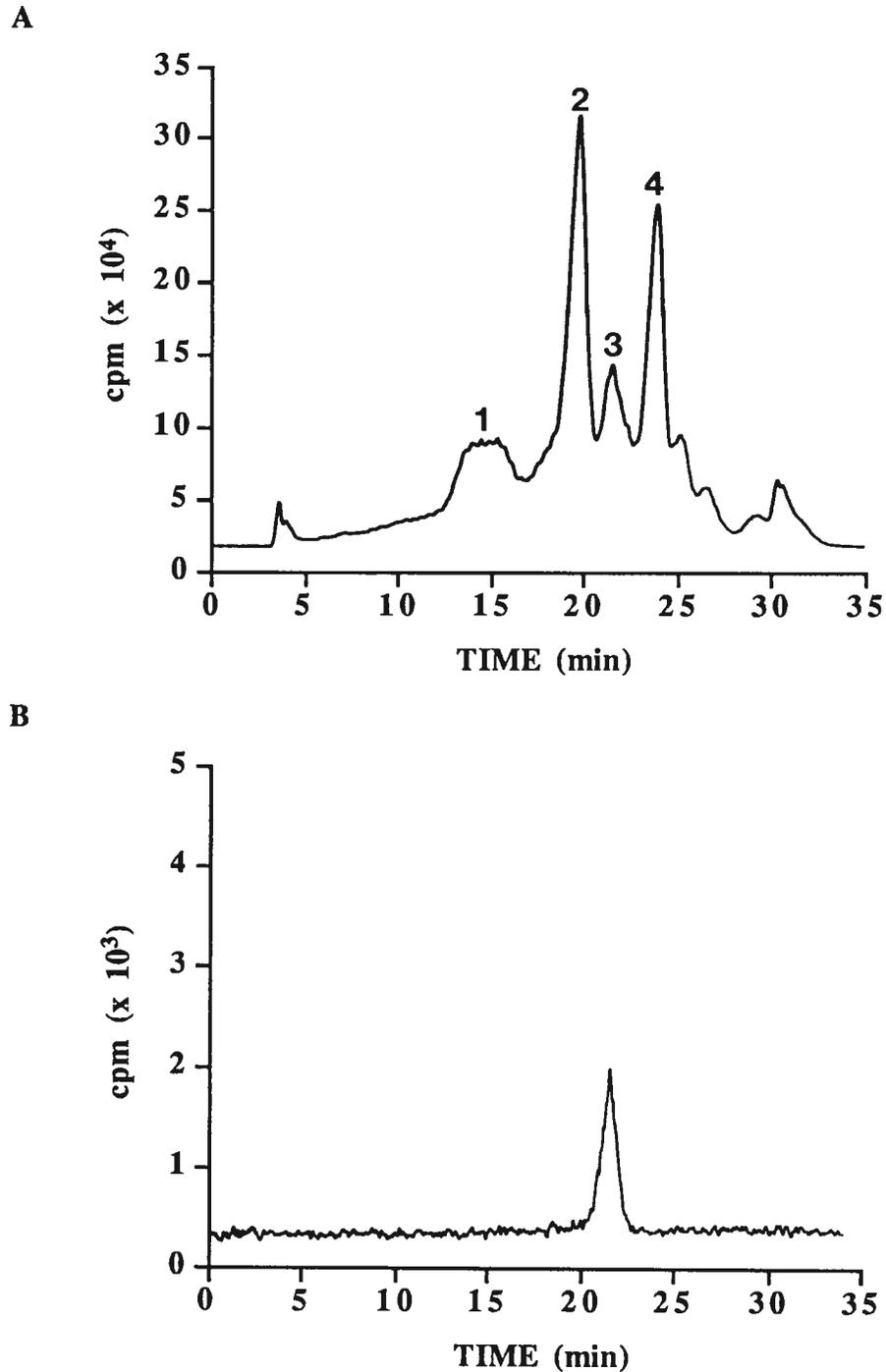
**FIGURE 22:** Determination of the relative levels of DPP IV in NIDDM (diabetic) and normal (control) subjects from the production of *p*-nitroaniline after 45 min. Average concentrations of *p*-nitroaniline were  $0.134 \pm 0.004$  mM ( $\sim 2.97$   $\mu\text{M}/\text{min}$ ) for controls ( $n = 12$ ), and  $0.128 \pm 0.004$  mM ( $\sim 2.84$   $\mu\text{M}/\text{min}$ ) for diabetics ( $n = 12$ ). These values are not significantly different.



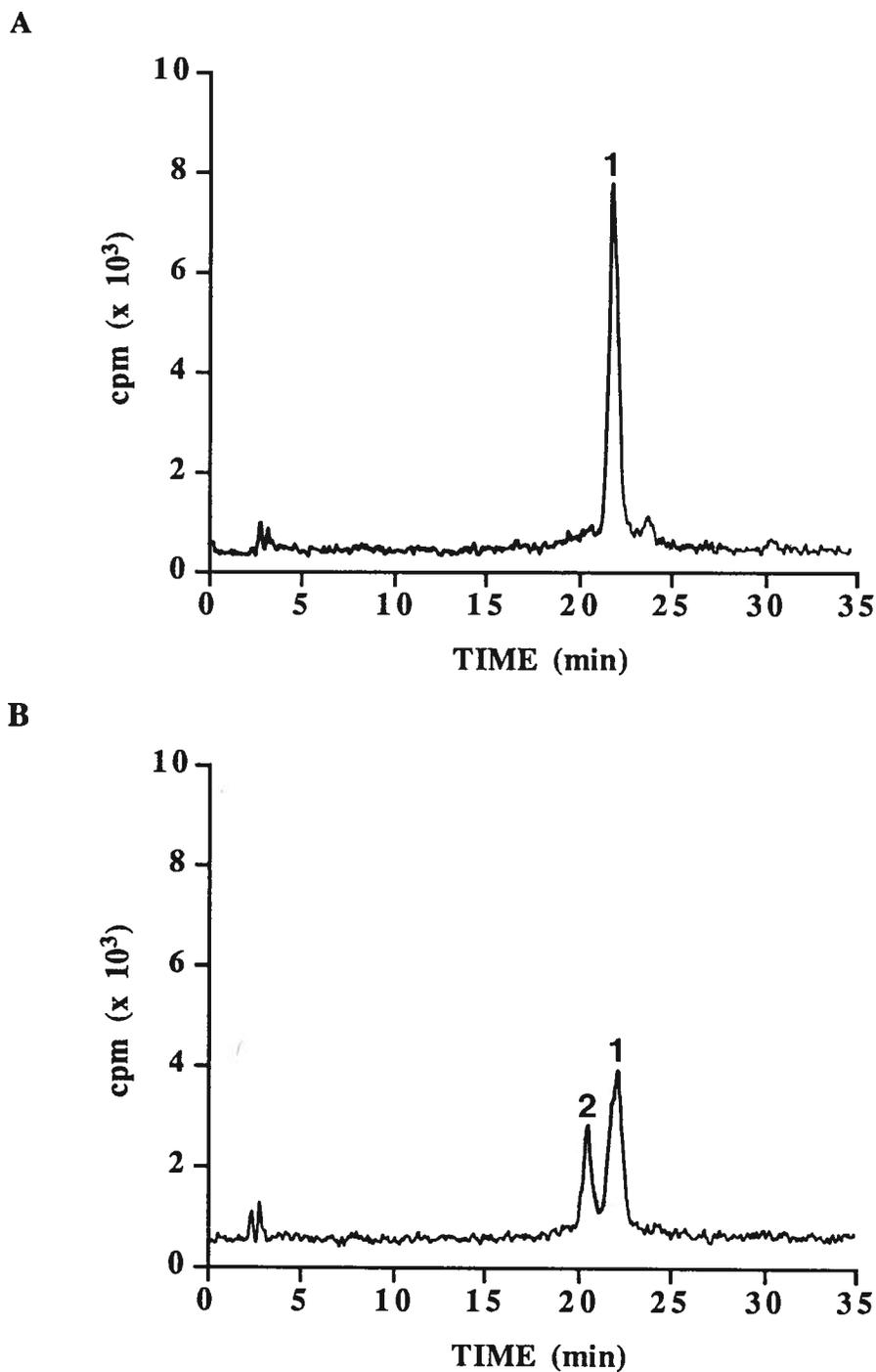
**FIGURE 23:** Profile of synthetic porcine GIP iodination mixture eluted on Sephadex G-15. The iodination mixture was applied to the column immediately following iodination, and fractions were collected every 1.5 min. <sup>125</sup>I-radioactivity was measured from 10 μl aliquots, to produce the profile. Free Na<sup>125</sup>I eluted after <sup>125</sup>I-GIP. \*denotes samples used.



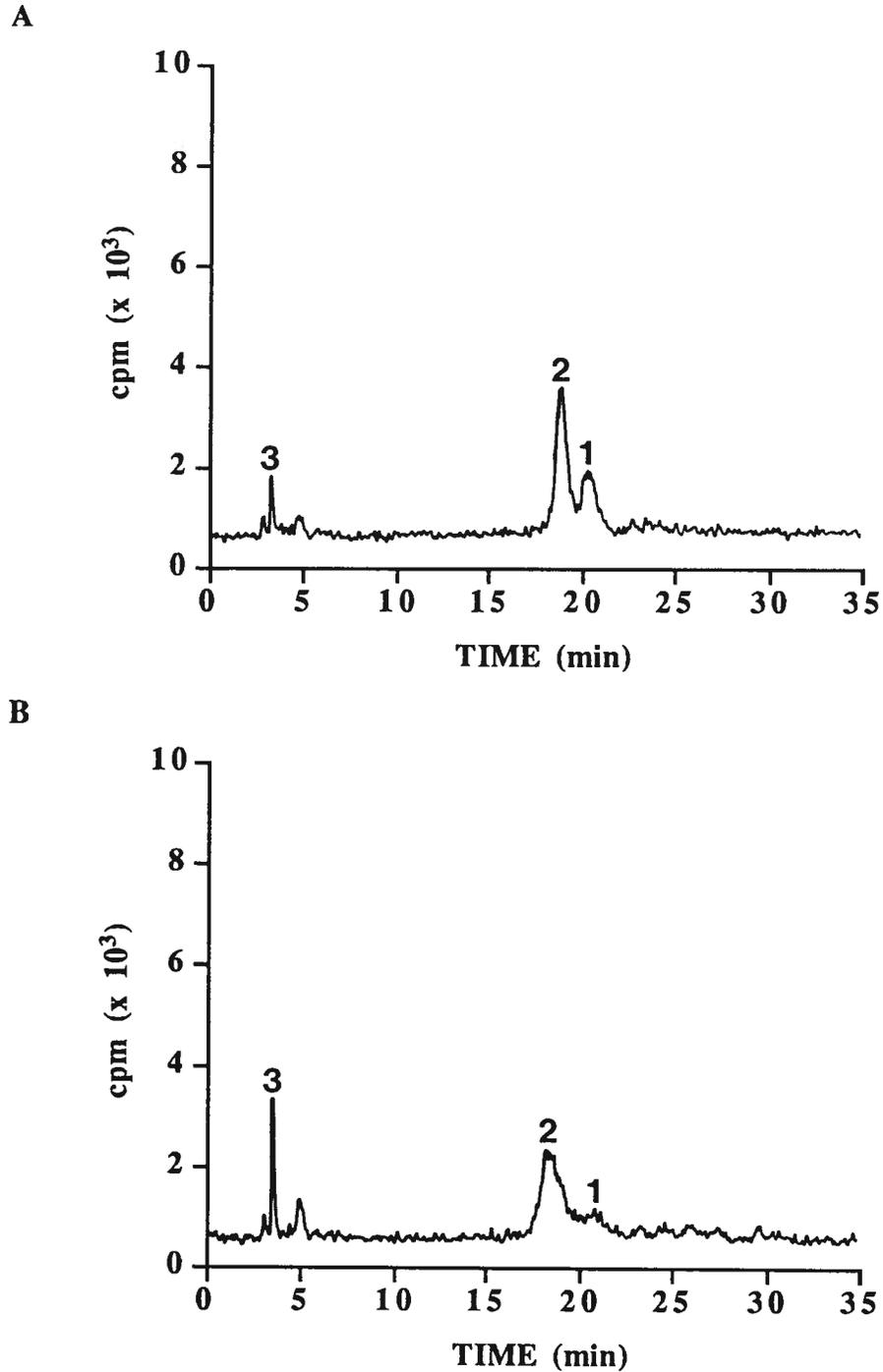
**FIGURE 24:** A) % bound (%B) for a GIP standard curve using increasing GIP concentrations (empty circles), and a self displacement curve using increasing amounts of <sup>125</sup>I-GIP (filled circles). B) Plot of mass of GIP standard vs. <sup>125</sup>I-GIP radioactivity as determined from A for a number of points. The inverse of the slope of this line was used to calculate the specific activity of HPLC purified <sup>125</sup>I-GIP (peak 2; ~100  $\mu$ Ci/ $\mu$ g).



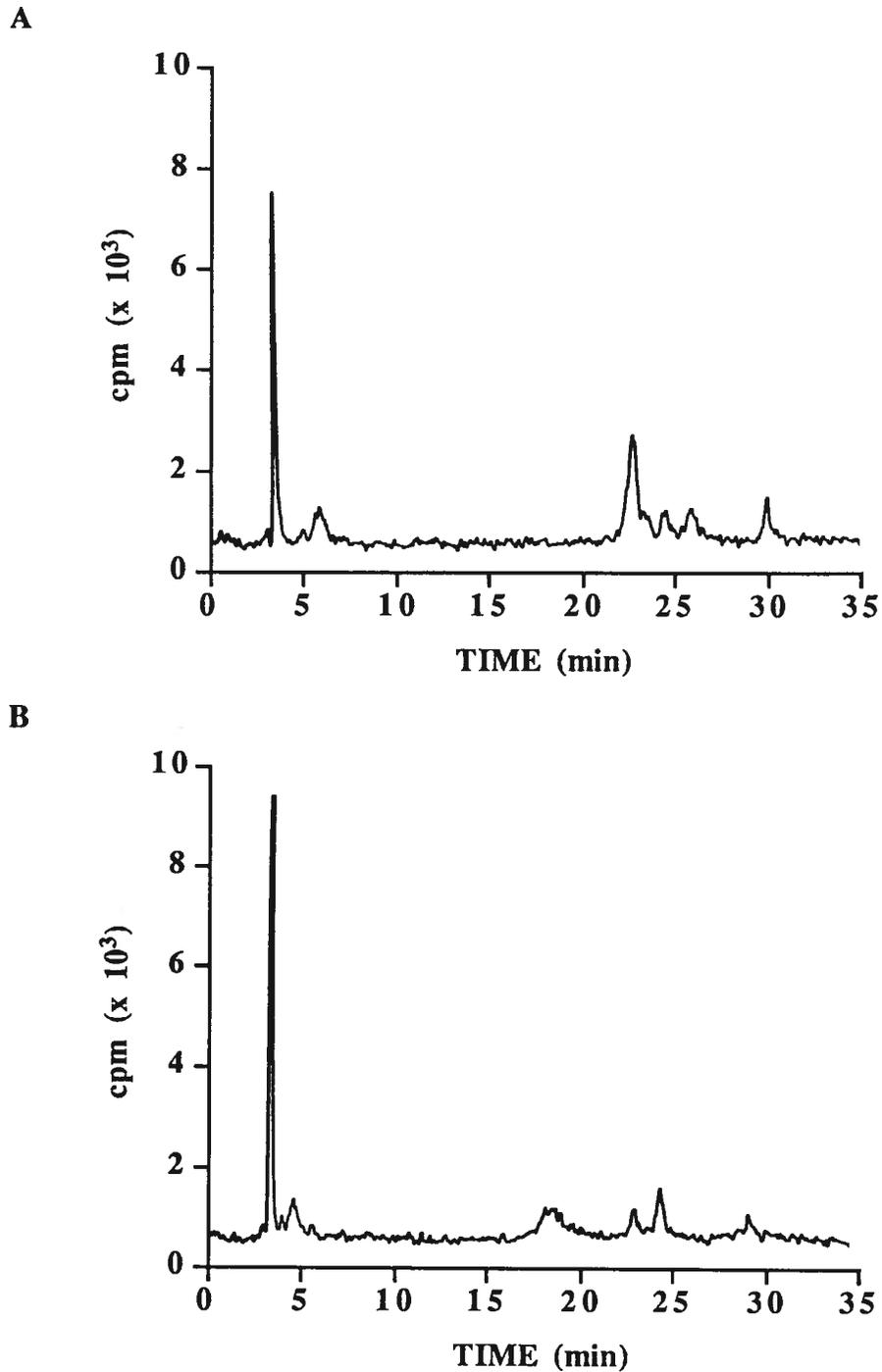
**FIGURE 25:** **A)** HPLC elution profile of  $^{125}\text{I}$ -GIP ( $\sim 10^7$  cpm) at 32% acetonitrile for 10 min, followed by a 10 min gradient up to 38%. After a further 5 min at 38%, the column was washed by a 5 min gradient up to 60%, and then at 30 min was returned to 32% over 5 min. Major peaks numbered 1-4 are discussed in the text. **B)** HPLC elution profile of peak 2 ( $^{125}\text{I}$ -GIP in subsequent figures) after 24 h incubation at 37°C ( $\sim 5 \times 10^5$  cpm).



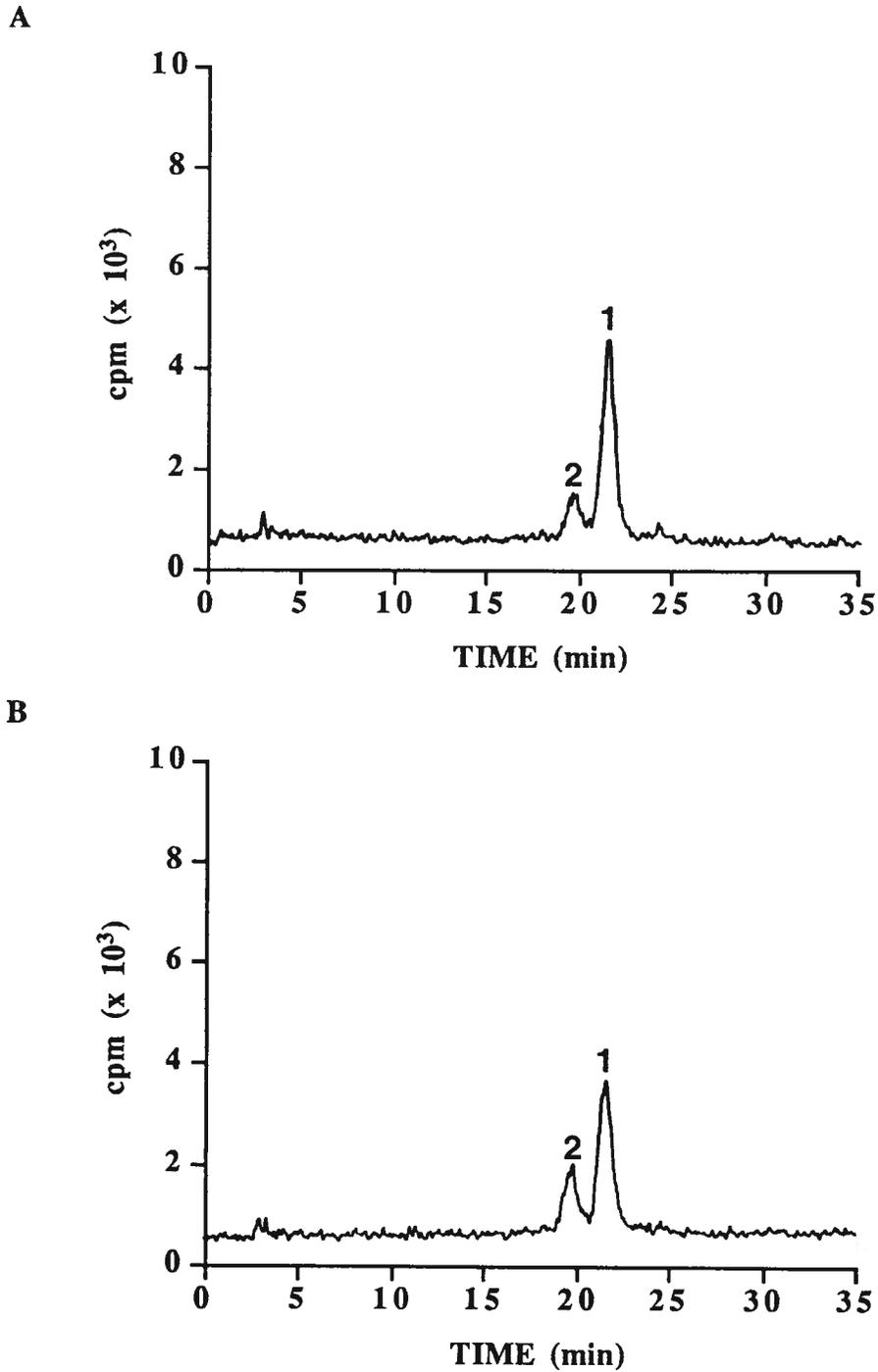
**FIGURE 26:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) at  $37^\circ\text{C}$ . A) duration = 10 min. The single peak corresponds to  $^{125}\text{I}$ -GIP 1-42. B) duration = 1 h. Peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42. In this and subsequent figures, the acetonitrile gradient is identical to that described for Figure 2.



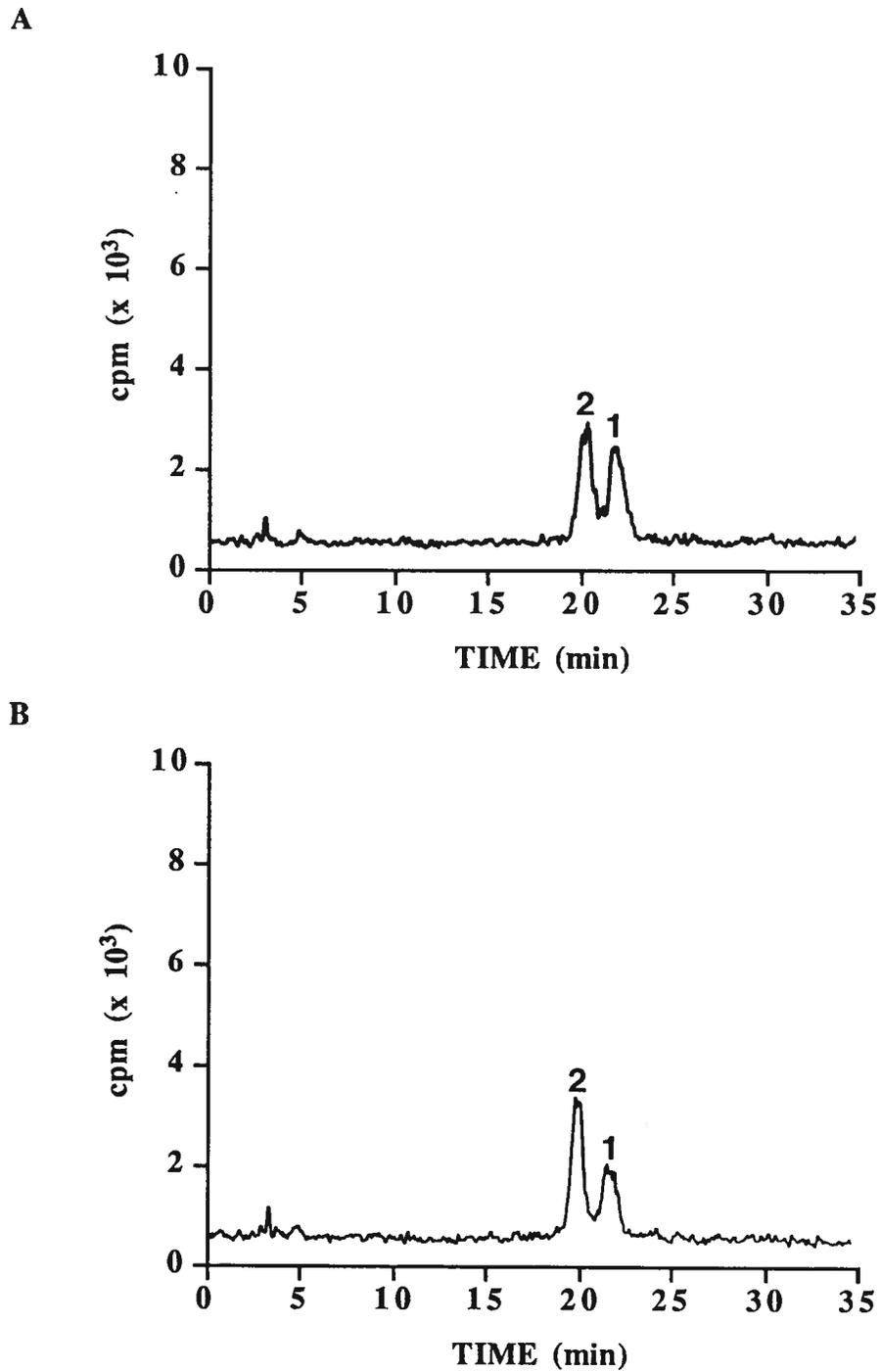
**FIGURE 27:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) at  $37^\circ\text{C}$ . A) duration = 3 h. B) duration = 6 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42, and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



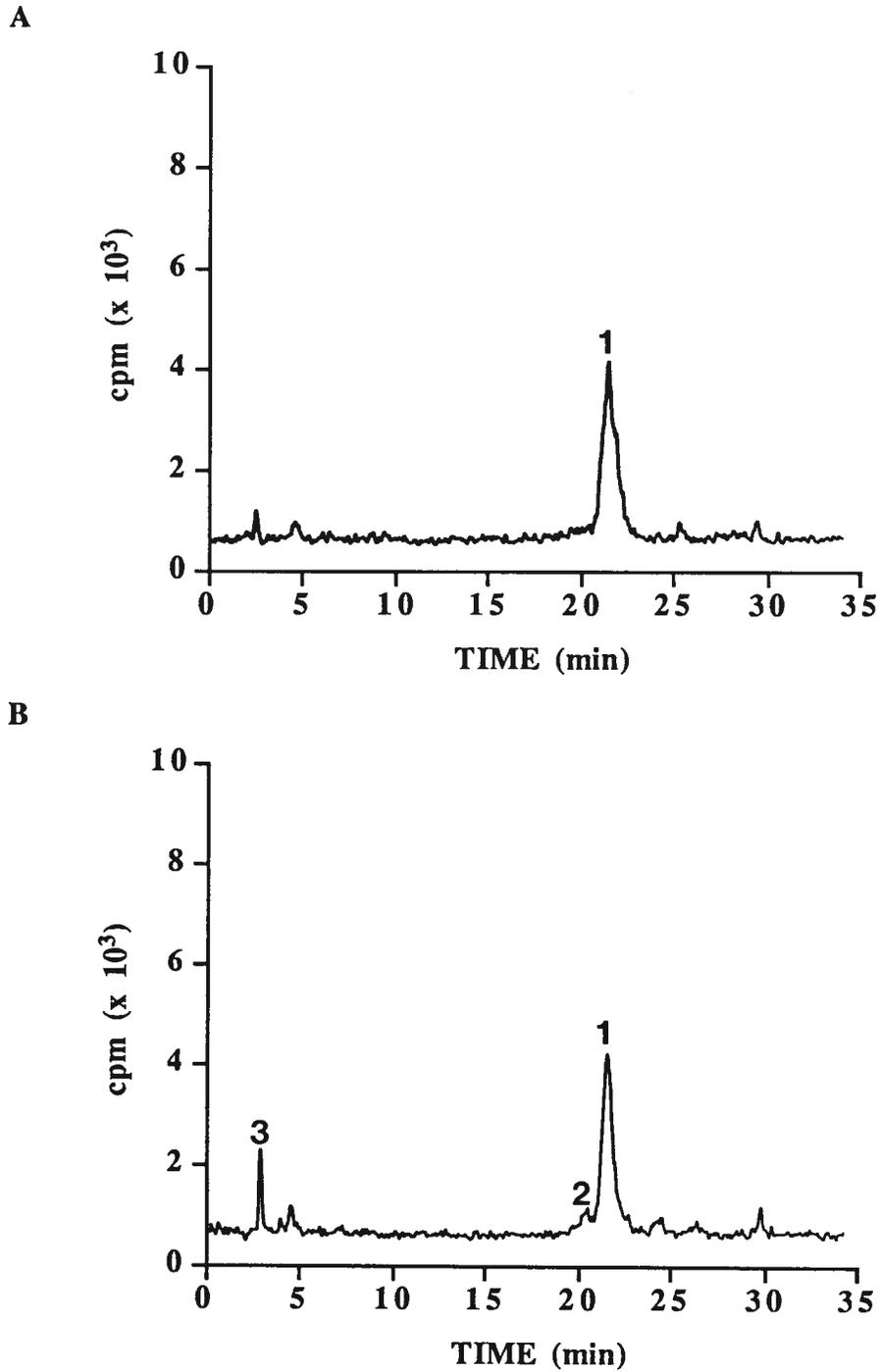
**FIGURE 28:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) at  $37^\circ\text{C}$ . A) duration = 15 h. B) duration = 24 h. In both figures, all peaks consist of unidentified  $^{125}\text{I}$ -GIP fragments.



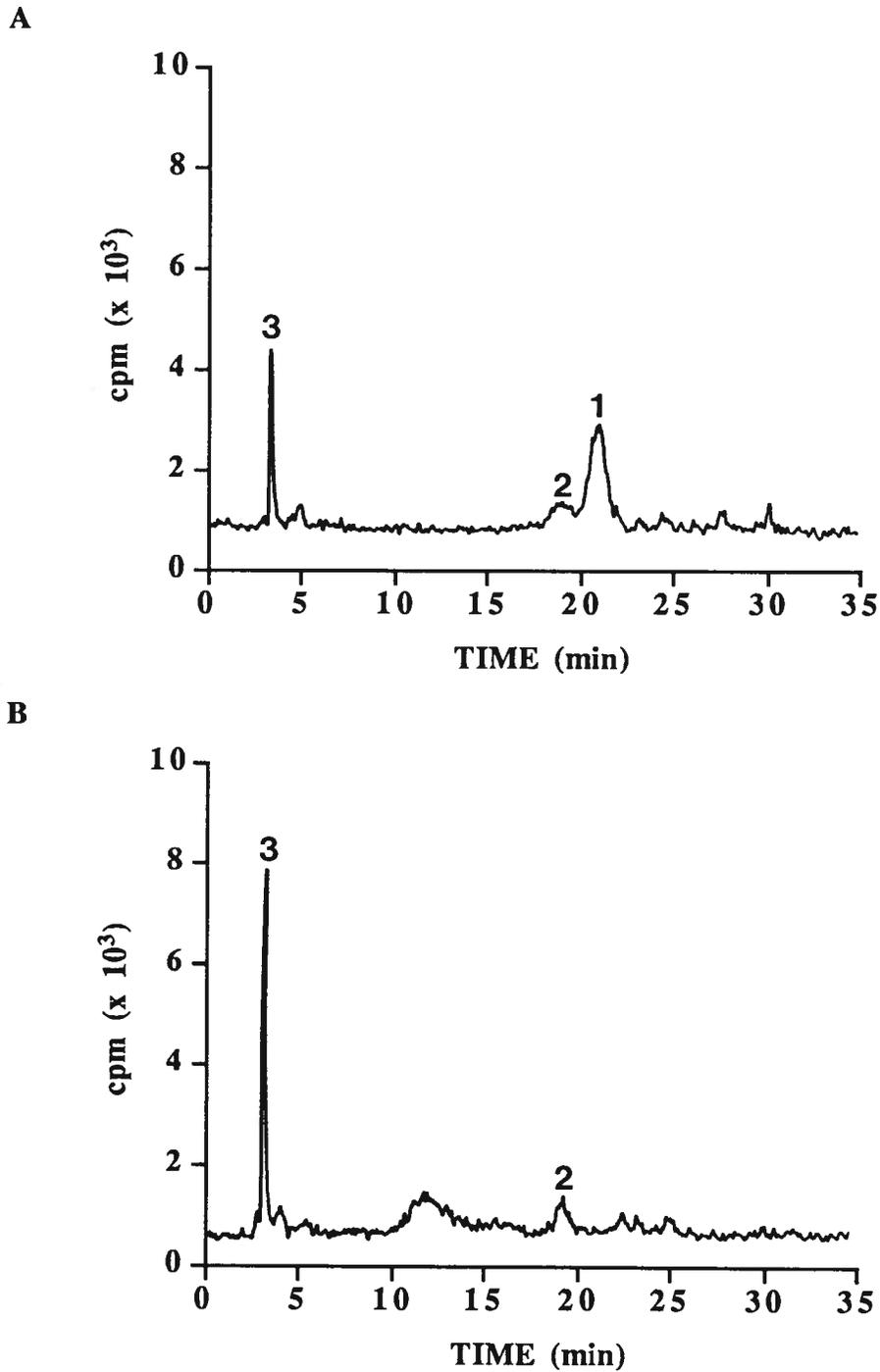
**FIGURE 29:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) at  $4^\circ\text{C}$ . **A)** duration = 4 h. **B)** duration = 7 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, and peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42.



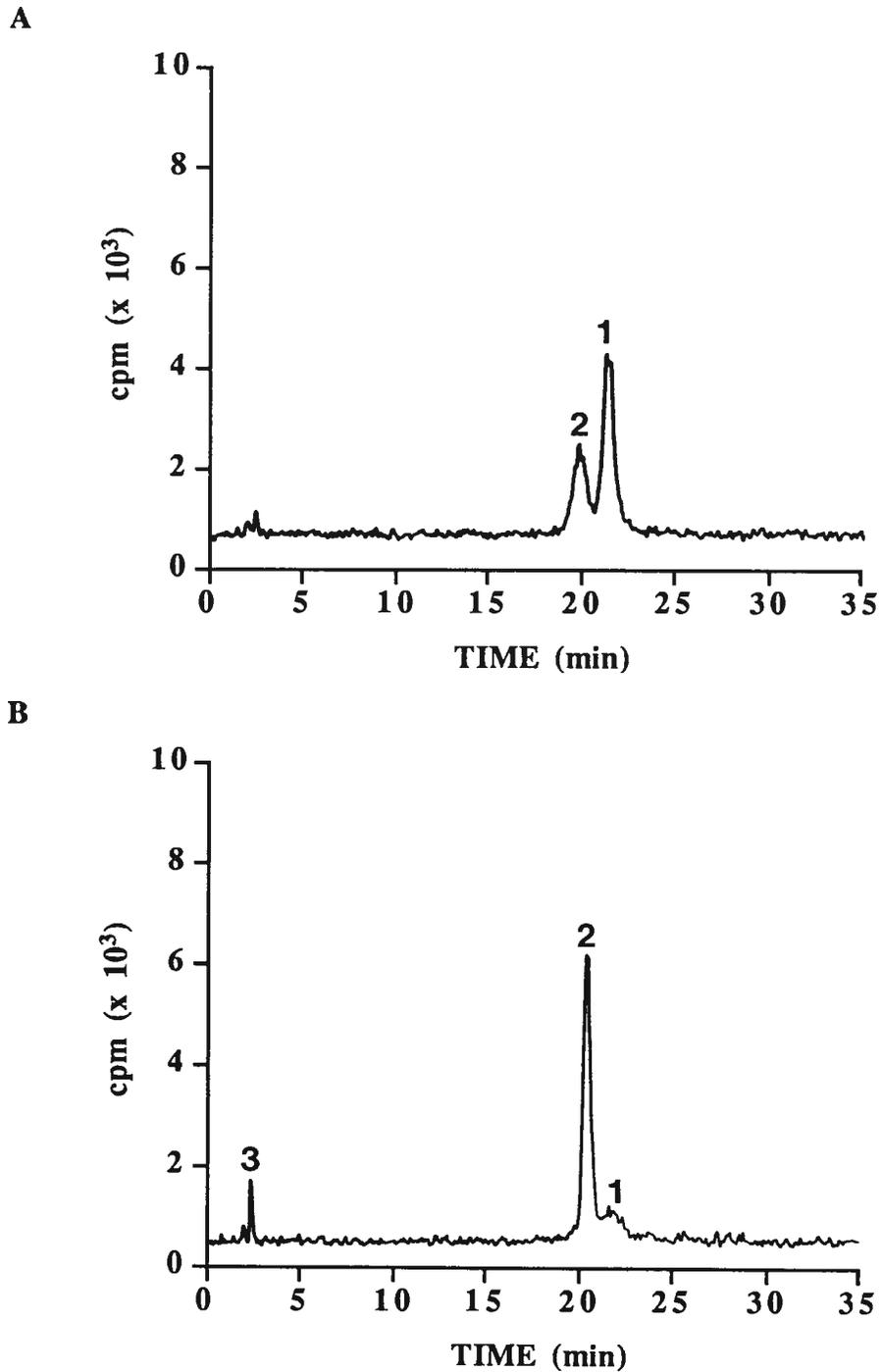
**FIGURE 30:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) at  $4^\circ\text{C}$ . **A)** duration = 19 h. **B)** duration = 24 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, and peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42.



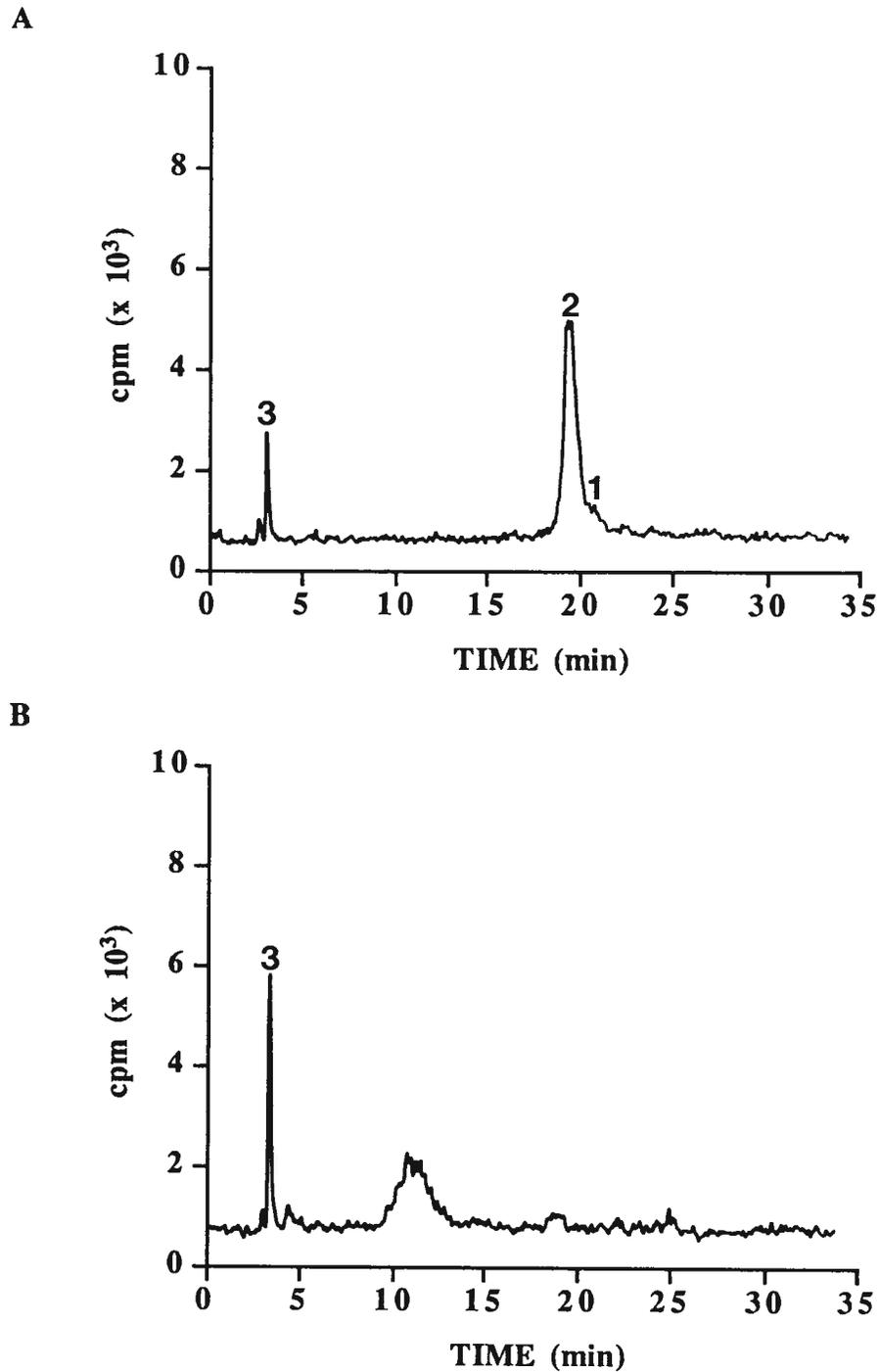
**FIGURE 31:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) and diprotin A (0.1 mM) at  $37^\circ\text{C}$ . **A)** duration = 2 h. **B)** duration = 6 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42, and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



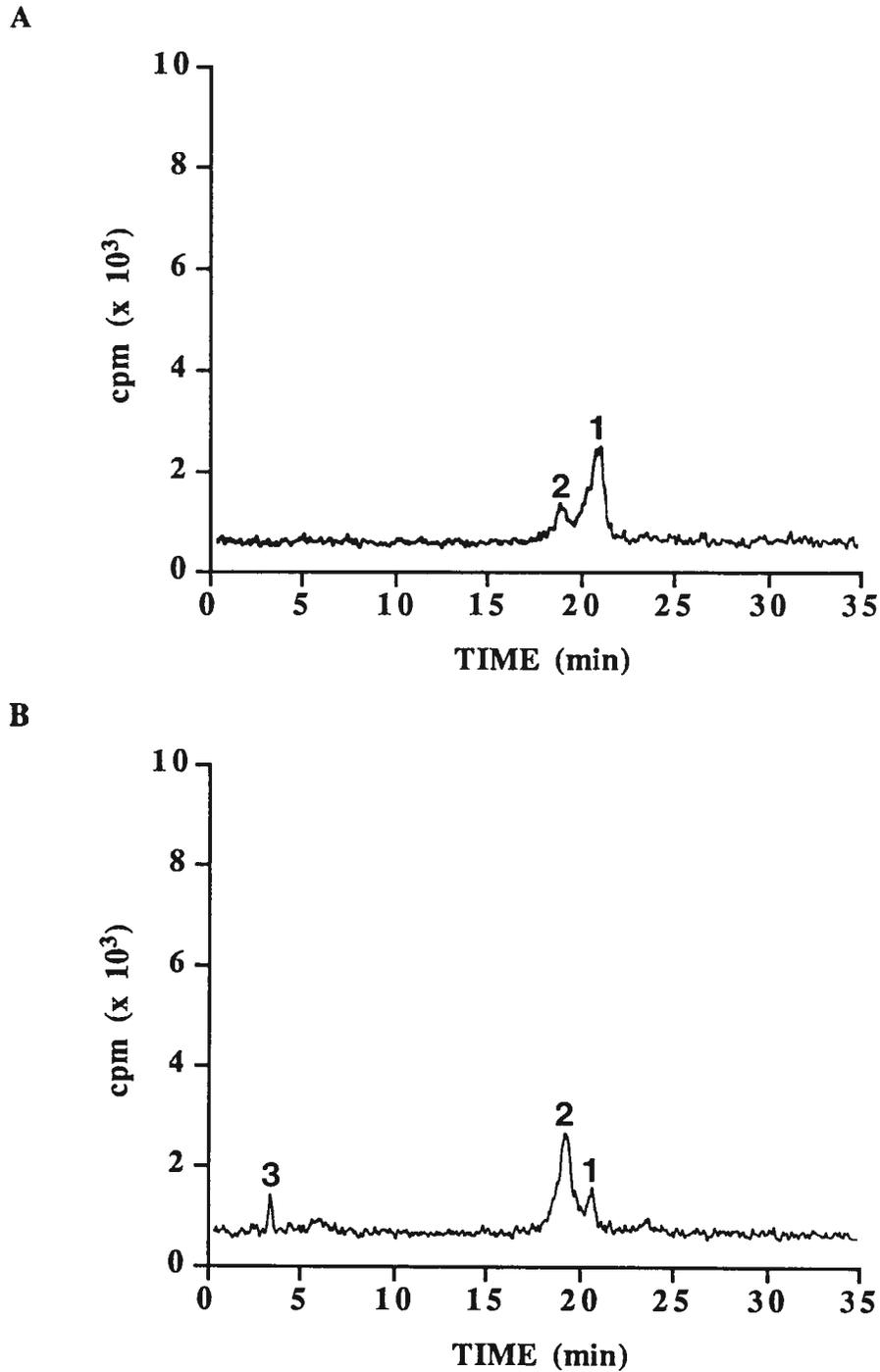
**FIGURE 32:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) and diprotin A (0.1 mM) at  $37^\circ\text{C}$ . **A)** duration = 12 h. **B)** duration = 26 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42, and peak 3 and others consist of unidentified  $^{125}\text{I}$ -GIP fragments.



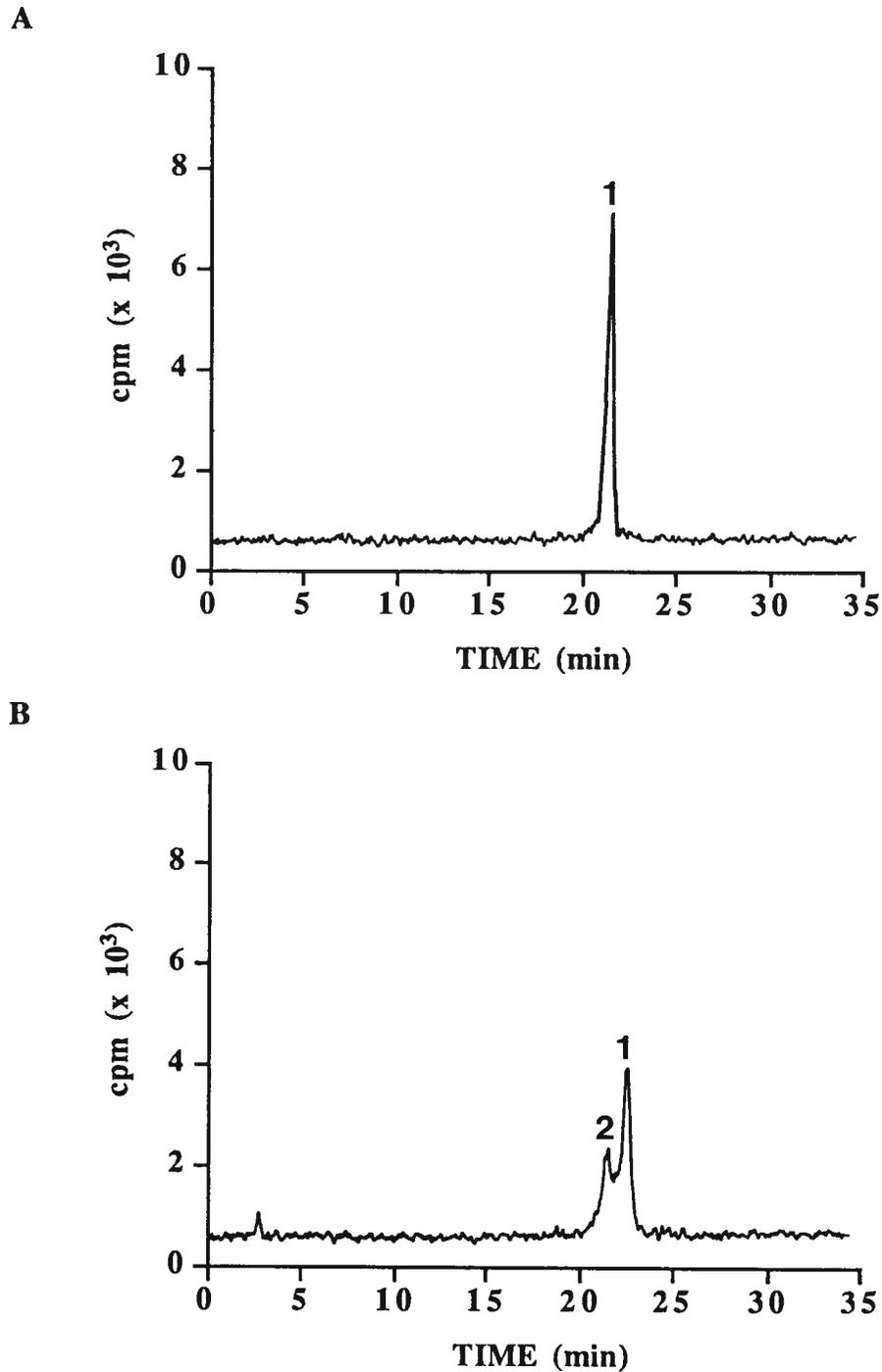
**FIGURE 33:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) and aprotinin (2%) at  $37^\circ\text{C}$ . **A)** duration = 1 h. **B)** duration = 5 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42, and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



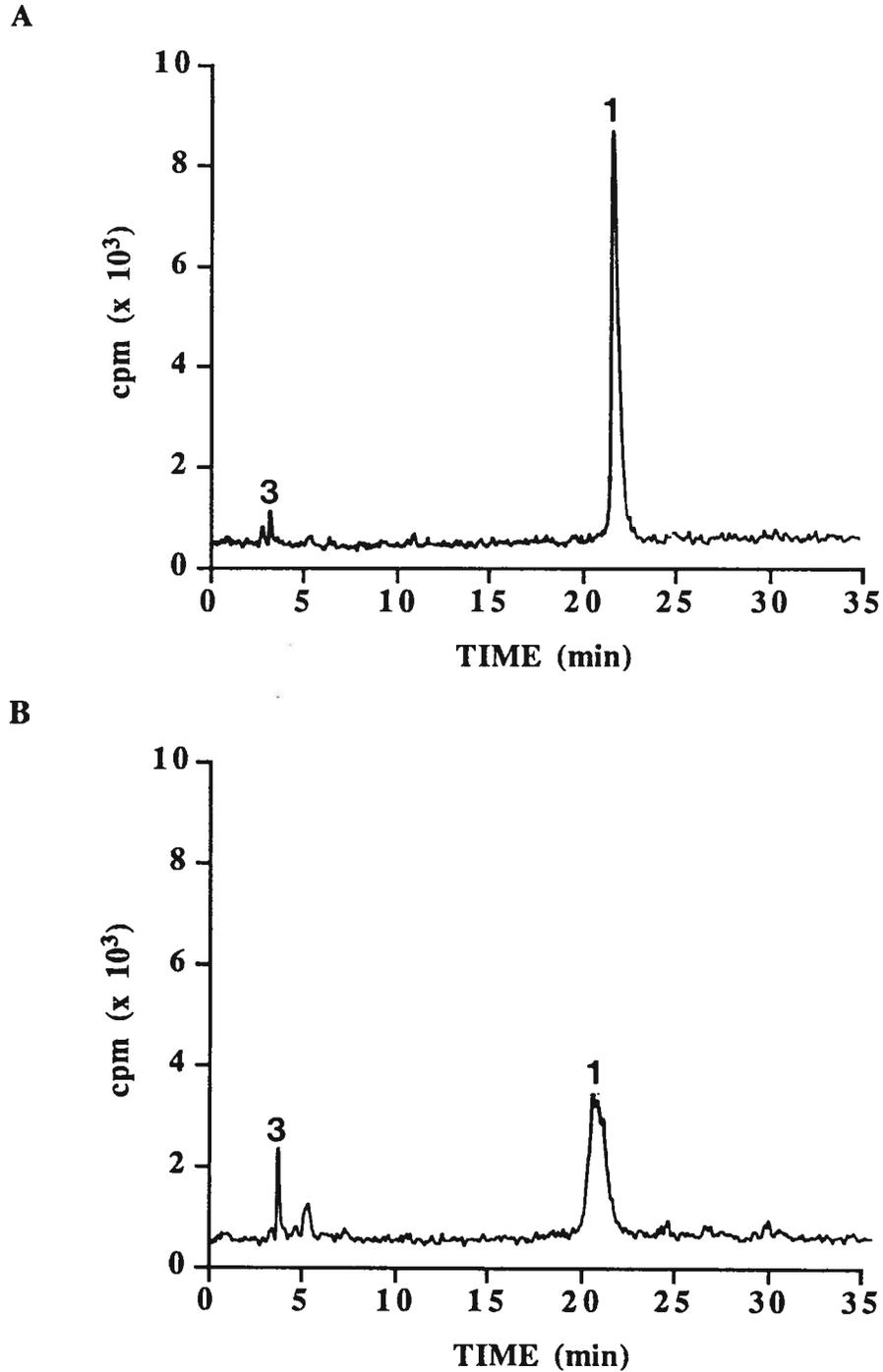
**FIGURE 34:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) and aprotinin (2%) at  $37^\circ\text{C}$ . A) duration = 11 h. B) duration = 25 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42, and peak 3 and others consist of unidentified  $^{125}\text{I}$ -GIP fragments.



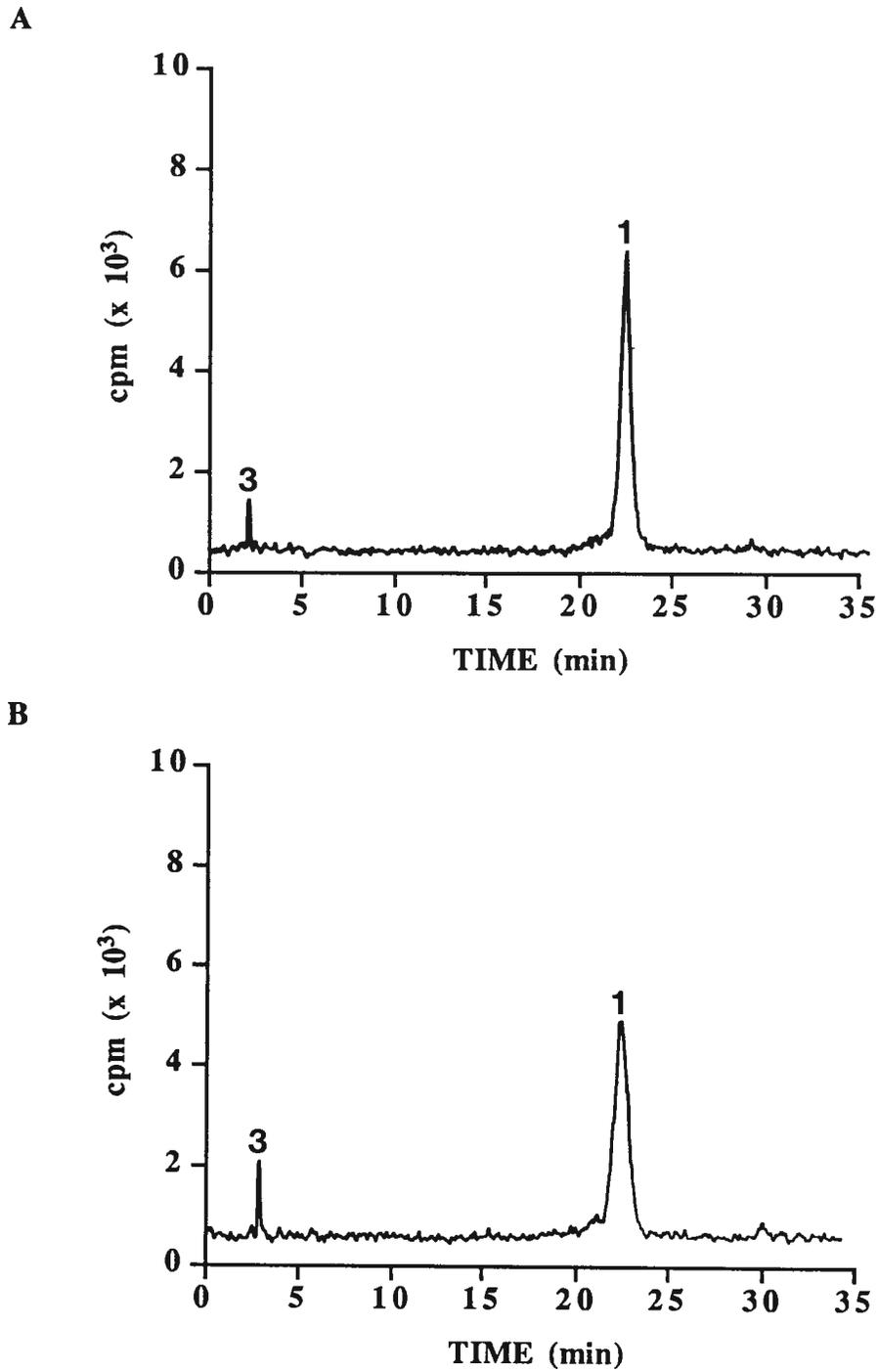
**FIGURE 35:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) and bacitracin (50 U/ml) at  $37^\circ\text{C}$ . **A)** duration = 2 h. **B)** duration = 19.5 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42, and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



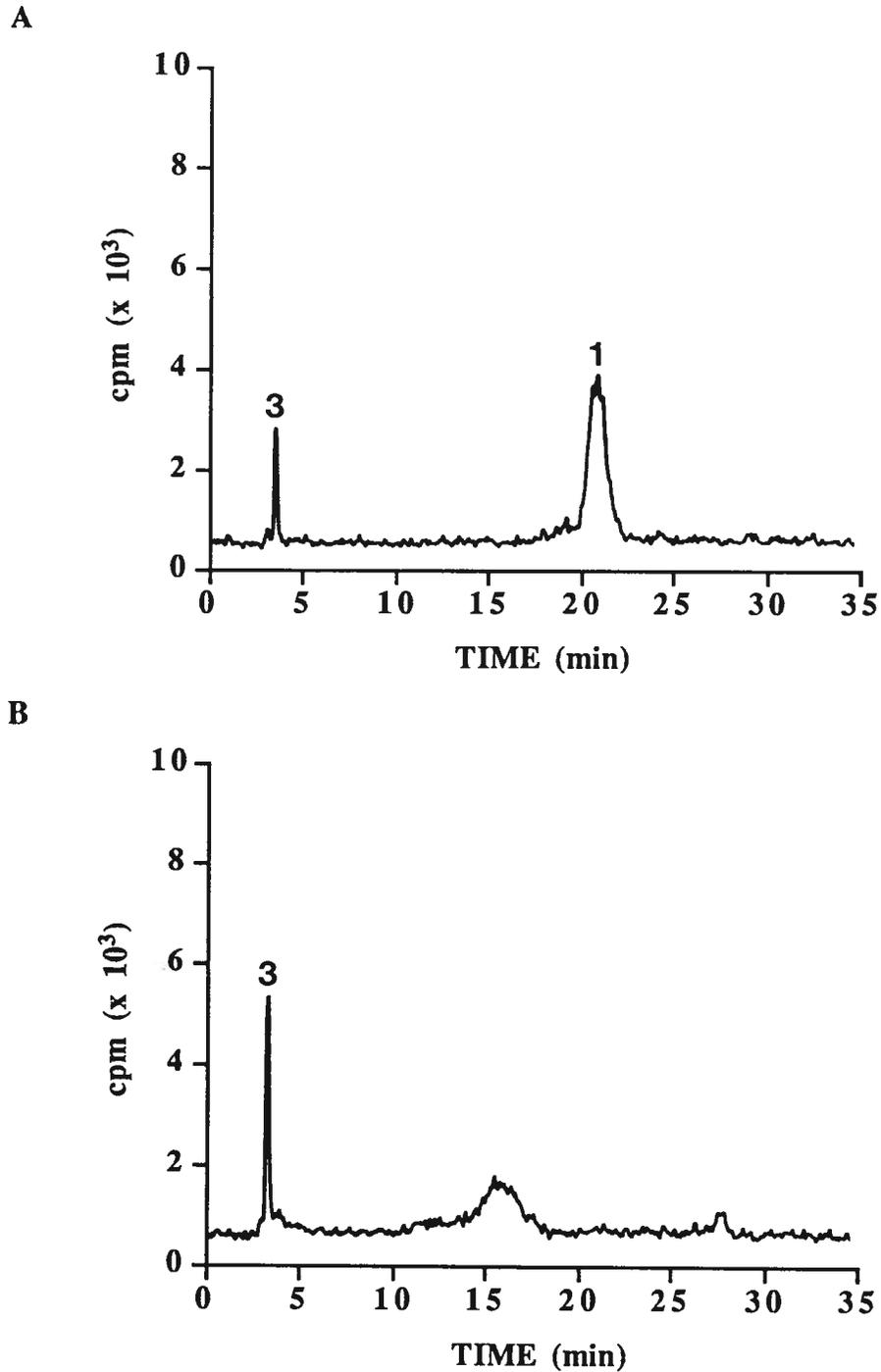
**FIGURE 36:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with Wistar rat serum (10%) containing diprotin A (0.1 mM), aprotinin (2%) and bacitracin (50 U/ml), at  $37^\circ\text{C}$ . **A)** duration = 3.25 h. **B)** duration = 20 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, and peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42.



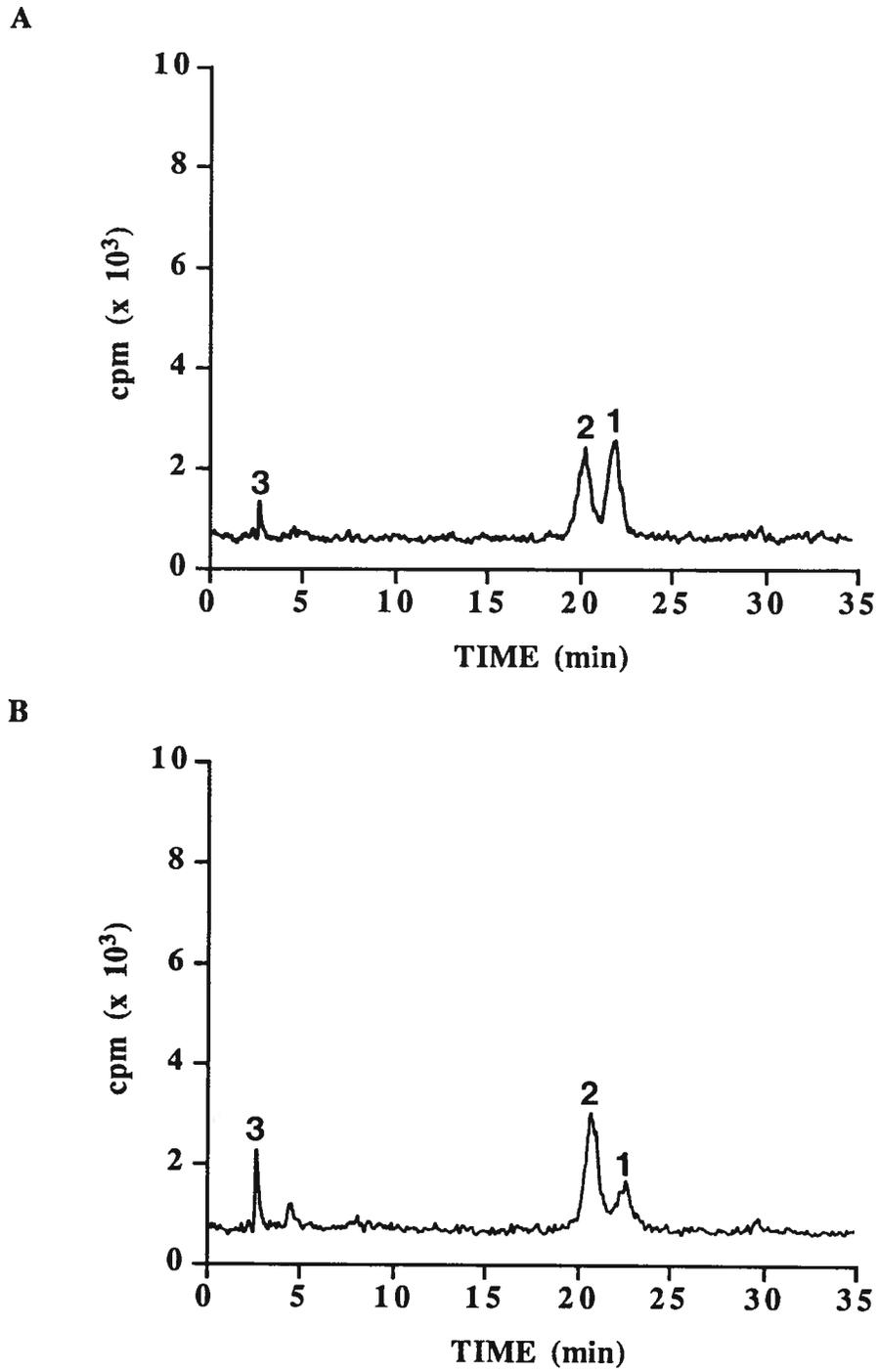
**FIGURE 37:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with DPP IV-negative rat serum (10%) at  $37^\circ\text{C}$ . **A)** duration = 2 h. **B)** duration = 5 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



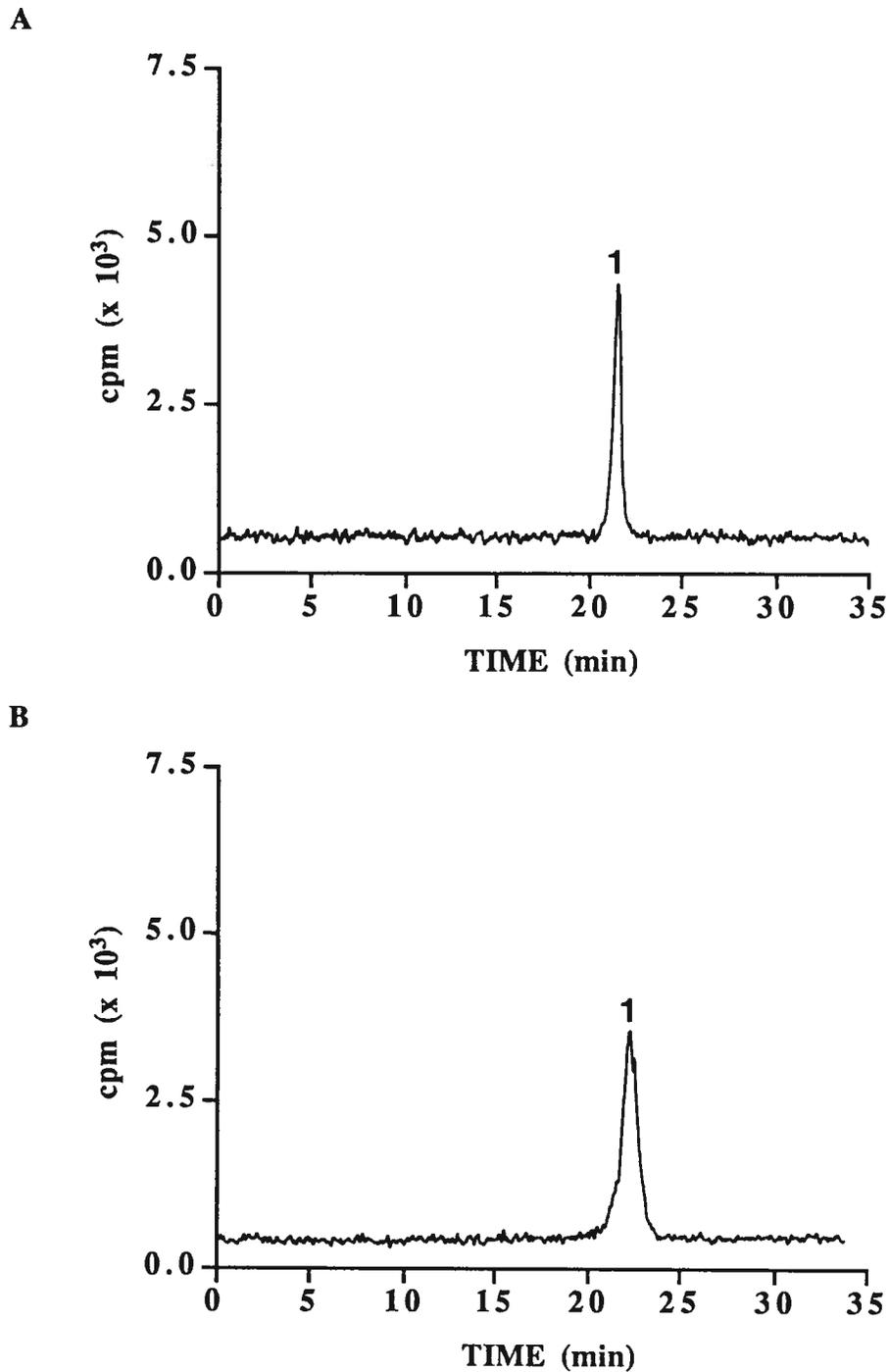
**FIGURE 38:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with DPP IV-negative rat serum (10%) and aprotinin (2%) at  $37^\circ\text{C}$ . **A)** duration = 4 h. **B)** duration = 8 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



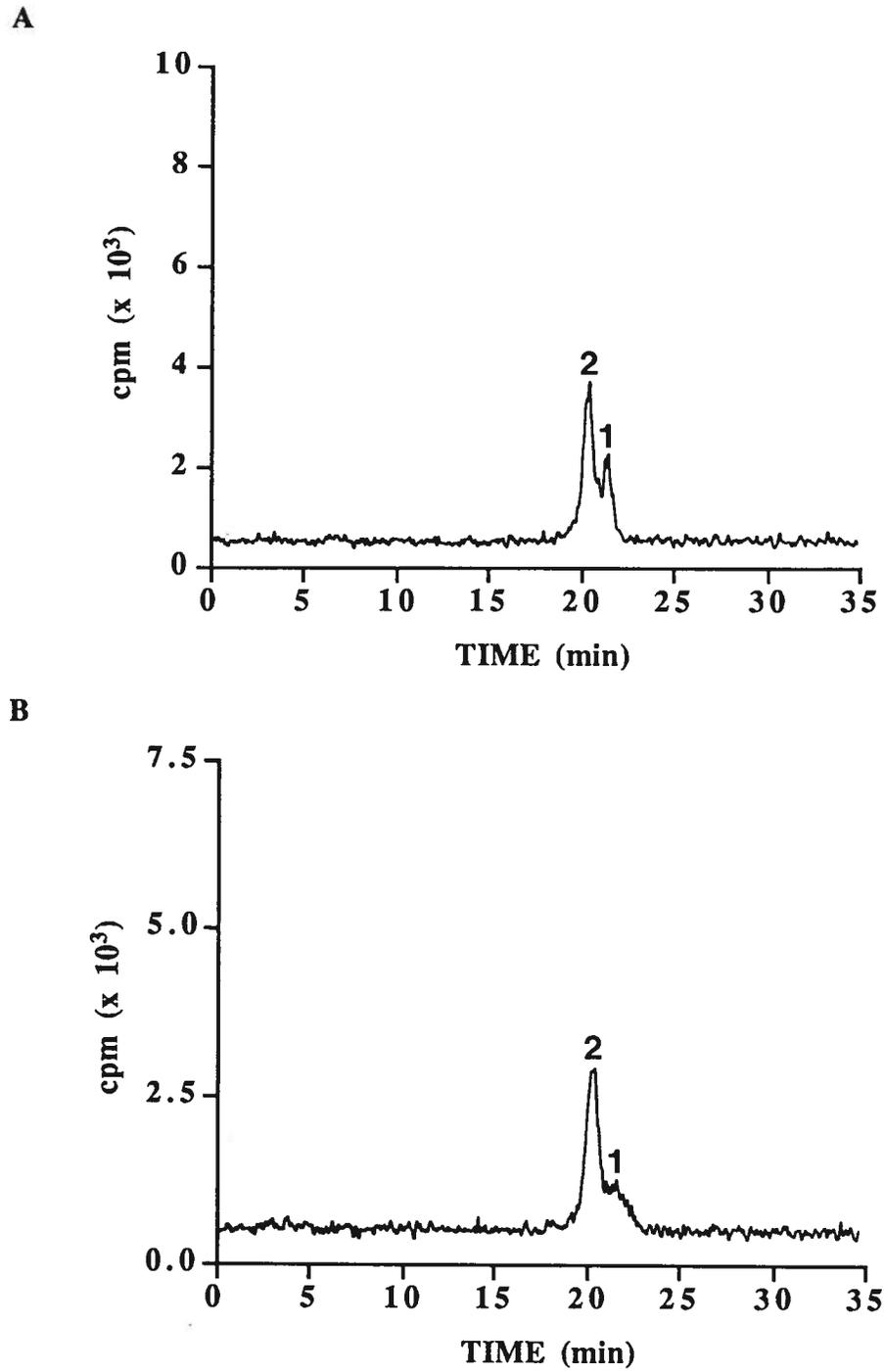
**FIGURE 39:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with DPP IV-negative rat serum (10%) and aprotinin (2%) at  $37^\circ\text{C}$ . A) duration = 13 h. B) duration = 27 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, and peak 3 and others consist of unidentified  $^{125}\text{I}$ -GIP fragments.



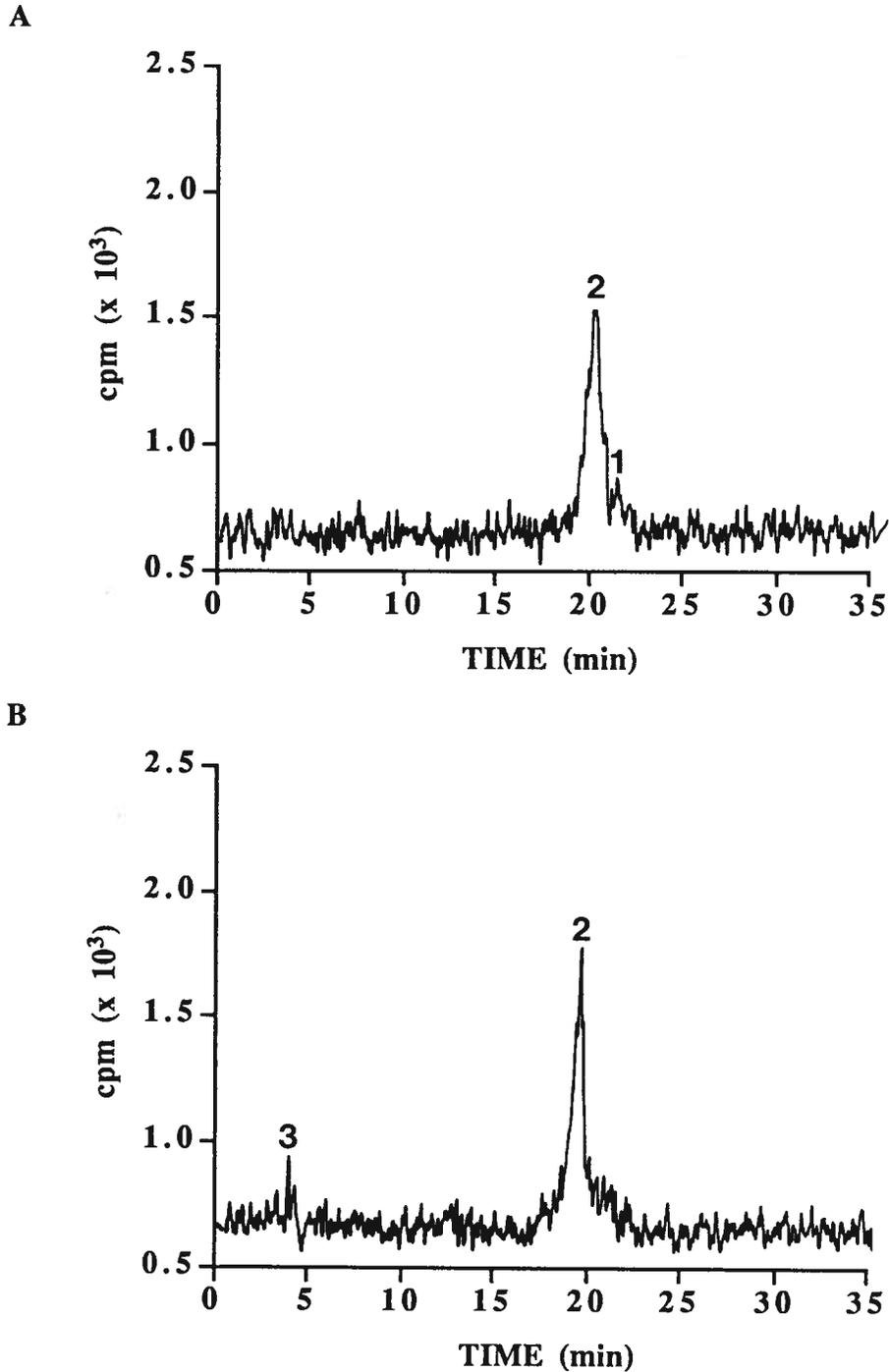
**FIGURE 40:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated with charcoal extracted human plasma (10%) used in the GIP RIA, at  $37^\circ\text{C}$ . **A)** duration = 3 h. **B)** duration = 7 h. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42, and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



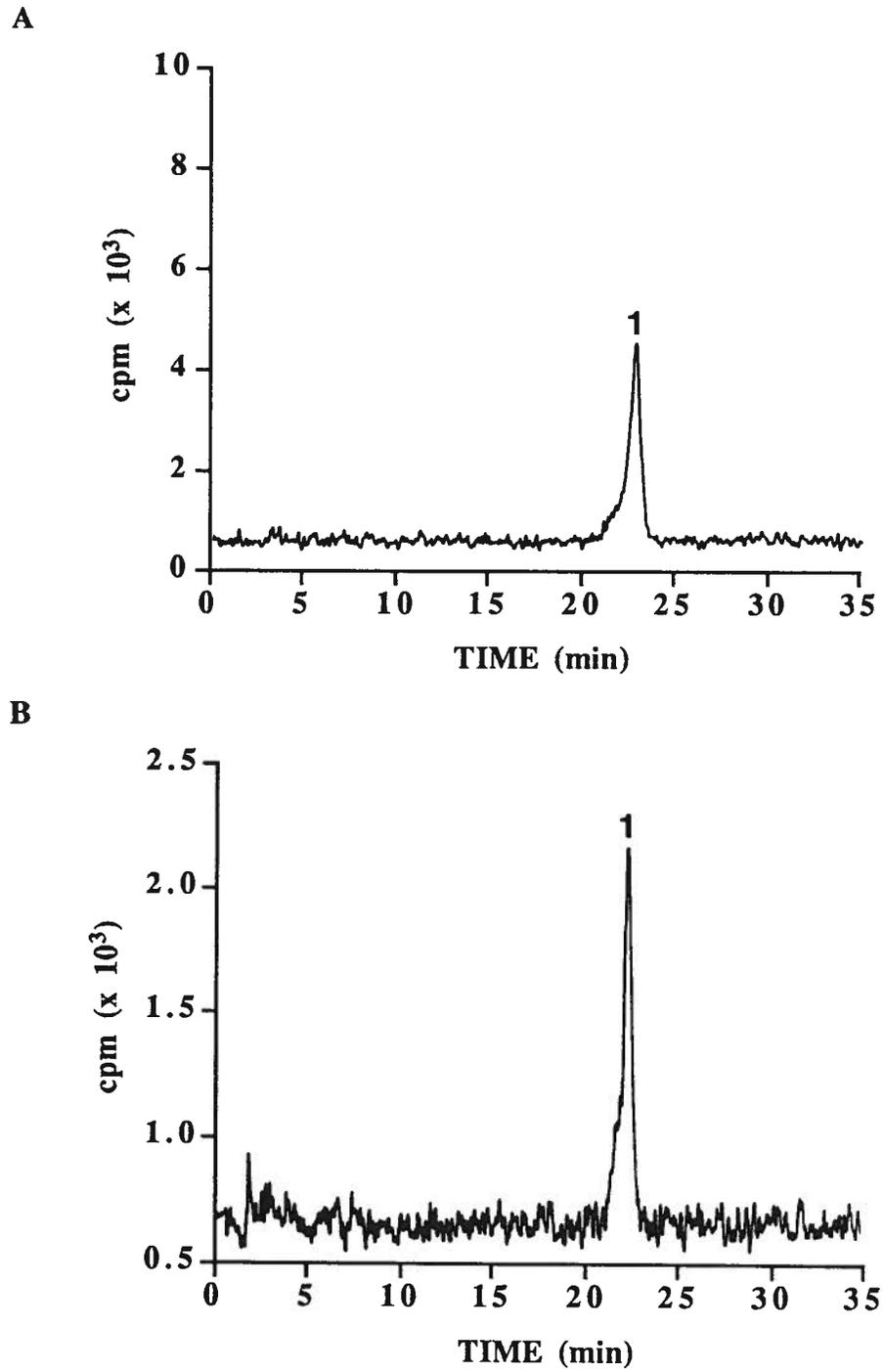
**FIGURE 41:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP ( $\sim 10^5$  cpm) incubated for 1 h with Wistar rat serum (neat) containing diprotin A (0.1 mM), aprotinin (2%), purified by SepPak, lyophilized, and reconstituted as described in section 4.2.6. A) incubation on ice. B) incubation at 37°C. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42.



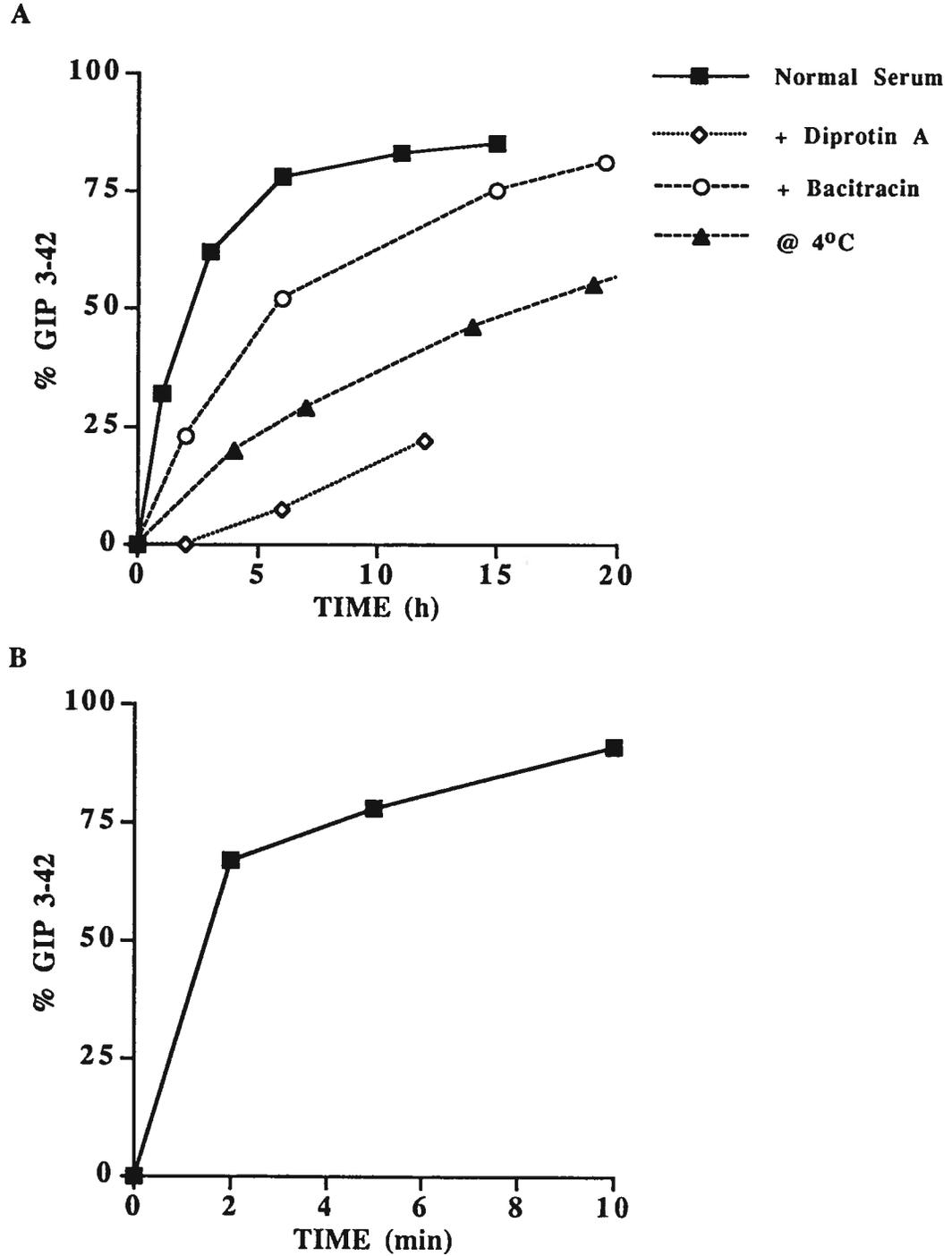
**FIGURE 42:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP infused into a Wistar rat, collected in 2 ml serum with diprotin A (0.1 mM) and aprotinin (2%), purified by SepPak, lyophilized and reconstituted, as described in section 4.2.6. A) duration = 2 min. B) duration = 5 min. In both figures,  $\sim 8 \times 10^5$  cpm was loaded, peak 1 is  $^{125}\text{I}$ -GIP 1-42 and peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42.



**FIGURE 43:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP infused into a Wistar rat, collected in 2 ml serum with diprotin A (0.1 mM) and aprotinin (2%), purified by SepPak, lyophilized and reconstituted, as described in section 4.2.6. A) duration = 10 min. B) duration = 20 min. In both figures,  $\sim 4 \times 10^4$  cpm was loaded, peak 1 is  $^{125}\text{I}$ -GIP 1-42, peak 2 corresponds to  $^{125}\text{I}$ -GIP 3-42, and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



**FIGURE 44:** Both figures are HPLC elution profiles of  $^{125}\text{I}$ -GIP infused into a DPP IV-negative rat, collected in 2 ml serum with diprotin A (0.1 mM) and aprotinin (2%), purified by SepPak, lyophilized and reconstituted, as described in section 4.2.6. **A)** duration = 2 min,  $\sim 10^5$  cpm loaded. **B)** duration = 10 min,  $\sim 5 \times 10^4$  cpm loaded. In both figures, peak 1 is  $^{125}\text{I}$ -GIP 1-42 and peak 3 consists of unidentified  $^{125}\text{I}$ -GIP fragments.



**FIGURE 45:** Graphs summarizing the conversion rate of  $^{125}\text{I}$ -GIP 1-42 to  $^{125}\text{I}$ -GIP 3-42 both *in vitro* and *in vivo* calculated from peak areas of Figures 5 to 22, plotted as % GIP 3-42 (% of GIP 1-42 + 3-42) vs. time. **A)** Relative %  $^{125}\text{I}$ -GIP 3-42 production in Wistar rat serum at 37°C (normal serum), + diprotin A (0.1 mM), + bacitracin (50 U/ml), and @ 4°C. **B)** Rate of formation of  $^{125}\text{I}$ -GIP 3-42 from  $^{125}\text{I}$ -GIP 1-42 infused in a Wistar rat.

#### 4.4 DISCUSSION

The enzyme DPP IV preferentially cleaves peptides and proteins having either X-Pro, X-Hyp or X-Ala at their N-termini (Walter *et al.*, 1980), and to a lesser extent X-Ser, X-Thr, and X-Val (Martin *et al.*, 1993). DPP IV was first identified in rat liver (Hopsu-Havu and Glenner, 1966), and has since been found ubiquitously distributed throughout mammalian tissues, with highest activity in the kidney and the intestinal brush-border membrane, as well as in insects, bacteria, and yeast (see Yaron and Naider, 1993 for review). The primary structure of rat liver DPP IV was deduced from its cDNA, with a calculated mass for the 767-residue polypeptide of 88,107 daltons (Ogata *et al.*, 1989). In keeping with the widespread occurrence of DPP IV, evidence suggests that the enzyme is involved in a number of biochemical processes. This list includes; renal transport and intestinal digestion of proline-containing peptides, immunological activation of immunocompetent cells, and fibronectin-mediated adhesion (Yaron and Naider, 1993). DPP IV also appears to have a role in the inactivation of biologically relevant peptides (Ahmad *et al.*, 1992; Frohman *et al.*, 1989; Mentlein *et al.*, 1993), and it is the involvement of DPP IV in the metabolism of GIP that was the focus of research discussed in this chapter.

The observation made in chapter 3 that IRGIP in STC 6-14 culture medium eluted differently than IRGIP extracted from the cells, suggested the possibility of GIP degradation. These observations were reproduced by incubating GIP in serum, yielding a time-dependent breakdown of GIP 1-42. The retention time of the major metabolic product, eluting just prior to GIP 1-42 standards, suggested it was GIP 3-42. GIP 3-42 has been sequenced, and shown to be a significant component of natural GIP preparations, eluting just prior to GIP 1-42 on reverse-phase HPLC (Jörnval *et al.*, 1981, Schmidt *et al.*, 1987). Further evidence that this component of GIP degradation by serum was GIP 3-42 was supplied by the observation that the inclusion of diprotin A, a competitive inhibitor of DPP IV, significantly reduced the production of the peak

believed to be GIP 3-42. The enzyme DPP IV, had previously been shown to be responsible for removal of the same two N-terminal residues from growth hormone releasing factor (GRF) in human serum (Frohman *et al.*, 1986; 1989).

Subsequent to these studies, a report confirming the degradation of GIP by DPP IV in serum was published (Mentlein *et al.*, 1993). These authors found that DPP IV purified from human placenta hydrolyzed the N-terminal dipeptide from GIP, GRF, tGLP-I, and PHM.  $K_m$  values of 4 - 34  $\mu\text{M}$  and  $V_{\text{max}}$  values of 0.6 - 3.8  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  protein were determined for the enzymatic degradation of the 3 peptides by the purified peptidase. The same fragments were identified when GIP or tGLP-I were incubated with human serum, and analyzed by HPLC. Furthermore, as was observed in the present study, this reaction could be inhibited by 0.1 mM diprotin A. Mentlein *et al.* (1993) concluded that DPP IV initiates the metabolism of GIP in serum.

Confirmation of a role for DPP IV in the metabolism of GIP required *in vivo* evidence. Frohman *et al.* (1986) proposed a role for DPP IV in GRF metabolism, by demonstrating degradation of GRF 1-44 to GRF 3-44 *in vivo*. These observations were accomplished by injecting subjects with GRF and analyzing serum by HPLC. There were, however, several disadvantages to this system, one being the requirement of infusing supraphysiological doses of hormone. Subjects were injected with GRF at 1  $\mu\text{g}/\text{kg}$ , and even at this dose, plasma concentrations were well beneath the concentrations required for UV detection after HPLC, thus necessitating the collection of eluted fractions and analysis by radioimmunoassay. Furthermore, the interpretation of these experiments were compounded by the presence of endogenous hormone. A primary objective of the present investigations was therefore to develop a highly sensitive assay to detect the conversion of GIP 1-42 to GIP 3-42 *in vivo*, to support the *in vitro* results.

The HPLC profile obtained with the  $^{125}\text{I}$ -GIP in the present study, is very similar to that previously reported by others (Maletti *et al.*, 1986; Verchere, 1991). Maletti *et al.* (1986) found that tracer from the two largest peaks was able to stimulate insulin release

from the isolated perfused rat pancreas similarly to natural GIP. These authors also had preliminary evidence from enzyme cleavage that both of these iodinated fractions were iodinated only at the tyrosine in position 10, and not at the tyrosine in position 1. Verchere (1991) investigated the iodination state of the four major peaks of  $^{125}\text{I}$ -GIP obtained after HPLC. Peaks 1-4 contained predominantly monoiodinated tyrosines (MIT), with peak 2 being the most pure, consisting of almost 100% MIT. Furthermore, in a binding assay using tumor derived  $\beta$ -cells ( $\beta\text{TC3}$ ), peak 2 had the greatest specific binding (Verchere, 1991). In combination, these investigations suggested that peak 2 consisted of GIP exclusively monoiodinated at the tyrosine in position 10. This peak was therefore deemed ideal for the present studies, as any iodination at tyrosine in position 1 could potentially alter the ability of DPP IV to cleave GIP between residues 2 and 3. Furthermore, this peak appeared to retain biological activity (Maletti *et al.*, 1986; Verchere, 1991), and made up the greatest proportion of the iodinated product, allowing for maximum yields. All subsequent experiments were therefore performed with peak 2 of the HPLC separated  $^{125}\text{I}$ -GIP.

In order to confirm that the purified  $^{125}\text{I}$ -GIP would still serve as a substrate for DPP IV, and that the products of enzyme degradation could be readily identified and quantified, experiments involving the incubation of GIP with serum followed by HPLC were repeated with the tracer. These *in vitro* experiments clearly demonstrated the time dependent metabolism of GIP into products that could be resolved. As had been observed with the metabolism of natural GIP in the present experiment and by Mentlein *et al.* (1993), there appeared to be one primary product which eluted just prior to  $^{125}\text{I}$ -GIP 1-42, that was considered  $^{125}\text{I}$ -GIP 3-42. The time required for conversion of 50% of the  $^{125}\text{I}$ -GIP to  $^{125}\text{I}$ -GIP 3-42 under the assay conditions was estimated at 2.2 h. Other peaks appeared indicating further degradation of the tracer, but these reactions occurred at a much slower rate. Further evidence that the primary product was  $^{125}\text{I}$ -GIP 3-42 was provided by the observation that the inclusion of diprotin A in the reaction significantly

reduced the rate of production of this peak (50%  $^{125}\text{I}$ -GIP 3-42 @ ~29 h). Furthermore, rats specifically lacking DPP IV, served as suitable controls, and serum from these animals was unable to metabolize  $^{125}\text{I}$ -GIP 1-42 to  $^{125}\text{I}$ -GIP 3-42.

This highly sensitive model also allowed for further characterization of the metabolism of  $^{125}\text{I}$ -GIP 1-42. As expected, the inhibitor aprotinin had no effect on the production of  $^{125}\text{I}$ -GIP 3-42. Aprotinin did however significantly reduce subsequent metabolism to other unidentified products, likely by inhibiting the action of other enzymes such as trypsin. Buckley and Lundquist (1992) reported that bacitracin was an effective inhibitor of enzymatic cleavage of tGLP-I, and therefore this inhibitor was also investigated. Bacitracin significantly reduced the degradation of  $^{125}\text{I}$ -GIP 1-42 to  $^{125}\text{I}$ -GIP 3-42, but not to the degree that was observed with diprotin A (50%  $^{125}\text{I}$ -GIP 3-42 @ ~5.6 h). However, while diprotin A did not appear to significantly affect subsequent label degradation, bacitracin was highly effective. Even after 19.5 h incubation at 37°C, very small amounts of other products were identifiable (other than  $^{125}\text{I}$ -GIP 3-42). In combination, diprotin A, aprotinin, and bacitracin provide highly effective protection against  $^{125}\text{I}$ -GIP degradation.

Interestingly, incubation with serum at 4°C resulted in significant degradation of  $^{125}\text{I}$ -GIP to  $^{125}\text{I}$ -GIP 3-42, although the rate was much slower than the same reaction at 37°C (50%  $^{125}\text{I}$ -GIP 3-42 @ ~16.1 h). Other enzymes capable of metabolizing GIP appeared to be completely ineffective at this temperature, however, as indicated by the lack of other significant peaks (products) even after 24 h incubation. Thus in addition to including the enzyme inhibitor 'cocktail', these results would suggest that storage at 4°C is an effective method to prevent degradation of GIP. While this study clearly supports the current practice of performing GIP assays at 4°C, the use of charcoal extracted plasma (CEP), a major assay constituent, requires further consideration. CEP was clearly able to degrade the tracer into multiple products, as indicated by the multiple peaks obtained by HPLC. This degradation of  $^{125}\text{I}$ -GIP, could very likely also extend to sample

degradation, which could affect the binding of the products by the antiserum. These studies, therefore, clearly do not support the use of plasma in the GIP radioimmunoassay.

This model thus appeared to fulfill the requirements of a suitable assay for the metabolism of GIP, and therefore *in vivo* experiments were performed. Approximately  $5 \times 10^6$  cpm  $^{125}\text{I}$ -GIP with a specific activity of  $\sim 100 \mu\text{Ci}/\mu\text{g}$  (equivalent to 22.5 ng/GIP), was injected in each rat. Thus for a plasma volume of  $\sim 10$  ml, the GIP concentration would be  $\sim 2$  ng/ml, a value within the physiological range. This method thus allows the use of concentrations at least 10-fold lower than those used by Frohman *et al.* (1986) in the investigation of *in vivo* metabolism of GRF. As expected, the rate for  $^{125}\text{I}$ -GIP degradation was much more rapid *in vivo* than was observed *in vitro* with diluted serum. Only  $\sim 1.5$  min was required for half of the  $^{125}\text{I}$ -GIP 1-42 to be converted to  $^{125}\text{I}$ -GIP 3-42. This represents an 88-fold reduction compared to the time observed *in vitro* for equivalent degradation. This result is comparable to that obtained by Frohman *et al.* (1986) who found that 1 min after injection of  $1 \mu\text{g}/\text{kg}$  GRF 1-44,  $43 \pm 7 \%$  of the total immunoreactive GRH was GRH 3-44. This short half-life of DPP IV substrates is likely a reflection of the fact that DPP IV is found in high concentrations as an ectoenzyme of the plasma membrane of numerous cell types, in addition to plasma (Yaron and Naider, 1993).

There are many implications of these results that should be considered. The data presented suggest that DPP IV is a primary enzyme involved in the degradation of GIP *in vivo*. The product of this reaction is GIP 3-42, a polypeptide that has been previously demonstrated to lack somatostatinotropic and insulinotropic activity (Brown *et al.*, 1981; Moody *et al.*, 1981; Schmidt *et al.*, 1986; 1987). There have been no assays reported to date using antisera that can distinguish between GIP 1-42 and GIP 3-42. Given this information, the significance of sustained elevation of circulating GIP levels must be re-evaluated. For example, reports of GIP half-lives of  $\sim 20$  min (see section 1.12) and circulating levels elevated for hours (see section 1.10.1) must be considered in light of the

time course of conversion of GIP 1-42 to GIP 3-42. Thus the insulinotropic activity appears regulated in such a fashion that secreted GIP only acts on the pancreas for one circulation and is then inactivated to prevent overproduction of insulin and hypoglycemia.

While GIP 3-42 is not an antagonist of the insulinotropic activity of GIP 1-42, other smaller fragments such as GIP 4-42 could act as antagonists, or may even be insulinotropic (see section 1.11.2). It remains possible, therefore, that the further enzymatic degradation of GIP that was observed to occur, albeit at a comparatively slow rate, might yield GIP fragments smaller than 3-42 with biological activity, or the ability to act as antagonists. It is also possible that the addition of  $^{125}\text{I}$  to the tyrosine at position 10 of the GIP polypeptide to produce the tracer used for metabolism studies hindered enzymatic cleavage near this residue. These queries can be addressed by the separation, identification, and determination of biological activities of further serum degradation products of GIP.

Over activity of DPP IV could result in increased metabolism of the insulinotropic hormones GIP and tGLP-I, and thus a reduced insulin response to the secretion of these hormones. This might then result in insufficient insulin secretion in response to a glucose load, yielding diabetic-type symptoms. Interestingly, urinary concentrations of DPP IV have been shown to be elevated in NIDDM patients (Nagata *et al.*, 1988; Takasawa *et al.*, 1990; Nukada *et al.*, 1992). As this elevation preceded the onset of microalbuminuria that is associated with nephropathy in diabetics, it was suggested that the alteration of the proximal tubules occurs prior to pathological changes of the glomeruli and brings about enzyme leakage from the brush border. If this enzyme dissociation is a response to the elevated glucose levels, then it is possible that maintained high glucose could also result in DPP IV dissociation from other tissues, such as endothelium, and lead to elevated levels in the plasma. However, neither in the obese Zucker rat, nor in the 12 NIDDM subjects tested, were DPP IV activity rates significantly different from controls. The

possibility of altered DPP IV activity in subjects with diabetes requires further investigation.

The mechanisms that regulate serum levels of DPP IV are not currently understood. In light of the metabolic role of this enzyme for degradation of incretins, it is possible that DPP IV, like incretins, is influenced by nutrient absorption. Suzuki *et al.* (1993) recently reported that rat intestinal DPP IV levels could be modified by diet. After 7 days on a high proline (gelatin) diet, DPP IV activity and mRNA levels in brush-border membranes were three- to six-fold greater. No analysis of serum concentrations were made in this study. Mentlein *et al.* (1993) measured serum DPP IV levels in subjects pre- and postprandial and found no significant difference with an  $n = 3$ . Clearly, regulation of serum DPP IV levels requires further investigation.

In conclusion, separation of the cleavage products of GIP *in vitro* and *in vivo*, combined with the influence of enzyme inhibitors and serum from DPP IV-negative rats indicates that dipeptidyl peptidase IV is a principal enzyme resulting in the degradation and biological inactivation of GIP. Preliminary data indicate no significant difference in DPP IV activity for either obese Zucker rats or NIDDM subjects when compared to controls. The ubiquitous nature of this enzyme, including epithelial cells of the intestine, might explain the isolation of considerable quantities of GIP 3-42 from natural GIP preparations. This and smaller enzyme fragments of GIP undoubtedly contribute to the overall immunoreactivity measured by GIP radioimmunoassays currently in use. Furthermore, the inclusion of plasma in assay buffer, results in significant degradation of  $^{125}\text{I}$ -GIP as well as perhaps GIP in standards and unknowns. This can be prevented by including diprotin A, aprotinin, and bacitracin in the assay buffer, in addition to maintaining all samples at 4°C.

## CHAPTER 5

### SUMMARY AND FUTURE DIRECTIONS

The role of gastric inhibitory polypeptide as a hormone has been well established. The polypeptide has been isolated, purified, and chemically identified, and its cells of origin located. Secretagogues for GIP release *in vivo* have been identified. The gene coding GIP has been isolated, sequenced, and the regulation of its transcription investigated. Induction of endogenous hormone release is accompanied by insulin release, and GIP injection in amounts that result in physiologically attainable levels mimic the biological actions. A GIP receptor has been cloned, sequenced, and located on target tissues, and the signal transduction system has been studied. Finally, putative roles for GIP in pathophysiological conditions, such as diabetes mellitus and obesity have been suggested, although data are equivocal. What is least well understood about GIP, are the mechanisms controlling its release, and its duration of biological activity i.e. metabolism. The objectives of investigations reported in this thesis were, therefore, firstly to develop methods to investigate GIP release at the cellular level, and secondly, to develop a sensitive assay to monitor the degradation of GIP in plasma.

A major difficulty in studying the release of GIP at the cellular level is the diffuse distribution of the endocrine cells of origin in the upper small intestine. Methodology was therefore developed involving preferential enrichment of intestinal endocrine cell preparations by centrifugal elutriation and short-term tissue culture in order to enable the study of the local regulation of IRGIP secretion from isolated endocrine cells. Canine intestinal duodenal and jejunal epithelial cell preparations enriched for endocrine cells released IRGIP in response to depolarization, as well as the principal GIP secretagogue identified in studies *in vivo*, glucose. It was demonstrated indirectly that glucose-stimulated IRGIP release was inhibited by somatostatin, which is also likely a mediator of GIP release *in vivo*. Increasing intracellular levels of cAMP or Ca<sup>2+</sup> by

pharmacological activators resulted in significant secretion of IRGIP, indicating that these intracellular compounds are likely involved as messengers in the signal transduction system regulating GIP release. Finally, receptor-dependent activation of intracellular  $\text{Ca}^{2+}$  messenger systems by the neuropeptide GRP yielded concentration-dependent increases in IRGIP output. This provides one of possibly many examples of neuropeptide control mechanisms for GIP release. While most of these observations were made with isolated canine epithelial cells, preliminary data suggests that porcine tissue is suitable for the continuation of these studies.

These studies represent the first data on GIP release at the cellular level. Other *in vitro* models, such as mucosal sections or isolated bowel preparations, have for the most part proven unsuccessful for studying GIP release, due to the problems associated with ischemia in intestinal tissues, and the diffuse distribution of the GIP cells. With the ability to culture the isolated cells, comes the ability to readily control their environment - a key factor in the investigation of stimulus-secretion coupling. Understanding the mechanism by which a compound acts on the GIP cell, whether it be luminal, endocrine, paracrine, or neuronal, is very difficult to establish from *in vivo* studies. The methods used in these investigations resulting in cultured GIP cells, allowed for observations of the effects of luminal (glucose), endocrine/paracrine (somatostatin) and neuronal (GRP) stimuli on GIP release.

There are many unanswered questions which remain concerning GIP release, which may be addressed using this model. For example, it might now be possible to investigate the influence of insulin on GIP release to resolve the potential role of insulin as a feedback-inhibitor of GIP secretion. The mechanisms by which nutrients, such as glucose and fat, act to cause GIP release may also be investigated. It is not known what intracellular processes in the metabolism of these nutrients result in GIP expression. Techniques are available in this laboratory to directly examine  $\text{Ca}^{2+}$  flux in individual cells in response to secretagogues. This could facilitate answering these remaining

questions, and corroborate data presented in this thesis suggesting that changes in intracellular  $\text{Ca}^{2+}$  are important in the regulation of GIP release.

It also remains to be determined whether nutrients or their digestion products act directly on the GIP cell, or indirectly on neighbouring cells to stimulate GIP release. If the spatial organization of cells is important in allowing signal transduction from nutrient sensitive cells to GIP cells, then it can be speculated that the reaggregation of dispersed cells during the culture process might influence the GIP response to certain stimuli. With the identification of the glucose transporter responsible for the activation of GIP release, and its localization on specific cell types, it will also be important to examine its distribution in the cultured cells. Specifically, it is of interest whether the cells attach and aggregate on the collagen coated wells in similar patterns that occur *in vivo*. To this end, a small chamber was constructed to enable culture of isolated cells on a microscope stage from which time-lapse photographs may be taken to observe the process of cell aggregation, growth of cellular projections, formation of cell to cell connections etc. which might facilitate these studies.

One negative aspect of the static release studies described in this thesis is that metabolites, and other secretory products of cells in the cultures may influence the release of GIP. Evidence for this was supplied by the ability of somatostatin immunoneutralizing antibody to increase IRGIP release. It would therefore be desirable to examine the release of GIP from cells in a perfusion apparatus, where fresh medium is continually flowing over the cells and thus removing any secreted compounds. Such a system would also allow investigation of the dynamics of the GIP response to various secretagogues. For example, the IRGIP response may be biphasic, or exhibit desensitization to prolonged stimuli. Finally, a molecular approach would also be beneficial, allowing determination of the action of secretagogues on GIP mRNA levels. It would then be possible to address how much of the observed release of GIP is from GIP stores, and how much is from newly synthesized hormone.

Tumor cell lines were also investigated as an alternate source of GIP cells for study. A cell line derived from intestinal tumors of transgenic mice (STC-1) was subcloned to produce a stable cell line with approximately 30% IRGIP and 30% IRSS cells. This new clone (STC 6-14) was used to study the release of IRGIP in response to glucose and interactions with somatostatin. HPLC of extracts of STC 6-14 cells indicated that the tumor cell derived IRGIP had the same retention time as natural porcine GIP 1-42. Release of IRGIP from STC 6-14 cells; increased in a concentration dependent fashion by glucose, was attenuated by the addition of somatostatin, and was augmented by addition of a somatostatin neutralizing antibody, presumably by immunoneutralizing endogenously released somatostatin. The addition of exogenous porcine GIP in the presence of 5 mM glucose produced a concentration dependent increase in IRSS release. These represent the first reported data on GIP release from a tumor cell line. The STC 6-14 cells may be useful to further investigate the cellular mechanisms controlling the release of GIP and somatostatin, in addition to their interactions.

This cell line provides a readily available, inexpensive supply of GIP cells. Studies on the cellular regulation of GIP secretion should be done concomitantly with studies on primary cells. Caution must be used when interpreting data from transformed cells, which might be regulated in a significantly different fashion than their natural counterpart. For example, in most malignant epithelial cells, there is an impairment of the metabolism of glucose with high rates of glucose consumption, aerobic glycolysis and an unusual accumulation of glycogen as compared with the normal tissue (see section 3.4 and Zweibaum *et al.*, 1991 for review). Such cellular alterations might therefore result in aberrant hormone regulation and release mechanisms. A multidisciplinary approach including parallel studies with primary cells is therefore desirable.

Most intestinal receptors were initially identified in isolated intestinal epithelial cell preparations from various species (Laburthe and Amiranoff, 1989). Cell lines offer several advantages over other tissue preparations for studying neurohormonal receptors

(Zweibaum *et al.*, 1991). Studies of the GIP receptor on pancreatic tumor derived  $\beta$ -cells are reviewed in section 1.11.2. In light of the fact that the GIP receptor on somatostatin cells may be different than that on  $\beta$ -cells (see section 1.11.1), the somatostatin secreting STC 6-14 cell line may be a useful model to further investigate this hypothesis, particularly in view of the recent cloning of the GIP receptor (Usdin *et al.*, 1993). Previous studies have used somatostatin-secreting tumor cell lines to study the binding characteristics of tGLP-I (Fehmann and Habener, 1991b; Gros *et al.*, 1993), but no such reports on GIP binding have been forthcoming to date.

All of the studies suggested for the primary GIP cells should also be performed on the STC 6-14 cells. However, more importantly, further experiments should first be aimed at investigating characteristics of the tumor cells. In particular, it is important to determine if the intracellular organization of these cells, as studied by electron microscopy, is characteristic of normal endocrine cells, e.g. secretory granule size and arrangement. Specifically, as this population of cells secretes a multitude of peptides (see section 3.3.1), it is of interest to determine if there are cells expressing more than one hormone. These studies are currently in progress at the Department of Physiology, UBC. In addition, the STC 6-14 cell line provides a source of murine GIP for sequencing and assessment of the biological activity.

Studies described in chapter 4 implicate DPP IV as the main degradation and inactivating enzyme for GIP in the circulation. This hypothesis is based on the observations of the degradation of GIP 1-42 to putative GIP 3-42. Evidence that the DPP IV product is GIP 3-42 is supplied by its elution position just prior to GIP 1-42, and the finding that this inactive peptide was absent or reduced by including diprotin A, a specific inhibitor of DPP IV or by incubation with serum from rats DPP IV deficient. Furthermore, a recent report by Mentlein *et al.* (1993) confirmed that GIP 1-42 is degraded to GIP 3-42 *in vitro* by purified DPP IV or serum, by sequencing the reaction products. In order to confirm that GIP 3-42 is the main product of experiments

performed in this thesis, sequencing analysis should also be performed. In order to avoid sequencing radioactive peptide,  $^{127}\text{I}$ -GIP could be prepared and purified similar to that which was performed for  $^{125}\text{I}$ -GIP. After incubation with serum, the products could be separated using the same HPLC protocols, collected and then sequenced.

Studies performed *in vivo* suggest that 50% of a bolus injection of GIP can be inactivated to GIP 3-42 in as little as 1.5 min. Rapid hormone inactivation in conjunction with the glucose-dependency of GIP, may act to ensure the prevention of hyperinsulinemia and subsequent hypoglycemia. Since GIP 3-42 is biologically inactive, yet still immunoreactive, reports of circulating IRGIP levels in health and disease must be re-considered. Information regarding the circulating levels of biologically inactive GIP fragments can be misleading. Therefore, it is desirable to obtain N-terminal directed antibodies to establish an RIA, that can be used to distinguish between GIP 1-42 and GIP 3-42. Such an assay would provide a clearer picture of circulating levels of biologically active GIP secreted.

As discussed in section 4.4, there is very little currently known about the mechanisms that control the levels of the enzyme DPP IV. If diet can alter intestinal brush border levels of this enzyme, this might lead to modulation of serum concentrations. Alterations in the levels of DPP IV could potentially have profound effects on the biological actions of GIP and its other substrates eg. tGLP-I. Elevated DPP IV levels would be expected to reduce the half-life of biologically active GIP, and thus reduce the incretin effect, potentially leading to hyperglycemia. Initial studies investigating the DPP IV activity in serum from obese Zucker rats and NIDDM subjects revealed no abnormalities. However, it does remain possible that other factors, such as diet or medical treatment, might alter DPP IV activity. Furthermore, serum samples were collected from NIDDM patients in the fasting state, during which GIP is normally neither secreted nor insulinotropic. In order to test the hypothesis that DPP IV serum activity is altered in NIDDM subjects, it is important, therefore, to also examine the enzyme activity

during the post-absorptive state, when GIP is insulinotropic. In addition, due to the ubiquitous distribution of DPP IV, with many potential sites for the metabolism of GIP other than in serum, *in vivo* GIP degradation studies should also be performed with NIDDM subjects. As diabetes is a multifactorial disorder, with many potential contributing abnormalities, further investigation into a potential role for DPP IV is warranted.

The DPP IV-negative rats provide a model for the investigation of the consequences of a deficiency of this enzyme. The half-life for biological activity of GIP in these animals is considerably longer than controls, limited only by degradation by other enzymes and extraction by organs such as the kidney. It might be conceived that the greater duration of insulinotropic activity of GIP (and tGLP-I) could potentially result in over production of insulin and hypoglycemia. However, as glucose is the primary source of cell metabolic energy, it might be expected that these animals adapt physiologically to ensure an appropriate insulin response to ingestion of a meal. Preliminary investigations indicate that these animals do in fact have a normal insulin response to an oral glucose tolerance test, resulting in the appropriate maintenance of blood glucose levels. One protective measure that could explain this observation is the glucose-dependent nature of GIP. As soon as elevated plasma glucose levels (following an oral glucose challenge) return to basal (below threshold for GIP action), any non-metabolized GIP 1-42 will not be insulinotropic, thus preventing hypoglycemia. Alternatively, or additionally, desensitization of  $\beta$ -cells to GIP could result from elevated levels of GIP 1-42. In light of the fact that elevated GIP or tGLP-I levels have been demonstrated to cause  $\beta$ -cell desensitization to these hormones (see section 1.11.2), it is of interest to determine if similar phenomena are also occurring in the DPP IV-negative rats. Preliminary studies suggest that the pancreas from these animals is in fact desensitized to GIP, as evident from a reduced insulin response from the perfused

pancreas to GIP in DPP IV-negative rats when compared to controls (Pederson and Kieffer, unpublished observations).

Desensitization of the DPP IV-negative rat  $\beta$ -cells to the incretins GIP and tGLP-I may occur as a result of down-regulation of the receptors to these incretins. This hypothesis could be tested by examining the binding of these peptides to  $\beta$ -cells from DPP IV-negative and control rats. Methods for rat  $\beta$ -cell isolation and examination of GIP binding have been previously described (Verchere *et al.*, 1991). It would also be interesting to examine the effect of total parenteral nutrition (TPN) on the enteroinsular axis in these rats. In previous studies with normal rats, Pederson *et al.* (1985) showed that while the IRGIP response to oral glucose was normal following TPN, the isolated perfused pancreas showed a 30% increase in the insulin release in response to GIP after TPN. It was hypothesized that the increase in  $\beta$ -cell sensitivity to GIP may be causally connected to the exposure of the pancreas to chronically low levels of GIP during TPN. Perhaps this procedure could reverse the  $\beta$ -cell desensitization to GIP observed in the DPP IV-negative rats.

Evidence provided in this thesis that implicates DPP IV as a principal inactivating enzyme of GIP suggests a strategy for the development of GIP analogs with a longer half-life in the circulation. DPP IV has an absolute requirement for the L configuration of the amino acid residue, both in the penultimate and the N-terminal position (Yaron and Naider, 1993). Thus substitution of D-amino acids for the L-amino acids His-Ala at the N-terminus of tGLP-I has been reported to result in resistance to DPP IV cleavage (Buckley and Lundquist, 1992). It is, therefore, likely that substitution of the GIP N-terminal amino acids Tyr-Ala with corresponding D configurations might make the resulting analogue resistant to degradation by DPP IV, with an attendant increase in insulinotropic potency. Such a compound could potentially have therapeutic actions in subjects with NIDDM who have insufficient insulin release to regulate glucose metabolism. This concept is supported by recent observations that tGLP-I (which may be

more insulinotropic than native GIP; see section 1.5.4) might have antidiabetogenic effects in subjects with NIDDM (Gutniak *et al.*, 1992; Holz *et al.*, 1993; Nathan *et al.*, 1992; Nauk *et al.*, 1993).

In conclusion, GIP is a hormone with important anabolic functions, of which the most extensively studied is undoubtedly its insulinotropic action. The role of GIP as an incretin in the enteroinsular axis has been well established, and potential links with obesity and NIDDM have been made. The experiments outlined in this thesis have been directed towards gaining a better understanding of the cellular mechanisms controlling GIP release and biological inactivation of this hormone *in vivo*. Data have been presented which implicate both the cAMP and Ca<sup>2+</sup> intracellular messenger systems in the release of GIP, and the enzyme dipeptidyl peptidase IV in the rapid inactivation of GIP *in vivo*. The models developed to examine the release and metabolism of GIP that are presented in this thesis should enable further future investigation of these aspects of GIP physiology in both normal and pathophysiological states.

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