INVESTIGATION OF THE METABOLISM OF GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP) AND GLUCAGON-LIKE PEPTIDE-1 (GLP-1) BY DIPEPTIDYL PEPTIDASE IV (DP IV)

by

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B.Sc., The University of British Columbia, 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Physiology

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1996

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Date Oct. 4, 1996

DE-6 (2/88)

Abstract

The incretins glucose-dependent insulinotropic polypeptide (GIP_{1-42}) and truncated forms of glucagon-like peptide-1 ($GLP\text{-}1_{7-36}$ and $GLP\text{-}1_{7-37}$) are hormones released from the gut in response to ingested nutrients and act on the endocrine pancreas to potentiate glucose-induced insulin secretion. GIP_{1-42} and $GLP\text{-}1_{7-36}$ are known substrates of the circulating exopeptidase dipeptidyl peptidase IV (DP IV, CD26, EC 3.4.14.5) which selectively hydrolyzes peptides after penultimate N-terminal proline or alanine. Hydrolysis of GIP_{1-42} and $GLP\text{-}1_{7-36}$ by DP IV yields the biologically inactive polypeptides GIP_{3-42} and $GLP\text{-}1_{9-36}$, and the dipeptides Tyr-Ala and His-Ala respectively. It has been speculated that DP IV-catalyzed incretin hydrolysis is the primary step in the metabolism of these hormones. This thesis reports the use of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to study incretin hydrolysis *in vitro*, including enzyme kinetics, and the establishment of a protocol for the inhibition of DP IV *in vivo*, allowing study of the influence of endogenous DP IV on the enteroinsular axis. Analysis of MS spectra indicated that human serum-incubated GIP_{1-42} and $GLP\text{-}1_{7-36}$ were cleaved by DP IV with only minor secondary degradation due to other serum protease activity. Kinetic constants of incretin hydrolysis by purified porcine kidney-derived enzyme and by human serum DP IV activity suggest that DP IV-mediated hydrolysis of these peptides is significant at physiological incretin concentrations. Ile-thiazolidide, one of a new class of competitive reversible transition state analogue inhibitors of DP IV was used to block DP IV activity *in vitro* and *in vivo*. Endogenous DP IV inhibition resulted in an earlier rise and peak of plasma insulin and more rapid clearance of blood glucose in

response to an intraduodenal glucose challenge. High performance liquid chromatography (HPLC) analysis revealed that inhibition of DP IV *in vivo* was able to prevent the hydrolysis of radiolabelled GLP-1_{7-36}, indicating that the altered insulin profile is likely an incretin-mediated response. On the basis of the studies described in this thesis it was concluded that DP IV is the principal protease responsible for the degradation of GIP_{1-42} and GLP-1_{7-36} and manipulation of endogenous DP IV activity was able to improve glucose tolerance in the rat.

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Acknowledgments

I was first introduced to Dr. Ray Pederson, my research supervisor, during the Department of Physiology Wine & Cheese event in the autumn of my third year of undergraduate studies. Since then, I have had the good fortune to have completed a B.Sc. graduating essay, and now an M.Sc. thesis, under his supervision. It is easy to see why Ray Pederson is such a popular supervisor by his encouragement and support of his students and all other graduate students as well. I would like to extend my sincerest gratitude to Ray for continually supporting my endeavors and for allowing me the freedom in experimentation in our own laboratory as well as the opportunity to conduct research abroad. All graduate students should be as lucky to have as excellent a supervisor as I had.

I would also like to recognize the constant support and encouragement I received from Dr. Chris McIntosh while this Master's project was being carried out. It is with a great deal of gratitude and respect that I acknowledge the many hours Chris spent answering my questions, providing me with current literature, editing my assignments and papers, as well as guiding me through my PHYL 548 project.

I would like to thank the remaining members of my supervisory committee, Drs. David Mathers and Lawrence McIntosh, for showing interest in my research project and for ensuring that the scope of that project remained realistic. Many thanks to both of them.

A great deal of this research was made possible by the generosity of Dr. Uli Demuth of the Hans-Knöll Institute in Halle, Germany. It was in his laboratory where much of the research presented in this thesis was carried out. His excitement for science is almost infectious, and I am grateful for his kindness and friendship. I would also like to thank the graduate students at HKI-Halle who made my stay there memorable.

I would like to thank John Sanker and Giuseppe Tay (with his vintage wine) for their expert preparation of the many slides, posters, and photo proofs I asked them to make. Their technical assistance was greatly appreciated.

I am also grateful for the assistance of Heather Ann White in carrying out the *in vivo* work described in this thesis. Her sense of humour and knowledge of movie trivia made the days in the lab during the last year of this project seem to go by more quickly. I thank Heather especially for her friendship and good cheer.

I would like to thank my family in Kelowna and Kamloops for their constant encouragement and interest in my education.

Finally, I would like to acknowledge my indebtedness to Andrea Buker for the time and effort she devoted to me. I can't even begin to describe the countless ways she has supported me.

Preface

The study presented in Chapter 1 of this thesis was carried out in the laboratory of Dr. Hans-Ulrich Demuth at the Hans-Knöll Institute of Natural Product Research Jena, in Halle, Germany between July and October, 1995. This work is currently in press as:

Pauly, R.P., Rosche, F., Wermann, M., McIntosh, C.H.S., Pederson, R.A. & Demuth, H.-U. (1996) Investigation of GIP_{1-42} and $GLP\text{-}1_{7-36}$ degradation *in vitro* by dipeptidyl peptidase IV (DP IV) using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS): a novel kinetic approach, *J. Biol. Chem.* **271**, 23222-23229.

The study presented in Chapter 2 was made possible by the availability of a specific DP IV inhibitor synthesized in the laboratory of Dr. Demuth. The mass spectrometric analysis was also conducted under his supervision. All other components of this study were conducted in the laboratory of Dr. Raymond Pederson at UBC. This study is currently under review as:

Pauly, R.P., Demuth, H.-U., Rosche, F., Schmidt, J., White, H.A., McIntosh, C.H.S. & Pederson, R.A. Improved glucose tolerance in rats treated with the dipeptidyl peptidase IV (DP IV, CD26) inhibitor Ile-thiazolidide (under review).

INTRODUCTION

The Incretin Concept

The first demonstration that a substance originating in the gut could influence the function of the pancreas was reported in 1902 by Bayliss and Starling. They observed that the introduction of hydrochloric acid into the duodenum of a completely denervated small intestine of a dog resulted in the release of pancreatic juice into the small intestine. Intravenous (*i.v.*) injection of a jejunal extract produced the same result, leading these investigators to conclude that an active substance was released into the blood stream from the small intestine in response to an acid stimulus in the duodenum. They called this substance ***secretin***, and with its discovery and characterization (Bayliss and Starling, 1902; 1903) arose the study of endocrinology. Long before the discovery of insulin by Banting and Best in 1921, it had been recognized that the pancreas was the source of an ***internal secretion*** which contributes to the regulation of blood sugar levels. Whereas secretin was shown to stimulate the ***external secretion*** of the pancreas - secretion of pancreatic juice into the lumen of the gut - it was not until 1906 that Moore, Eddie and Abram postulated "*...that the internal secretion of the pancreas might be stimulated and initiated (similar to external secretion) by a substance of the nature of a hormone or secretin yielded by the duodenal mucous membrane....*" In fact, Moore *et al.* (1906) even suggested "*...that in certain cases of diabetes the appearance of sugar in the urine might be due to the functional disturbance occasioned by the absence of such an intestinal excitant of the internal secretion.*" Due to the small number of subjects in their study,

however, these investigators were unable to conclusively state whether administration of their acid extract from porcine small intestine was able to normalize diabetic hyperglycemia.

It wasn't until the 1920's and 1930's that much attention was devoted to characterizing this hypoglycemic phenomenon. During this time, La Barre and colleagues found that *i.v.* injection of a crude secretin extract into the dog produced hypoglycemia (Zunz and La Barre, 1929; La Barre and Still, 1930). It was concluded that crude secretin contained a second active substance, which they called ***incretin*** (La Barre, 1932), able to stimulate the release of insulin from the endocrine pancreas. Similar results were reported by Heller (1929; 1935), and Elrick *at al.* (1964) reported that no less than forty-six publications between 1923 and 1936 described the hypoglycemic effects of intestinal mucosal extracts.

However, a series of studies by Loew, Gray and Ivy (1939; 1940a; 1940b) were deemed decisive in demonstrating that an intestinal substance was not responsible for the blood glucose lowering response reported by previous investigators, and it was not until the development of an insulin radioimmunoassay (RIA) (Yallow and Berson, 1960) that the existence of a potential hypoglycemic hormone was again considered in the mid 1960s. McIntyre *et al.* (1964) reported that intrajejunal administration of glucose in two healthy subjects resulted in a greater insulin response and more rapid blood glucose clearance than when the same glucose dose was given intravenously, suggesting that an insulinotropic substance may be released from the intestine in response to luminal glucose. In 1965, McIntyre, Holdsworth and Turner reported that the response to intrajejunal infusion of glucose in patients having end-to-side portacaval shunts due to liver cirrhosis was the

same as in healthy control subjects suggesting that prior circulation of glucose through the liver was not a prerequisite for an augmented insulin response. This evidence suggested that the source of La Barre's incretin was the intestinal mucosa and not the liver.

By 1967, Perley and Kipnis quantified the insulin responses to oral and *i.v.* glucose and reported that the response to *i.v.* glucose was 30 - 40 % of that observed for oral glucose in healthy and obese, diabetic and nondiabetic subjects. By 1969, enough evidence of a physiological connection between the gut and the endocrine pancreas had accumulated for Unger and Eisentraut to coin the term ***enteroinsular axis*** to describe a "... *regulatory system in which the secretion of pancreatic islet cell hormones is under the partial influence of hormones of the gastrointestinal tract.*" Though the term originally included only hormonal factors, neural and substrate influences (Fig. 1) were later incorporated into the definition (Creutzfeldt, 1979). Due to nature of the studies described in this thesis, only the endocrine component of the enteroinsular axis will be considered, and in particular, those hormones which influence insulin secretion.

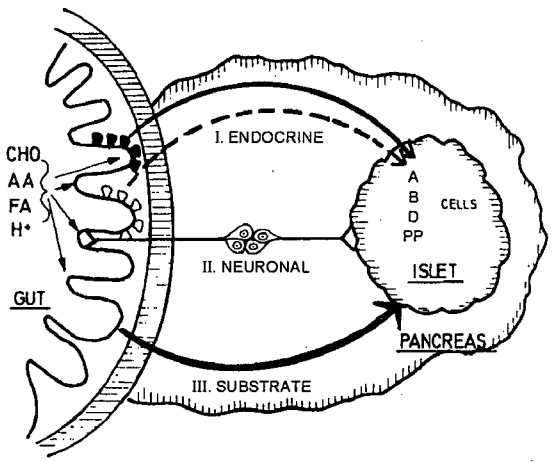

Fig. 1. The enteroinsular axis. This axis reflects the endocrine, neural and substrate factors originating in the gut which influence the secretion of hormones from the endocrine cells of pancreatic islets of Langerhans. This figure was reproduced from Creutzfeldt, 1979.

The term incretin does not refer to a single hormone, but rather to any endocrine substance released from the intestinal mucosa which potentiates the secretion of insulin (Creutzfeldt, 1979) and thus contributes to the greater insulin response after oral versus *i.v.* glucose. Before a substance can be considered an incretin, Creutzfeldt (1979) outlined two criteria that must be met:

- *the substance must be released in response to nutrients in the lumen of the gut, and*
- *the insulinotropic action of the incretin must be glucose concentration-dependent when administered exogenously at physiological concentrations.*

The glucose-dependence of incretin action offers a unique advantage in preventing inappropriate insulin secretion and subsequent hypoglycemia so that even in the presence of elevated circulating incretin concentrations, insulin is secreted only when required (i.e. in the presence of glucose). Thus, an incretin cannot, by itself, stimulate the secretion of insulin, but only potentiate the insulinotropic actions of nutrients.

Though a number of intestinal peptide hormones have been considered as incretin candidates, most have been rejected since they are not glucose-dependent or not insulinotropic at physiological levels (reviewed in Creutzfeldt, 1979; Creutzfeldt and Ebert, 1985, Creutzfeldt and Nauck, 1992). Of the gut factors considered to date, only glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide (GIP_{1-42}) and truncated forms of glucagon-like peptide-1 ($GLP\text{-}1_{7-36}$ and $GLP\text{-}1_{7-37}$) are considered true incretins (Fehmann *et al.*, 1995a).

Glucose-dependent Insulinotropic Polypeptide (GIP)

GIP* is a hormone of the glucagon superfamily of hormones including glucagon, glucagon-like peptides 1 and 2, glicentin, secretin, vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), and growth hormone releasing hormone (GHRH), all of which exhibit considerable sequence homology (Dockray, 1989).

GIP was initially isolated by Brown and Pederson (1970) based on its ability to inhibit gastric acid secretion. Two preparations of cholecystokinin-pancreozymin (CCK-PZ), one designated as 10 % pure and other as 40 % pure, were tested for their ability to stimulate gastric acid secretion in a vagally and sympathetically denervated stomach of the dog (Bickel pouches). The 40 % pure CCK-PZ extract was a more effective stimulant of acid secretion than the 10 % pure preparation, leading the investigators to conclude that a stimulant of gastric acid secretion could have been concentrated, or an inhibitor of acid secretion removed during the purification of CCK-PZ. Though both extracts were effective in inhibiting pentagastrin-induced acid secretion in the dog, the efficacy of the 10 % pure preparation was greater, leading Pederson (1971) to conclude that the CCK-PZ extracts contained a second active substance: an inhibitor of gastric acid secretion which they called gastric inhibitory polypeptide. The insulin stimulating action of GIP was discovered shortly thereafter in 1973 (Dupré *et al.*, 1973) leading to its alternate designation as Glucose-dependent Insulinotropic Polypeptide (Brown and Pederson, 1976).

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* For simplicity, GIP_{1-42} will be referred to as GIP, and $GLP\text{-}1_{7-36}$ will be referred to as GLP-1, unless a specific prohormone sequence or hormone metabolite is being referred to.

Initial immunocytochemical studies of GIP secreting cells indicated this peptide was localized in cells of the duodenum and jejunum in man and dog (Polak *et al.*, 1973). Later studies identified the specific cells of origin in man as being the K cell, a typical endocrine cell of the intestinal mucosa (Buchan *et al.*, 1978). Evidence suggests that GIP secreting cells are confined exclusively to the alimentary tract in mammals (review by Pederson, 1994).

Secretion

The development of the first RIA of GIP in 1974 (Kuzio *et al.*, 1974) allowed investigators to study the endogenous release of this peptide. Kuzio and colleagues (1974) reported fasting GIP** concentrations of 237 ± 14 pg/ml in 48 healthy subjects. This level rose to greater than 1200 pg/ml after a mixed meal stimulus, and remained elevated for several hours. Subsequent RIAs have measured basal levels ranging from 60 - 460 pg/ml and rising to 170 - 1470 pg/ml within an hour after the ingestion of a meal (Morgan *et al.*, 1978; Sarson *et al.*, 1980; Burhol *et al.*, 1980; Jorde *et al.*, 1983b). Though the absolute hormone concentrations between RIA's utilizing different antisera vary considerably, all indicate a significant increase in GIP in response to an ingested meal. GIP release by specific nutrients was investigated concurrently. It is important to note for later discussion of GIP metabolism that the antibodies utilized in these assays cross-react with N-terminally truncated GIP (ie. the antisera are directed against a C-terminal epitope of the GIP molecule).

** Circulating hormone levels determined by radioimmunoassay are most accurately described as *immuno-reactive (IR)* peptide concentrations (eg. IR-GIP or IR-insulin). For the sake of brevity, the prefix *IR-* has been omitted from all peptide concentrations cited in this text.

It makes physiological sense that the release of a gut hormone having insulinotropic action be stimulated by ingested carbohydrates. This hypothesis was confirmed by Cataland and colleagues (1974) who administered an oral glucose tolerance test (OGTT) to a group of healthy subjects. Concomitant to the rise and fall in serum glucose and insulin concentrations typical of an OGTT, was a parallel serum GIP secretory profile. By sampling blood from the portal vein of a patient undergoing treatment for portal hypertension, Cataland *et al.* (1974) determined that GIP rose within 2 min after oral glucose administration while an increase in serum insulin remained undetectable until 5 min after oral glucose. These experiments suggested a causal link between luminal glucose, GIP and insulin secretion. At the same time, Pederson *et al.* (1975a) reported a dose-dependent relationship between ingested glucose concentration and serum GIP levels in the dog while Falko *et al.* (1980) determined the same effect in man, thus clearly establishing glucose as a potent stimulant of GIP release. Galactose and sucrose, but not fructose, were also shown to stimulate the release of GIP in both man and rat (Morgan, 1979; Sykes *et al.*, 1979). The precise carbohydrate sensing mechanism is not clear, though evidence suggests that active absorption of hexoses by a Na^+-dependent pathway is necessary (reviewed in Creutzfeld and Ebert, 1993; Hopfer, 1987).

In 1974, Brown reported that oral Lipomul, a fat suspension, produced a significant rise in GIP reaching a peak at approximately 2 h after ingestion. Similar results were reported by Falko *et al.* (1975), and Cleator and Gourlay (1975), confirming that fat is a potent stimulant of endogenous GIP release in man. Pederson *et al.* (1975a) reported the same results in the dog and demonstrated a dose-dependent increase in GIP in response to triglycerides. It was later determined that short and medium chain length

triglycerides and free fatty acids resulted in insignificant GIP release, whereas long chain triglycerides and fatty acids yielded significant stimulation (O'Dorisio *et al.*, 1976; Williams *et al.*, 1981; Ohneda *et al.*, 1984; Kwasowski *et al.*, 1985).

Initial reports suggested that protein was unable to stimulate GIP secretion in humans (Brown, 1974; Cleater and Gourlay, 1975). However, later studies indicated that amino acids do stimulate an increase in serum GIP (O'Dorisio *et al.*, 1976; Schulz *et al.*, 1982a). Thomas *et al.* (1976) also demonstrated that duodenal perfusion of arginine, histidine, isoleucine, lysine and threonine in man resulted in significant increases in circulating GIP and insulin concentration, while perfusate containing phenylalanine and tryptophan did not (Thomas *et al.*, 1978). As is thought to be the case for glucose, endogenous GIP release in response to amino acids is likely to occur after nutrient absorption, and not simply due to their presence in the lumen of the gut (Schulz *et al.*, 1982b).

There is no clear indication as to the role of autonomic control of GIP secretion. Conflicting reports indicate that the sympathetic and parasympathetic nervous systems increase, decrease, or have no effect on GIP release (Kieffer, 1995).

Enterogastrone Action of GIP

The term ***enterogastrone*** was originally used to describe an endocrine substance, which is released from the intestine in response to fat, and inhibits the secretion of gastric acid (Farrell and Ivy, 1926; Kosaka and Lim, 1930). On the basis of this definition, GIP became an important enterogastrone candidate when it was isolated on the basis of its acid-inhibiting ability (Brown and Pederson, 1970; Pederson and Brown, 1972)

and when intraduodenal fat was demonstrated to be a potent stimulant of GIP release (Pederson *et al.*, 1975a; Cleator and Gourlay, 1975; Falko *et al.*, 1975, Martin *et al.*, 1980). However, these early reports were challenged by mounting evidence that GIP was a poor inhibitor of gastric acid secretion in the innervated dog stomach and in humans (Soon-Shiong *et al.*, 1979; Maxwell *et al.*, 1980). In addressing this disparity, Soon-Shiong *et al.* (1984) reported that GIP was able to inhibit acid secretion from vagally denervated pouches in the dog, but not from the innervated stomach in the same animal model. Though these results call into question the physiological relevance of GIP as a true enterogastrone, it remains clear that under certain conditions GIP can influence gastric acid secretion. Evidence suggests that this enterogastrone-like effect is mediated by gastric somatostatin (McIntosh *et al.*,1981) which exerts inhibitory actions on gastrin, histamine and acid secreting cells of the stomach by a paracrine mechanism.

GIP Action on Islet Hormones

Dupré *et al.* (1973) administered a highly purified GIP preparation into healthy volunteers and observed that, in the presence of glucose, GIP was able to stimulate insulin secretion to a greater extent than glucose alone. In the absence of glucose, GIP was ineffective as an insulinotropic substance and thus Dupré and colleagues had also described the glucose-dependence of GIP stimulation of the endocrine pancreas. Pederson *et al.* (1975a) made similar conclusions by demonstrating that fat-stimulated GIP in the dog was only insulinotropic in the presence of *i.v.* glucose. This study also concluded that *i.v.* glucose by itself did not result in changes in circulating GIP levels and did not enhance insulin secretion. The glucose concentration threshold for the insulinotropic action of GIP

has been reported to be between 4.4 and 5.5 mM (Pederson and Brown, 1976; Jia, *et al.*, 1995). Pederson and co-workers went on to describe a dose-dependent relationship between exogenously delivered GIP and increases in insulin secretion in the dog and in the isolated perfused pancreas of the rat (Pederson *et al.*, 1975b; Pederson and Brown, 1976). In a series of experiments using a glucose clamp technique in man to maintain circulating glucose concentrations at a fixed level, Andersen *et al.* (1978) conclusively demonstrated that endogenous GIP was released in response to oral glucose and served to potentiate glucose-induced insulin secretion. Studies employing a similar hyperglycemic clamp demonstrated that exogenously administered GIP was able to mimic these effects (Elahi *et al.*, 1979).

Several investigators have studied the effect of GIP on glucagon secretion from the islet α cells. Pederson and Brown (1978) reported that in the perfused rat pancreas GIP was able to stimulate glucagon secretion when the prevailing glucose concentration was less than 5.5 mM, while stimulating insulin secretion at higher glucose concentrations. Elahi *et al.* (1979) demonstrated that glucose-suppressed glucagon release in man was not reversed by the addition of GIP under mild (3.0 mM above basal) and moderate (7.9 mM above basal) hyperglycemic clamps; glucose clamps below basal glucose concentrations could not be performed to determine the effect of GIP on glucagon under hypoglycemic conditions. In the mouse, however, glucose-suppressed glucagon secretion from isolated perifused islets was reversed in a GIP concentration-dependent manner even at 11.1 mM glucose (Opara and Go, 1991). Thus, it seems that GIP is able to stimulate glucagon release but this action is dependent on the experimental conditions and the animal model used. Recently, GIP receptors have been identified on rat pancreatic α cells, thus

providing evidence for direct stimulation of glucagon secretion by GIP (Moens *et al.*, 1996).

GIP has been reported to have only weak stimulatory effects on islet somatostatin release (Schmid *et al.*, 1990). The exact pancreatic cell types which express GIP receptors remains to be determined (reviewed in Fehmann *et al.*, 1995a).

Extrapancreatic Actions of GIP

Though specific GIP binding has been reported in a variety of tissues including the liver, skeletal muscle, small intestine and stomach (reviewed in Morgan, 1996), the biological significance is only beginning to be investigated. Several investigators have reported that GIP possesses direct effects on glucose metabolism in conjunction with a well established role as an incretin. In 1984, Andersen *et al.* demonstrated that intravenous infusion of GIP in combination with insulin resulted in augmented suppression of insulin-induced decreases in hepatic glucose production as well as blunting glucagon-mediated glucose production in the dog. The same response was reported in man by Elahi and colleagues in 1986. Hartmann *et al.* (1986) demonstrated this effect in the rat while reporting that in the presence of insulin at a concentration too low to antagonize the effects of glucagon by itself, GIP was able to reverse and suppress glucagon-induced hepatic glycogenolysis in a dose-dependent manner. The mechanism by which this occurs is still unknown.

Since the discovery of GIP as a hormone released from the proximal intestine by the metabolism of luminal fat, investigators have postulated a direct effect of GIP on fat metabolism especially since fat-stimulated GIP release was shown to be essentially non-

insulinotropic (Falko *et al.*, 1975; Pederson *et al.*, 1975a). Eckel and colleagues (1979) demonstrated that GIP was able to stimulate lipoprotein lipase activity directly in cultured preadipocytes while Wasada *et al.* (1981) were able to confirm this report *in vivo*. Similar results have been found by other investigators (Ebert *et al.*, 1991) thus linking GIP physiology with fat metabolism. In fact, high dietary fat has been shown to increase GIP mRNA expression as well as increase nutrient-stimulated GIP secretion (Higashimoto *et al.*, 1995; Morgan, 1996). In the presence of insulin, GIP enhanced insulin-induced lipogenesis in rat adipose tissue (Beck and Max, 1983; Oben *et al.*, 1991), as well as increasing insulin receptor affinity and sensitivity of insulin-stimulated glucose transport in isolated rat adipocytes (Starich *et al.*, 1985).

The demonstration that GIP has direct effects on both glucose and fat metabolism, independent of its insulinotropic function, suggests that GIP is a true anabolic hormone.

Glucagon-Like Peptide-1 (GLP-1)

Unlike GIP, which was discovered and characterized by classical extraction and biological assay, GLP-1 was discovered by molecular biological techniques before its biological functions were characterized. GLP-1 is encoded by the glucagon gene and is a product of post-translational processing of proglucagon ($PG_{1\text{-}160}$) (Fig. 2) (Bell *et al.*, 1983).

$PG_{1\text{-}160}$ has been localized to pancreatic α cells of the islets of Langerhans and intestinal L cells, concentrated in the ileum but is also present in the jejunum and colon, where it undergoes tissue-specific processing to form a series of unique biologically active

polypeptides (Varndell *et al.*, 1985; Mojsov *et al.*, 1986; Vaillant *et al.*, 1986; Kauth and Metz, 1987; Ørskov *et al.*, 1987; Holst, 1994; Deacon *et al.*, 1995c). The most prominent product of pancreatic processing is the glucoregulatory hormone glucagon (PG_{33-61}); the other polypeptides are glicentin-related pancreatic polypeptide (PG_{1-30}), intervening peptide-1 (PG_{64-69}), and the major proglucagon fragment (PG_{72-158}) (Holst and Ørskov, 1994). It is believed that α cells secrete equimolar amounts of these post-translational products (Yanaihara *et al.*, 1985; Ørskov *et al.*, 1986). Approximately 30 % of the major proglucagon fragment is further processed to GLP-1 (PG_{72-107}, PG_{72-108}, or PG_{78-107}) and GLP-2 ($PG_{126-158}$) (Mojsov *et al.*, 1990; Holst, 1994; Ørskov *et al.*, 1994).

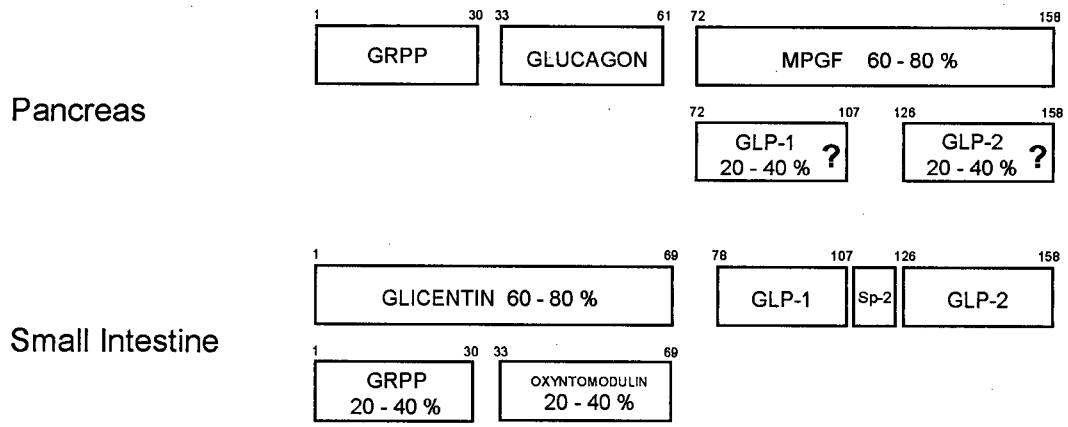

Fig. 2. Differential post-tranlational processing of proglucagon. See text for explanation. This figure was adapted from Holst and Ørskov, 1994.

Intestinal processing of proglucagon, on the other hand, differs markedly from pancreatic processing. Glucagon is not formed in intestinal L cells; instead, the glucagon sequence (PG_{33-61}) is contained within the larger polypeptide sequence of glicentin (PG_{1-69}), a hormone believed to contribute to the enterogastrone effect (Thim and Moody,

1981; Holst and Ørskov, 1994). Approximately 20 - 40 % of glicentin is further hydrolyzed to form glicentin-related pancreatic polypeptide (PG_{1-30}) and oxyntomodulin (PG_{33-69}) (Holst, 1994). To date, no biological actions have been reported for the former, while oxyntomodulin has exhibited glucagon-like effects on hepatic glucose production and gastric acid secretion (Holst and Ørskov, 1994). The remaining three processing products of intestinal proglucagon are GLP-1 (PG_{78-107}, or PG_{78-108}), GLP-2 ($PG_{126-158}$), and an intervening oligopeptide ($PG_{111-123}$) (Buhl *et al.*, 1988; Ørskov *et al.*, 1994). GLP-2 is thought to mediate intestinal mucosal growth (Drucker *et al.*, 1996).

GLP-1_{1-36} (PG_{72-107}) is the predominant molecular form of this hormone extracted from the pancreas whereas the majority of the intestinal hormone is secreted as an N-terminally truncated form, GLP-1_{7-36} (PG_{78-107}) (Ørskov *et al.*, 1994). Both of these polypeptides are amidated at their C-terminus (Holst, 1994). GLP-1_{7-36} represents approximately 80 % of the hormone secreted from the intestine while 20 % exists in a glycine-extended form as GLP-1_{7-37} (Ørskov *et al.*, 1994). However, both the amidated and the glycine-extended forms are equipotent with respect to insulin secreting, and glucose, glucagon and free fatty acid lowering effects (Ørskov *et al.*, 1989; Suzuki *et al.*, 1989; Weir *et al.*, 1989; Ørskov *et al.*, 1993).

Secretion

Intestinal GLP-1 levels have been observed to rise rapidly in response to a mixed meal, resulting in peak postprandial concentrations after 15 - 30 min (D'Alessio *et al.*, 1993; Elliot *et al.*, 1993; Morgan *et al.*, 1993; Herrmann *et al.*, 1995). Reported GLP-1 levels vary between investigators depending on the selectivity and specificity of the

antisera used in the respective radioimmunoassays; however, fasting and meal-stimulated GLP-1 levels are typically reported in the low picomolar range (Holst and Ørskov, 1994). Little is known about the precise stimuli which evoke the release of GLP-1 or the mechanism(s) involved. Studies of the effects of individual nutrients on hormone secretion have been directed at clarifying this issue.

A rapid biphasic GLP-1 secretory response to oral glucose has been demonstrated in man, yielding increases in circulating GLP-1 concentrations of up to 300 % (D'Alessio *et al.*, 1993; Göke *et al.*, 1993; Morgan *et al.*, 1993; Herrmann *et al.*, 1995). Similar results were reported with oral sucrose and galactose (Fukase *et al.*, 1992; Göke *et al.*, 1993; Herrmann *et al.*, 1995; Qualmann *et al.*, 1995). Shima and colleagues (1990) found that glucose, galactose, 3-0-methyl-D-glucose, maltose, sucrose and maltitol all stimulated the release of GLP-1 from isolated ileal loops of the dog, while fructose, fucose, mannose, xylose and lactose did not. A rapid, but less pronounced increase in GLP-1was observed in response to oral amino acids (Göke *et al.*, 1993; Morgan *et al.*, 1993; Herrmann *et al.*, 1995), while oral fats elicited a much stronger and prolonged rise in GLP-1 (Roberge and Brubaker, 1991; D'Alessio *et al.*, 1993; Göke *et al.*, 1993; Morgan *et al.*, 1993). Plaisancié *et al.* (1995) reported that luminal stimulation of colonic L cells by some dietary fibers and certain bile salts contributed to GLP-1 release, though the functional significance of this observation is not clear.

Considerable evidence suggests that direct stimulation of intestinal L cells by ingested nutrients alone cannot account for circulating GLP-1 levels after an oral nutrient load. The aforementioned studies report significant increases in GLP-1 within minutes of nutrient ingestion even though GLP-1 is released from the distal gut. In addition, it was

demonstrated that the GLP-1 response to intraduodenal fat was equipotent to the response to intra-ileal fat (Roberge and Brubaker, 1991), and that ileostomy patients were still able to secrete appreciable levels of GLP-1 even though ingested nutrients bypassed the majority of the ileum (D'Alessio *et al.*, 1993). Such observations led investigators to speculate that neural and/or hormonal factors originating in the proximal gut feed forward to stimulate the release of GLP-1 from the distal intestine and colon. In this regard it has been reported that GIP was able to stimulate the release of GLP-1 from cultured rat intestinal cells as well as *in vivo* in the rat, providing strong evidence that GIP, secreted primarily from the duodenum, is a stimulant of GLP-1 secretion in an enteroendocrine loop (Brubaker, 1991; Roberge and Brubaker, 1993). Subsequent studies have confirmed that GIP is the most potent hormonal stimulant of endogenous GLP-1 in the rat though a variety of other endocrine and neuroendocrine substances may act in a similar manner (Plaisancié *et al.*, 1994; Herrmann-Rinke *et al.*, 1995). However, studies in humans have found no evidence for neural or endocrine substances which influence the secretion of GLP-1 from the distal gut, and factors evoking the early rise in GLP-1 secretion *in vivo* remain the subject of ongoing research.

GLP-1 Action on Islet Hormones

As with GIP, the primary biological function of GLP-1 is believed to be its ability to potentiate glucose-stimulated insulin secretion. This insulinotropic action was first demonstrated in the isolated perfused pancreas of the rat and pig (Holst *et al.*, 1987; Mojsov *et al.*, 1987) and later in the dog (Kawai *et al.*, 1989). Kreymann and colleagues (1987) were able to mimic the insulin response in man observed following oral glucose by

administering *i.v.* glucose and GLP-1. The insulinotropic action of GLP-1 could also be demonstrated in isolated rat or human islets (D'Alessio *et al.*, 1989; Fridolf and Ahren, 1991; Fehmann *et al.*, 1995b). Soon after the identification of GLP-1 as a hormone able to influence the secretion of insulin from pancreatic β cells, studies were designed to investigate the glucose-dependence of this action. As would be expected from any incretin, the levels of GLP-1 rise in response to oral glucose but not to *i.v.* glucose administration (Göke *et al.*, 1993a; Herrmann *et al.*, 1995). Shima *et al.* (1988) demonstrated the glucose concentration-dependence of 0.1 nM GLP-1 on insulin secretion from the isolated perfused rat pancreas. This was confirmed in isolated rat islets by D'Alessio and colleagues (1989). The glucose threshold for GLP-1 action has been reported to be 2.8 mM in the isolated rat pancreas (Göke *et al.*, 1993a), 3.3 mM in isolated rat islets (Fridolf and Ahrén, 1991), and 5.0 mM in the rat *in vivo* (Hargrove *et al.*, 1995). This disparity in glucose threshold may be explained by an observation made by Ahrén (1995) who demonstrated that the glucose concentration threshold of GLP-1 depends on the concentration of GLP-1. In the mouse, an exogenously administered dose of GLP-1 at 1 nmol·kg^{-1} required a glucose threshold of ~ 25 mM while a peptide dose of 32 nmol·kg^{-1} required a threshold of only 5 mM (Ahren, 1995). A similar report had previously been published in 1990 by Schmid *et al.* who demonstrated a potentiating effect of 0.01 nM GLP-1 at ~ 8.3 mM glucose, which could only be mimicked by 1 nM GLP-1 at ~ 4 mM glucose.

In addition to its direct effect on the insulin secreting cells of the pancreas, GLP-1 also influences the secretion of other islet hormones. The glucose lowering effect of GLP-1 is not only a consequence of its insulinotropic action since GLP-1 is also a gluca-

gonostatic hormone. In 1988, Ørskov and colleagues demonstrated a 50 % decrease in pancreatic glucagon output from the perfused porcine pancreas in response to 0.1 nM GLP-1; a 70 - 80 % decrease from basal output was observed with 1 nM GLP-1. This effect was subsequently confirmed in the isolated perfused rat pancreas (Komatsu *et al.*, 1989), the conscious dog (Kawai *et al.*, 1990), and in isolated human islets (Fehmann *et al.*, 1995b). GLP-1 has also been shown to stimulate the secretion of islet somatostatin from δ cells in the pM range (Ørskov *et al.*, 1988; D'Alessio *et al.*, 1989; Schmid *et al.*, 1990; Fehmann *et al.*, 1995a). Fehmann *et al.* (1995b) even demonstrated that 0.1 nM GLP-1 in the presence of 2.8 mM glucose was able to stimulate the release of pancreatic polypeptide from isolated human islets. The significance of this observation is not clear.

It is widely believed that the intestinal GLP-1 is responsible for the described incretin effect; however, Heller *et al.* (1995) reported that GLP-1 was also secreted from isolated rat islets in a glucose concentration-dependent manner. These investigators suggest that α cell-derived GLP-1 may play a unique role in intra-islet hormone regulation.

Extrapancreatic Actions of GLP-1

GLP-1 is believed to have a number of biological functions which are independent of its influence on islet hormones. GLP-1 has been reported to inhibit pentagastrin-stimulated acid secretion in the stomach, as well as gastric emptying (Schjoldager *et al.*, 1989; O'Halloran *et al.*, 1990). Ørskov and colleagues (1988) reported that GLP-1 had no effect on antral nor non-antral somatostatin secretion, and a unique GLP-1 receptor was recently identified on gastric parietal cells (Gros *et al.*, 1995). Evidence has been

presented that GLP-1 may also contribute to the hormonal signal mediating the ***ileal brake***, a term used to describe the phenomenon whereby unabsorbed nutrients in the ileum and colon feed back to slow intestinal transit of ingested food from the proximal gut. Ileal perfusion by carbohydrates, fats, and proteins, in both man and dog, indicate that GLP-1 in conjunction with peptide YY, may play the most active role in decreasing gastric acid secretion and inhibiting the motility of the proximal gut (Layer *et al.*, 1995; Wen *et al.*, 1995).

Conflicting data suggest that GLP-1 may also have an insulin-independent effect on hepatic glucose metabolism. In 1994, D'Alessio *et al.* reported an increased rate of glucose disposal in the presence of GLP-1 even at basal circulating insulin concentrations, suggesting this hormone is able to facilitate insulin-independent glucose absorption. However, Toft-Nielsen and coworkers (1996) were unable to observe any change in hepatic glucose disposal. Other investigators had previously been unable to demonstrate a GLP-1-induced effect on hepatic glycogenolysis and ketogenesis in the isolated perfused rat liver (Murayama *et al.*, 1990), but a later study demonstrated that GLP-1 was able to inhibit glucagon-induced glycogenolysis in a dose-dependent manner in a subpopulation of rat hepatocytes (Yamatani *et al.*, 1996). GLP-1 receptors have also been identified in adipose and skeletal muscle tissues (Valverde *et al.*, 1993; Villanueva-Penacarillo *et al.*, 1994), and it has been suggested that GLP-1 may play a direct role in fatty acid synthesis *in vivo* (Oben *et al.*, 1991). Although GLP-1 may demonstrate a number of extrapancreatic effects, is seems clear that its primary function is the potentiation of nutrient-induced hormone secretion from the endocrine pancreas.

Relative Contribution of GIP and GLP-1 to the Incretin Effect

GIP_{1-42} and truncated forms of glucagon-like peptide-1 ($GLP\text{-}1_{7-36}$ and $GLP\text{-}1_{7-37}$) are the only gut peptides identified to date which satisfy the incretin criteria. What remains controversial is the relative contribution of these peptides to the enteroinsular axis. Even before the insulinotropic role of GLP-1 had been characterized, Ebert and colleagues had reported that GIP antiserum was able to block only the early phase of the incretin response (Ebert *et al.*, 1979b; Ebert and Creutzfeldt, 1982). Immuno-neutralization and immunoabsorption of GIP_{1-42} from gut extracts was able to suppress the incretin effect by 30 - 50 % (Ebert *et al.*, 1983). Intravenous or subcutaneous administration of exendin-[9-39], a specific GLP-1 receptor antagonist, prior to an enteral glucose infusion or a mixed meal, reduced the incretin effect by 50 - 60 % (Kolligs *et al.*, 1995; Wang *et al.*, 1995). Collectively, these studies indicate that GIP and GLP-1 could account for the entire incretin response.

Several studies have attempted to determine which incretin is more effective. Suzuki *et al.* (1990; 1992) reported that GIP and GLP-1 exhibited comparable insulinotropic effects on a molar basis in the perfused rat pancreas. Jia and colleagues (1995) reported similar results and demonstrated a similar glucose threshold for both peptides. These investigators predicted that since the postprandial GIP concentration in the rat is ~ 6 times that of GLP-1, the former may be capable of potentiating glucose-induced insulin secretion several times greater than GLP-1. By monitoring endogenous incretin concentrations in man, and mimicking these with an isoglycemic clamp and concomitant infusion of exogenous incretins, Nauck *et al.* (1993a) also found that GIP

may be the more important incretin under physiological conditions. Other studies, however, have suggested that GLP-1 is likely the more important contributor to the incretin response since its insulinotropic effects were reported at both lower peptide concentrations than for GIP in isolated rat islets (Siegel *et al.*, 1992) and the perfused rat pancreas (Shima *et al.*, 1988), along with a lower glucose threshold in perifused canine islets (van der Burg *et al.*, 1995). Using a hyperglycemic clamp, Elahi *et al.* (1994) reported a much greater insulin potentiating effect of 1.5 $pmol \cdot kg^{-1} \cdot min^{-1}$ GLP-1 as compared to 4 $pmol \cdot kg^{-1} \cdot min^{-1}$ GIP in man (increased insulin concentrations of 2105 $pmol \cdot l^{-1}$ versus only 920 $pmol \cdot l^{-1}$). However, many investigators caution that greater efficacy under experimental conditions may not accurately reflect physiological relevance *in vivo*.

Incretins and Diabetes Mellitus

Abnormal circulating GIP and GLP-1 levels may contribute to the pathophysiology of dysfunctional insulin secretion of non-insulin dependent diabetes mellitus (NIDDM) and other conditions involving glucose intolerance. Fasting GIP concentrations have been reported as elevated (Ebert *et al.*, 1976a; Crockett *et al.*, 1976; Coxe *et al.*, 1981) or unchanged (Bloom, 1975; Ross *et al.*, 1977; Ross *et al.*, 1978; Mazzaferri *et al.*, 1985; Osei *et al.*, 1986) in NIDDM patients relative to healthy control subjects. Many investigators found meal stimulated GIP levels elevated in diabetics even though fasting levels may have been normal (Ebert *et al.*, 1976a; Crockett *et al.*, 1976; Ross *et al.*, 1977; Coxe *et al.*, 1981; Salera *et al.*, 1982; Mazzaferri *et al.*, 1985; Osei *et al.*, 1986) while

others have reported no such increases in meal stimulated GIP (Bloom, 1975; Service *et al.*, 1984; Nauck *et al.*, 1986). Elahi *et al.* (1984) noted that even though fasting and meal-stimulated GIP levels were elevated in their patients with NIDDM, the degree to which GIP was increased relative to basal in diabetics was less than in healthy individuals. Service *et al.* (1984) also reported a decreased GIP response, indicating a decreased incretin response in NIDDM. Nauck and colleagues (1986) found similar GIP levels in diabetics in response to oral glucose, but noted a diminished overall incretin effect. This was attributed to a decreased responsiveness of pancreatic β cells to GIP. In fact, in a more thorough study of the incretin effect in disease states, it was found that the overall incretin response was blunted regardless of the status of GIP secretion (ie. with either hyper- or hypoGIPemia) (Creutzfeldt *et al.*, 1983).

GLP-1 was reported to be elevated in diabetics (Hiroto *et al.*, 1990), though Ørskov *et al.* (1991) demonstrated that the increase in GLP-1 was due primarily to increases in $PG_{72\text{-}158}$ (MPGF) and not $PG_{78\text{-}107}$ ($GLP\text{-}1_{7\text{-}36}$). The significance of this observation is not known. Genetic studies involving the GLP-1 receptors have shown that mutations on or near the receptor in pancreatic β cells are not indicative of NIDDM susceptibility (Tanizawa *et al.*, 1994; Zhang *et al.*, 1994).

Incretin levels have also been investigated in obesity, where fasting GIP was also observed to be increased (Bloom, 1975; Ebert *et al.*, 1976b; Willms *et al.*, 1978), or unchanged (Lauristen *et al.*, 1980; Jorde *et al.*, 1983a; Elahi *et al.*, 1984; Service *et al.*, 1984; Mazzaferri *et al.*, 1985). When obese subjects were subclassified as having either normal or pathological oral glucose tolerance tests, those with impaired glucose tolerance always exhibited an exaggerated GIP response, whereas glucose tolerant obese subjects

exhibited normal (Creutzfeldt *et al.*, 1978) or elevated (Salera *et al.*, 1982) GIP levels. It has been demonstrated that lowering caloric intake in hyperGIPemic obese subjects reversed their elevated incretin levels (Willms *et al.*, 1978; Ebert *et al.*, 1979a; Deschamps, 1980).

There is no clear consensus pertaining to the role of incretins in pathophysiological states. It is unknown whether abnormal circulating incretin concentrations contribute to the etiology of these conditions or whether their over-secretion is simply a compensatory measure for decreased islet cell responsiveness to GIP and GLP-1.

In spite of the varied circulating incretin levels observed in NIDDM patients, exogenous GIP and GLP-1 have been considered in the treatment of diabetic hyperglycemia. Since incretins do not exert their insulinotropic effects in the presence of sub-threshold glucose concentrations, these peptides or suitable analogues would likely not induce the hypoglycemia associated with inappropriate administration of insulin or oral hypoglycemics (Creutzfeldt and Ebert, 1985; Gerich, 1989; Creutzfeldt and Nauck, 1992; Amiel, 1994; Hargrove *et al.*, 1996). GLP-$1_{7\text{-}36}$ and GLP-$1_{7\text{-}37}$ in particular have been investigated as potential antidiabetogenic hormones which were able to increase peak insulin secretion significantly in diabetics and non-diabetics alike (Gutniak *et al.*, 1992; Nathan *et al.*, 1992). Exogenous GLP-1 has even been effective in normalizing fasting hyperglycemia in NIDDM patients (Nauck *et al.*, 1993c). This is thought to be due not only to potentiated insulin secretion, but also to the inhibition of glucagon release from pancreatic α cells (Willms *et al.*, 1996). In fact, the GLP-1-mediated reduction in glucagon is believed to be responsible for lowering fasting glycemia in insulin-dependent diabetics (IDDM) (Creutzfeldt *et al.*, 1996).

Several investigators have demonstrated that the insulinotropic effect of exogenous GIP was also preserved in patients with NIDDM as well as in patients with IDDM, suggesting that GIP may also be of therapeutic value (Jones *et al.*, 1987; Krarup *et al.*, 1987). Though GIP administration to patients with untreated NIDDM was able to potentiate insulin secretion, Jones *et al.* (1989) later reported that this augmented insulin response was insufficient to normalize fasting hyperglycemia.

Nauck *et al.* (1993b) compared the glucose-lowering effect of GIP and GLP-1 and found that the former was not able to potentiate insulin secretion in diabetics to the extent observed in healthy control subjects, while the latter retained its full insulinotropic effects. Similar results were reported by Elahi *et al.* (1994). These studies support the use of GLP-1 as a hypoglycemic agent, and stimulate further research to clarify the role of exogenous GIP in treating hyperglycemia.

Dipeptidyl Peptidase IV (DP IV)

It has been demonstrated recently that both GIP and GLP-1 are substrates of the circulating protease dipeptidyl peptidase IV (DP IV, CD26, EC 3.4.14.5) which by removing an N-terminal dipeptide (Mentlein *et al.*, 1993b), renders these hormones biologically inactive (Brown *et al.*, 1981; Schmidt *et al.*, 1986; Suzuki *et al.*, 1989; Gefel *et al.*, 1990). It has been speculated that DP IV-mediated GIP and GLP-1 hydrolysis is the primary mechanism of inactivation of these hormones *in vivo* (Mentlein *et al.*, 1993b; Kieffer *et al.*, 1995; Deacon *et al.*, 1995a).

DP IV is a serine protease which was first identified in the liver (Hopsu-Havu and Glenner, 1966), although DP IV activity was later found in many tissues including the stomach, spleen, lung, bone, testes, thyroid, gall bladder, large intestine, vascular endothelium and even in pancreatic Islets of Langerhans (Vanhoof *et al.*, 1992; Poulsen *et al.*, 1993; Mentzel *et al.*, 1996). The greatest concentration of DP IV activity, however, has been detected on the brush border membranes of intestinal enterocytes and proximal tubule cells of the kidney, as well as in placental tissue (Yaron and Naider, 1993). The cell differentiation marker CD26, expressed on the surface of a subpopulation of T-lymphocytes, was also shown to have DP IV catalytic activity, and was later determined to be the same protein (Bauvois, 1995).

DP IV is a 105 - 130 kDa intrinsic membrane glycoprotein which consists of a homodimer in its active form. Monomer size depends not only on the species but also on the tissue of origin within a given species (Yaron and Naider, 1993; Reutter *et al.*, 1995). There is approximately 85 % amino acid sequence identity between rat and human DP IV, and approximately 92 % homology between rat and mouse (Reutter *et al.*, 1995). The DP IV amino acid sequence is divided into five structural domains (Reutter *et al.*, 1995). The intracellular N-terminus consists of only 6 amino acids and is followed by a single 22 amino acid transmembrane spanning region which serves to anchor the bulky extracellular portion of the protein (739 amino acids) to the cell surface. The majority of glycosylation occurs in the extracellular domain adjacent to the transmembrane region. This is followed by a cysteine-rich region whose functional significance is not clear, while the C-terminal domain contains the proteolytic active site. Duke-Cohan and colleagues (1995) have recently identified a 175 kDa soluble form of DP IV which exists as a trimer in human

serum. Even in its unglycosylated state, this protein is larger than membrane-associated DP IV, excluding the belief that serum DP IV is exclusively derived from the membrane bound form.

Though DP IV is constituitively expressed in most endothelial and epithelial cells, evidence was recently presented that activity of soluble DP IV rises within days after T-lymphocyte activation *in vitro* (Duke-Cohan *et al.*, 1996), suggesting that serum DP IV activity may be regulated. What impact regulated serum DP IV levels has on the activation or inactivation of peptides *in vivo* is not known. It is also unclear whether the hydrolysis of circulating peptides is mediated primarily by cell bound DP IV, freely circulating enzyme, or both.

Catalytic Mechanism and Inhibition of DP IV

DP IV is a highly selective protease which preferentially hydrolyzes peptides after a penultimate N-terminal proline or alanine residue (Heins *et al.*, 1988). Aromatic or aliphatic amino acids are preferred in the P_2 position (Demuth and Heins, 1995). Both P_1 and P_2 amino acids must be in the L-isomer conformation and the peptide bond between these amino acids must be *trans* for substrate recognition (Fig. 3) (Fischer *et al.*, 1983). A protonated N-terminus is also an absolute requirement for hydrolysis (Demuth and Heins, 1995) which is interfered with by the presence of proline and N-methylated amino acid analogues C-terminal to the scissile bond; there are no other such restrictions (Demuth and Heins, 1995).

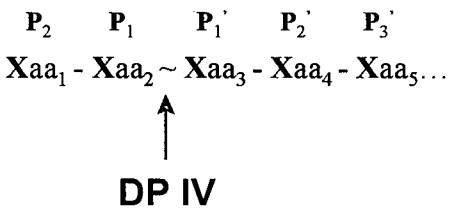

Fig. 3. The catalytic scheme of dipeptidyl peptidase IV (DP IV). DP IV is a proline-specific exopeptidase which hydrolyzes polypeptides after a penultimate proline or alanine residue.

It was reported recently that DP IV shares a conserved series of ~ 200 amino acids with a group of non-classical serine proteases including acylamino-acid hydrolase and prolyl endopeptidase (Marguet *et al.*, 1992). This conserved region contains the catalytic triad of Ser^{624}, Asp^{702}, and His^{734} (mouse sequence) in a unique order as compared to classical serine proteases (Marguet *et al.*, 1992). Substitution of any of these three amino acids with another abolished enzymatic activity, thereby demonstrating their necessity for catalysis (David *et al.*, 1996). Since elucidating a DP IV crystal structure has remained elusive, many insights into the catalytic mechanism of DP IV have had to be derived from studying the interaction between the protease and substances which affect its action. Though some inconsistencies have been described, it is assumed that the catalytic mechanism of DP IV is similar to that of classical serine proteases such as chymotrypsin (York, 1992; Demuth and Heins, 1995). An enzyme-substrate complex forms when an appropriate substrate positions itself into the catalytic pocket of the enzyme. The nucleophilic serine hydroxyl group, formed by protonating the adjacent histidine residue, attacks the carbonyl group of the P_1 amino acid, forming a tetrahedral intermediate. When this unstable intermediate collapses the N-terminally truncated peptide product is released and an acyl enzyme is formed. Water hydrolyzes the acyl-enzyme, forming a second tetrahedral intermediate which collapses and releases the N-terminal dipeptide and reforms

the enzyme. This deacylation step is rate limiting for the entire reaction, provided that a proline is in the P_2-position; this is not so in alanine containing substrates (Demuth and Heins, 1995). The aspartic acid residue is believed to contribute to proper orientation of the histidine residue, thus facilitating the stabilization of transition states. Brandt and colleagues (1996) have recently proposed a new mechanism for DP IV catalysis based on thermodynamic modeling. These investigators suggested that after the formation of the first tetrahedral intermediate, the protonated N-terminus of the substrate can donate a proton to the oxyanion of the intermediate, thus forming a neutral compound. This state is unstable and can continue to react in a manner similar to that described by the classical mechanism, except that the recently proposed mechanism explains the catalytic requirement for a substrate with a non-modified N-terminus (Demuth, 1988).

Inhibition of DP IV Activity

Several strategies for the inhibition of serine proteases, and DP IV in particular, have evolved over the past decades, and four distinct classes of inhibitors have emerged: affinity labels, transition-state analogues, acyl enzyme inhibitors, and enzyme-activated inhibitors (Demuth, 1990). Affinity labels are among the earliest artificial protease inhibitors and refer to compounds which resemble natural substrates but are able to irreversibly modify the enzyme. Peptidyl halomethylketones have been successful in this regard since they result in the irreversible alkylation of the active site histidine (Demuth, 1990). These inhibitors, however, have found little use as endogenous serine protease inhibitors due to their high degree of non-specific alkylation before reaching the target enzyme (Demuth, personal communication). Transition state analogues are substances

which lack a hydrolyzable peptide bond but are recognized by the active site of the enzyme and are susceptible to nucleophilic attack by the active site serine hydroxyl group. Typically, aldehyde, boronic acid, and nitrile derivatives form stable tetrahedral intermediates and thus, have been used to develop transition state analogues. However, Ile-thiazolidide (K_i = 130 nM) and Val-thiazolidide (K_i = 270 nM) belong to this class of inhibitors and are two of the most potent DP IV inhibitors described in the literature to date (Demuth and Heins, 1995). The ring structure of the thiazolidide moiety mimics the structure of proline, the amino acid after which DP IV preferentially hydrolyzes (Yaron and Naider, 1993). The other two inhibitor classes are mechanism-based inhibitors which require activation by the target enzyme and follow one of two schemes: either the inhibitor forms an acyl enzyme whose deacylation reaction is slow compared to an acyl enzyme formed from a natural substrate, or the inhibitor reacts with the enzyme in such a way that a latent chemically reactive intermediate is produced which can interfere with the catalytic triad. Several recent reports describe a series of inhibitors which form acyl enzymes by nucleophilic attack of the catalytic serine to the nitrile carbon atom of aminoacylpyrroline nitriles or the phosphorous atom of diphenyl phosphonate esters, highlighting the ongoing interest in this enzyme inhibition scheme (Boduszek *et al.*, 1994; Li *et al.*, 1995; Lambeir *et al.*, 1996). Diacylhydroxylamines represent a class of inhibitors which release a highly reactive nitrene or isocyanate group when activated by the protease. These reactive intermediates irreversibly bind to the active site and as such have been called *suicide inhibitors* (Yaron and Naider, 1993). Compounds of this type, however, have been demonstrated to be toxic for human lymphocytes at concentrations required to block DP IV activity (Schön *et al.*, 1991). Ongoing research is aimed at addressing this concern as

well as developing more effective compounds specifically targeted at proline specific peptidases.

Biological Role of DP IV

DP IV is present in highest concentrations in the small intestine and kidney where it contributes to the degradation of ingested proteins and the reabsorption of oligopeptides from the glomerular filtrate (Yaron and Naider, 1993; Bauvois, 1995). In fact, DP IV constitutes up to ~ 4 % of renal brush border protein (Yaron and Naider, 1993).

DP IV also acts as a cell adhesion factor by binding both fibronectin and collagen (Piazza *et al.*, 1989; Reutter *et al.*, 1995). The putative binding sites for these cell adhesion proteins are distinct from the active site (Hanski *et al.*, 1988; Piazza *et al.*, 1989) so that DP IV inhibition does not interfere with cell-to-cell and cell-to-extracellular matrix binding. Löster *et al.* (1995) recently described the extracellular cysteine rich domain as the collagen binding site.

Among the most intriguing but least understood functions of DP IV is its role in the immune system. By the early 1990s it was evident that the cell differentiation antigen CD26, located on the surface of T lymphocytes, was, in fact, the protease DP IV (Yaron and Naider, 1993). Later studies demonstrated that the majority of $CD26^+$ cells are also $CD4^+$, and that activation of such cells results in proliferation, differentiation, and an increase in DP IV activity (Hendriks *et al.*, 1991). Thus DP IV serves as a marker for T lymphocyte activation and initiation of memory cell activity (Hafler *et al.*, 1986; Subramanyam *et al.*, 1995). The extent to which DP IV enzyme activity is required for

intracellular signaling in T lymphocytes remains controversial. It has been demonstrated that $CD4^+$ $CD26^-$ cells still respond to mitogens but are unable to elicit helper T lymphocyte functions (Hegen *et al.*, 1993; Brandsch *et al.*, 1995), while other investigators have demonstrated that specific DP IV inhibitors impair mitogen-induced DNA synthesis (Schön *et al.*, 1989). It has been suggested that DP IV activity is not the sole prerequisite for CD26 signaling and that this protein may be co-associated with other integral membrane proteins (Brandsch *et al.*, 1995).

DP IV has also been implicated as a cofactor for the entry of the human immunodeficiency virus-1 (HIV-1) into $CD4^+$ T lymphocytes; however, contradictory observations leave the functional significance in question. Human DP IV was able to promote viral entry into lymphocytes while mouse DP IV was not, and monoclonal anti-DP IV antibodies and DP IV inhibitors were shown to prevent host cell infection (Callabaut *et al.*, 1993; Morimoto and Schlossmann, 1995). However, both $CD26^-$ DP IV^- and mutant $CD26^+$ DP IV^- transfected cells were infected by HIV-1, while wildtype $CD26^+$ DP IV^+ expressing cells were more resistant to viral invasion (Morimoto *et al.*, 1994). The latter results suggest that enzyme activity may actually protect host cells from infection. In fact, the HIV-1 Tat protein has observable DP IV binding properties and is able to partially inhibit DP IV activity (Callabaut *et al.*, 1993; Gutheil *et al.*, 1994; Wrenger *et al.*, 1996). Obviously, more research is required to clarify these issues.

DP IV-Mediated Hydrolysis of Regulatory Peptides

Since a number of prohormone and hormone sequences share an N-terminal X-Pro dipeptide and are thus resistant to proteolytic cleavage by most proteases, DP IV is also

believed to play an important role in the activation or inactivation of these biologically active polypeptides (Mentlein, 1988). Among the potential natural substrates of DP IV are substance P, corticotropin-like intermediate lobe peptide, human α-relaxin, human pancreatic polypeptide, human α-chorionic gonadotropin, prolactin, neuropeptide Y, peptide YY, and β-casomorphin (Mentlein, 1988; Nausch *et al.*, 1990; Wang *et al.*, 1991; Mentlein *et al.*, 1993a). It has been suggested that DP IV-mediated removal of N-terminal dipeptides from biologically active polypeptides need not in itself cause their inactivation, but that this hydrolysis may leave these hormones susceptible to proteolytic cleavage by other exopeptidases (Mentlein, 1988). Even though the Michaelis-Menton constants (K_m) of DP IV catalysis of many potential natural DP IV substrates are reported to be in the micromolar range, supporting the idea that DP IV may be involved in hormone processing *in vivo*; to date, this conclusion is derived exclusively from *in vitro* experiments. Thus, the search for biologically relevant DP IV substrates continues.

In 1986, Frohman and colleagues reported that $GHRH_{1-44}$ was rapidly degraded *in vitro* and *in vivo* to $GHRH_{3-44}$, which was found to have only 10^{-3} times the biological activity of the intact hormone. The enzyme responsible for this inactivation was later identified as DP IV, and it was demonstrated that $GHRH_{1-44}$ analogues resistant to DP IV catalysis possessed prolonged biological activity (Frohman *et al.*, 1989). The relevance of these findings to incretin physiology is that GIP, GLP-1 as well as GHRH belong to the same hormone family sharing the N-terminal X-Ala motif. On the basis of this observation, it was predicted that the gastrointestinal hormones GIP and GLP-1 could also be substrates of DP IV (Mentlein *et al.*, 1993b).

DP IV-Mediated incretin inactivation

It had been noted that intestinal GIP preparations were heterogeneous, containing a minor component comprising up to 20 % of the peptide content (Jörnvall *et al.*, 1981). When a revised sequence of GIP was published by Jörnvall *et al.* in 1981, the identity of the minor component was determined to be GIP_{3-42}. This truncated polypeptide was later shown to be biologically inactive (Brown *et al.*, 1981; Schmidt *et al.*, 1986). Similarly, N-terminally truncated forms of GLP-1 were also demonstrated to be biologically inactive (Suzuki *et al.*, 1989; Gefel *et al.*, 1990). Thus, if GIP and GLP-1 are hydrolyzed by DP IV, this catalysis would result in the loss of their biological activity.

In 1993(b) Mentlein and coworkers investigated the enzymatic degradation of GIP and GLP-1, by purified human placental DP IV using high performance liquid chromatography (HPLC). The K_m for GIP_{1-42} and $GLP\text{-}1_{7-36}$ were determined to be 34 ± 3 and 4.5 ± 0.6 μM respectively. The rate specificity constants (k_{cat}/K_m) were $2.2 \cdot 10^5$ for GIP_{1-42} and $4.3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ for $GLP\text{-}1_{7-36}$ suggesting that DP IV-mediated incretin metabolism at physiological concentrations (picomolar range) could be a significant mechanism of *in vivo* inactivation of these hormones (Mentlein *et al.*, 1993b).

Deacon *et al.* (1995a) confirmed that $GLP\text{-}1_{7-36}$ was degraded by a plasma protease to $GLP\text{-}1_{9-36}$, and that diprotin A, a competitive inhibitor of DP IV was able to prevent this degradation. It was subsequently shown that $GLP\text{-}1_{7-36}$ when administered intravenously or subcutaneously into healthy individuals or patients with Type II diabetes mellitus, was rapidly inactivated *in vivo* (Deacon *et al.*, 1995b). Using a combination of

HPLC, RIA, and enzyme-linked immunosorbent assay (ELISA), these investigators confirmed that *in vivo* degradation of GLP-1_{7-36} yielded the DP IV hydrolysis product.

In an effort to study further the relevance of DP IV-catalysis *in vivo*, Kieffer *et al.* (1995) administered physiological concentrations of intravenous ^{125}I-GIP_{1-42} and ^{125}I-GLP-1_{7-36} into anesthetized rats and monitored the fate of the injected label. HPLC analysis of plasma extracts revealed that over 50 % of both incretins were hydrolyzed into DP IV reaction products in less than 2 min (Kieffer *et al.*, 1995). This biological half-life was considerably shorter than previous estimates determined by radioimmunoassays utilizing C-terminally directed or side-viewing antibodies incapable of distinguishing between the biologically active peptides and their inactive N-terminally truncated metabolites. This has led to an over estimation of biological half-life since imunoreactivity of these peptides is not a true measure of their biological activity.

DP IV-mediated incretin degradation is undoubtedly an important component of GIP and GLP-1 metabolism which requires further study.

Thesis Investigation

The aim of this thesis investigation was twofold: to investigate the role of DP IV in the metabolism of GIP and GLP-1 and to study the effects of DP IV inhibition *in vivo* on the physiology of the enteroinsular axis.

Currently used methods for studying the degradation of biologically active peptides rely on RIA and/or measurement of radioligand metabolites by HPLC. Since these approaches offer only limited information on incretin metabolites, the study outlined in

Chapter 1 of this thesis was designed to use Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) to investigate incretin degradation in human serum, and study the kinetics of GIP and GLP-1 hydrolysis by human serum and purified porcine kidney DP IV. Since MALDI-TOF MS is tolerant of heterogeneous samples (containing buffers, salts, and contaminants) this technology is ideally suited for analysis of biological fluids such as serum. The accuracy of the instrumentation is such that all analyte metabolites can be accurately resolved on the basis of their mass-to-charge ratio (*m/z*), overcoming a significant limitation of other approaches. The importance of DP IV-mediated incretin degradation was assessed by monitoring the hydrolysis of intact GIP and GLP-1 and the formation and identity of metabolite appearance.

The study described in Chapter 2 was designed to investigate the physiological implications of DP IV inhibition on the enteroinsular axis. A protocol for the inhibition of endogenous DP IV in the anaethetized rat was developed using Ile-thiazolidide, a highly specific reversible competitive transition state analogue inhibitor of DP IV (K_i = 130 nM) (Schön *et al.*, 1991; Demuth and Heins, 1995). The availability of this inhibitor allowed the investigation of endogenous DP IV inhibition on exogenously administered radiolabeled GLP-$1_{7\text{-}36}$ as well as the effect on insulin secretion and glucose clearance in response to a glucose challenge. It was hypothesized that inhibition of DP IV increases the circulation time of biologically active incretins yielding a more rapid return to normoglycemia after a glucose challenge.

CHAPTER 1: *In vitro* DEGRADATION OF GIP AND GLP-1

Project Rationale

Enzymatic degradation of GIP and GLP-1 is undoubtedly an important first step in the metabolism of these hormones in the circulation. However, RIA and HPLC offer only limited information on incretin metabolites in serum. The present study was designed to investigate serum degradation of GIP and GLP-1 by establishing MALDI-TOF MS protocols to characterize the importance of serum DP IV in incretin metabolism and to investigate the kinetics of GIP and GLP-1 hydrolysis by DP IV, thereby introducing a novel application of MALDI-TOF MS: the study of enzyme kinetics.

Methodological Background

The present study investigates serum degradation of GIP and GLP-1, and clarifies the role of DP IV in the breakdown of these hormones. Protocols were developed to apply MALDI-TOF MS to the qualitative and quantitative analysis of incretin metabolism.

Mass spectrometry is an analytical tool able to differentiate accurately between components of an analyte solution on the basis of their mass to charge ratio (*m/z*). With the introduction of ElectroSpray Ionization (ESI) (Karas and Hillenkamp, 1988; Tanaka *et al.*, 1988) and MALDI (Yamashita and Fenn, 1984) as soft ionization methods which greatly decrease the fragmentation of fragile biomolecules, mass spectrometry has become an important tool in biological research. Subsequent to the development of these

techniques, mass spectrometry has been used to analyze a wide range of substances including polypeptides, proteins, oligonucleotides, polysaccharides, and other bio-organic compounds. An important feature of mass spectrometry is high sensitivity which allows detection of picomole to femtomole amounts of test substance. MALDI MS has a critical advantage over ESI MS in that it tolerates heterogeneous samples (including salts and buffers) and is thus well suited for direct analysis of biological solutions. ESI MS analysis dictates that samples are first purified by HPLC. Thus, MALDI-TOF MS was the method of choice in this study, where serum-incubated samples were analyzed by MS.

A linear MALDI-TOF mass spectrometer functions on the basis that a laser beam ionizes a matrix-embedded analyte molecule and allows it to desorb from the probe tip (Zaluzec *et al.*, 1995). This ionized particle is accelerated through an electric field before entering a field-free flight tube where its velocity remains constant; the time required to traverse this region can be measured and is a function of *m/z* (Zaluzec *et al.*, 1995). Such a system has been used to detect molecules in excess of 300 kDa with an accuracy of 0.1 to 0.01 % (Siuzdak, 1994).

In 1993 Chait and colleagues introduced a new approach for protein sequencing using MALDI-TOF MS (Chait *et al.*, 1993). Cycles of stepwise degradation with a small amount of terminating agent resulted in a protein ladder (similar to a Sanger DNA sequencing ladder) which was analyzed by mass spectrometry. Mass differences between successive peaks corresponded to specific amino acids. Similarly, acid hydrolysis followed by mass spectrometry has been used to determine the amino acid sequence of polypeptides up to a mass of ~3000 Da (Vorm and Roepstorff, 1994). Biochemists have also used

MALDI MS to generate *fingerprints* of large genomic proteins allowing them to be identified by comparison to a data base of known sequences (James *et al.*, 1993).

Efforts have recently been made to apply mass spectrometry to quantitative as well as qualitative analysis. Tang *et al.* (1993) reported that absolute quantification of peak area or height of biomolecules using MALDI-TOF MS is difficult due to the limitations imposed by poor signal reproducibility, and results in a nonlinear relationship between signal and quantity of analyte. Normalizing the MS signals by using an internal standard having similar chemical properties as the analyte, eliminated the effects of variable laser beam consistancy and sample preparation, resulting in linear relationships between analyte signal and quantity (Tang *et al.*, 1993; Duncan *et al.*, 1993).

Craig *et al.* (1994) employed MALDI-TOF MS to monitor peptide phosphorylation and dephosphorylation, without utilizing an internal standard for quantification. The relative amount of phosphorylated peptide was calculated as the peak intensity of the phosphorylated peptide divided by the sum of the peak intensities for both phosphorylated and dephosphorylated peptides. Since peaks were quantified relative to the total intensity of analyte signals of the same preparation exposed to identical laser beam condition and crystallization, an internal standard was not necessary.

Though mass spectrometry has been extensively used for analyzing protein degradation products, Hsieh and colleagues (1995) combined quantitative mass spectrometry with enzymatic degradation to demonstrate the feasibility of studying enzyme kinetics in real time using HPLC-coupled ESI MS. Kinetic constants (K_m and V_{max}) for RNase A and β-galactosidase-mediated hydrolysis of cytidylyl 3'-5'-guanosine and lactose respectively, were in good agreement with those determined using

conventional approaches to kinetic analysis. Classical methods for investigating enzyme kinetics such as refractive index monitoring, RIA, or HPLC are time consuming, use large amounts of substrate, and are often insensitive. In the case of colorimetric assays, chromogenic substrates must often be synthesized, and many of these do not adequately parallel the kinetics of the substrate they were designed to mimic. Mass spectrometry offers a rapid, accurate and easy approach to study enzyme kinetics. This is especially relevant for analyzing large biomolecules such as proteins.

Introduced here is the use of MALDI-TOF MS as an analytical tool to study the kinetics of DP IV-catalyzed hydrolysis of the insulin-releasing hormones GIP and GLP-1, and for quantitatively assessing the role of DP IV in serum metabolism of these hormones.

Experimental Procedures

Instrumentation and General Procedures

Matrix-assisted laser desorption/ionization mass spectrometry was carried out using a Hewlett-Packard G2025 mass spectrometer with a linear time of flight analyzer. The instrument was equipped with a 337 nm nitrogen laser, a high-potential acceleration source (5 kV) and a 1.0 m flight tube. Detector operation was in the positive-ion mode and signals were recorded and filtered using a LeCroy 9350 M digital storage oscilloscope linked to a personal computer. The spectrometer was externally calibrated using the Hewlett-Packard low molecular weight standard (G2051A).

The DP IV used in this study was purified from porcine kidney according to a previously described method (Wolf *et al.*, 1978). The specific activity measured using H-Gly-Pro-4-nitroanalide as a chromogenic substrate, was 45 $U \cdot mg^{-1}$.

To obtain mass spectra of GIP_{1-42} (Peninsula) and GLP-1_{7-36} (Bachem), in the presence or absence of DP IV, substrate was incubated at 30 °C with 0.1 mM TRICINE buffer pH 7.6 and either enzyme or water in a 2:2:1 ratio. Samples (4 µl) of the incubation mixture were removed at various time intervals and mixed with equal volumes of 2',6'-dihydroxyacetophenone as matrix solution (Aldrich). A small volume (< 1 µl) of this mixture was transferred to a probe tip and immediately evaporated in a vacuum chamber (Hewlett-Packard G2024A sample prep accessory) to ensure rapid and homogeneous sample crystallization. All spectra were obtained by accumulating data generated by 250 single shots with laser power between 1.5 and 4.5 µJ.

Dependence of MALDI-TOF MS Signal on the Concentration of GIP and GLP-1

Various concentrations of synthetic porcine GIP_{1-42} (Peninsula) and synthetic human GLP-1_{7-36} (Bachem) were mixed with buffer and water as described above, and 1 µl samples ranging from 0.5 to 6 pmol/sample of GIP_{1-42} and 3.75 to 10 pmol/sample GLP-1_{7-36} were analyzed by MS in order to determine the relationship between concentration of hormone versus MS signal intensity. Spectra for each peptide concentration were generated in triplicate. Quantification of GIP_{1-42} and GLP-1_{7-36} signals was accomplished by dividing the peak intensity by the baseline intensity resulting in a signal intensity normalized to spectra baselines.

Monitoring *in vitro* Degradation of GIP and GLP-1 by DP IV using MALDI-TOF MS

Incubation of Peptide with Purified Porcine Kidney DP IV.

To study the hydrolysis of GIP_{1-42} (5 μM) and GLP-1_{7-36} (15 μM) by DP IV, peptides were incubated in buffer and enzyme (0.58 nM for GIP_{1-42} incubations and 2.9 nM for GLP-1_{7-36} incubations) under the aforementioned standard conditions. Samples of GIP_{1-42} (2.5 pmol) and GLP-1_{7-36} samples (7.5 pmol) were removed from the incubation mixture at 4, 9 and 16 min and prepared for MS analysis as described above.

Incubation of Peptide in Human Serum.

In order to study proteolytic degradation of GIP_{1-42} (30 μM) and GLP-1_{7-36} (30 μM) in serum, peptides were incubated in buffer containing 40 % human serum under standard conditions. Serum was pooled from three individuals and obtained from the Medical Science Division (courtesy of Dr. S. Heins, Department of Child Diseases), Martin-Luther University, Halle-Wittenberg, Germany. Samples of the respective peptide (15 pmol) were removed from the incubation mixture at hourly intervals for 15 h and analyzed using the MS.

Kinetic Analysis of DP IV-mediated GIP and GLP-1 Hydrolysis using MALDI-TOF MS

Hydrolysis with Varying Concentrations of DP IV.

In order to determine the feasibility of studying the time dependence of an enzymatic reaction using MALDI-TOF MS and to establish a convenient DP IV concentration for subsequent kinetic analysis, GIP_{1-42} (5 μM) and GLP-1_{7-36} (15 μM) were incubated under standard conditions with varying concentrations of purified DP IV (ranging from 0.29 to 5.8 nM for GIP_{1-42} incubations and from 1.5 to 12 nM for GLP-1_{7-36}). Samples were removed at various time intervals after the start of the reaction and analyzed by MS. The relative amounts of GIP_{1-42} and GLP-1_{7-36} were calculated from net substrate peak intensity divided by the sum of the net substrate and net product peak intensities, and plotted versus time. Net peak height was defined as peak intensity minus baseline intensity. Before transferring to the probe for MS analysis, these samples were diluted so that the final amount of peptide on the probe tip was 2.5 pmol for GIP_{1-42} metabolites and 7.5 pmol for GLP-1_{7-36} metabolites. The linearity between rate of hydrolysis and enzymatic concentration was determined from a plot of the initial slopes of substrate turnover (μmol/l/min) versus enzyme concentration.

Determination of Kinetic Constants.

The kinetic constants of DP IV-catalyzed GIP_{1-42} and GLP-1_{7-36} hydrolysis were determined by introducing a specific and kinetically characterized DP IV inhibitor into the incubation mixture and observing the relative reaction rates of inhibited and uninhibited substrate hydrolysis as described by Crawford *et al.* (1988). GIP_{1-42} (20 μM) and GLP-1_{7-}

$_{36}$ (30 μM) were incubated with DP IV (0.59 and 2.9 nM respectively) under standard conditions, in the presence or absence of either Ala-thiazolidide (20 μM - K_i of 3.4 μM) or Ile-thiazolidide (20 μM - K_i of 0.126 μM). Both are specific, competitive inhibitors of DP IV synthesized in the laboratory of Dr. Hans-Ulrich Demuth at the Hans-Knöll Institute of Natural Products Research in Halle, Germany (Demuth, 1990). Similarly, GIP_{1-42} (30 μM) and GLP-1_{7-36} (30 μM) were incubated with 20 % human serum in the presence or absence of inhibitors. Samples were appropriately diluted and assayed by MS. Quantification of relative amounts of substrate after various time intervals was calculated as described in the previous section. The initial slopes of peptide turnover with purified DP IV or human serum DP IV activity in the presence and absence of inhibitors were used to calculate reaction velocities. The K_m of DP IV-catalyzed peptide hydrolysis was calculated according to the equation:

$$Km = \frac{\left(vo/vi\right)[S] - [S]}{1 + \left([I]/Ki\right) + \left(vo/vi\right)}$$

where v_o and v_i are the uninhibited and inhibited relative reaction rates respectively, [S] is the substrate concentration, [I] is the inhibitor concentration and K_i is the inhibition binding constant. V_{max} was then calculated according to the equation:

$$V\,max = v/[S]\left(K\,m + [S]\right)$$

Values for k_{cat} were calculated using M = 110 kDa per catalytically active subunit as the molar mass of DP IV.

To estimate these kinetic constants for serum DP IV activity it was necessary to determine the concentration of purified DP IV equivalent to human serum DP IV activity. A standard curve of DP IV activity versus DP IV concentration was generated by incubating 50 µl of various DP IV concentrations (ranging from 29.3 to 293 pM) in 0.04 M HEPES buffer pH 7.6 at 30 °C and monitoring the rate of H-Gly-Pro-4-nitroanalide hydrolysis. Data acquisition was carried out using a Kontron 930 Uvicon *uv-vis* spectrophotometer at 390 nm (ε = 11 500 $M^{-1} \cdot cm^{-1}$) equipped with thermostated cells. An equivalent volume of serum was assayed under identical conditions allowing the purified DP IV concentration equivalence of serum DP IV activity to be determined using the standard curve. Although considerable controversy still surrounds the exact nature of serum DP IV, it was assumed that the major serum DP IV iso-enzyme has M = 110 kDa per catalytically active subunit. For the sake of simplicity, all calculations were made using this, the most accepted molecular weight of DP IV.

Confirmation of MS-derived K_m Values using a Spectrophotometric Competition Assay

To confirm the kinetic constants determined using MALDI-TOF MS, the inhibition constant of GIP_{1-42} as a competitive effector of the DP IV-catalyzed hydrolysis of a chromogenic substrate was determined spectrophotometrically. Three concentrations of H-Gly-Pro-4-nitroanilide, corresponding to $K_m/2$, K_m and $2K_m$ ($5.0 \cdot 10^{-5}$, $1.0 \cdot 10^{-4}$ and

$2.0 \cdot 10^{-4}$ M) were incubated in 0.04 HEPES buffer pH 7.6 at 30 °C in the presence of a range of GIP_{1-42} concentrations ($1.0 \cdot 10^{-7}$ to $1.0 \cdot 10^{-5}$ M). Hydrolysis of the chromogenic substrate was monitored using the Kontron 930 Uvicon *uv-vis* spectrophotometer as outlined above. Data were analyzed using nonlinear regression (Graphfit 3.01) yielding an inhibition binding constant (K_i) for GIP_{1-42}. Since GIP_{1-42} is simultaneously an inhibitor to DP IV-catalyzed H-Gly-Pro-4-nitroanalide hydrolysis, as well as a substrate of DP IV, this inhibition binding constant should be an approximation to the K_m for DP IV-catalyzed GIP_{1-42} hydrolysis.

Results

GIP and GLP-1 Concentration Dependence of MS Signal Intensities

Polypeptide concentration was plotted versus GIP_{1-42} and GLP-1_{7-36} signal intensities normalized to spectra baselines (Fig. 4). This simple approach resulted in graphs indicating the concentration range of polypeptide during which signal intensity increased with increasing concentration of substance without the necessity of internal standards. By knowing this unique concentration *window*, bound by the limit of detection versus the highest normalized signal intensity, the optimum analyte to matrix ratio for subsequent sample dilution, was chosen. The molar GIP_{1-42}:matrix ratio was optimum at $2.5{:}10^5$, while the GLP-1_{7-36} to matrix optimum was $7.5{:}10^5$.

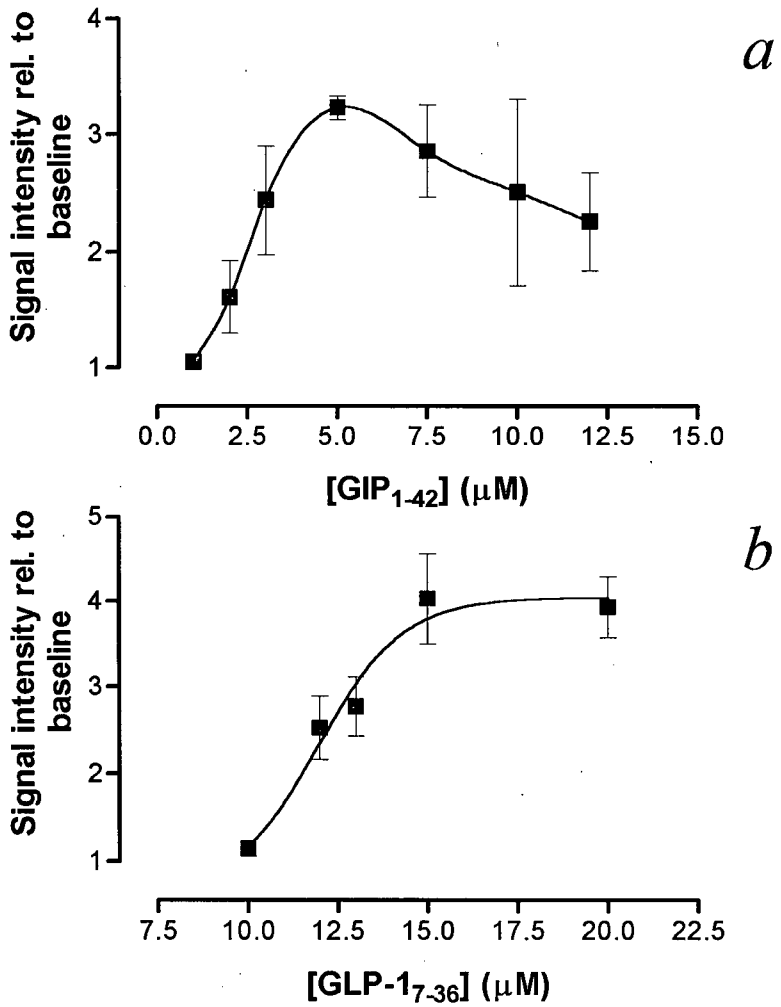

Fig. 4. Concentration dependence of MS signal intensity. (*a*) GIP_{1-42} concentrations ranging from 1 to 12 μM correspond to 0.5 to 6 pmol per MS analysis, while (*b*) GLP-1_{7-36} concentrations ranging from 7.5 to 20 μM correspond to 3.75 to 10 pmol. Spectra were collected and analyzed as outlined in the Experimental Procedures. Data are presented as mean signal intensity relative to spectrum baseline ± s.e.m. (n = 3).

In vitro Degradation of GIP and GLP-1 by DP IV

Fig. 5 shows the MS spectra of GIP_{1-42} and GLP-1_{7-36} and their DP IV reaction products at various time intervals during incubation with purified DP IV. The relative heights of the substrate signal (GIP_{1-42} and GLP-1_{7-36}) decreased as the relative heights of the peaks corresponding to the DP IV hydrolysis products (GIP_{3-42} and GLP-1_{9-36}) increased. The average *m/z* of GIP_{1-42} and GIP_{3-42} were 4980.1 and 4745.2 representing

an error of 0.09 and 0.10 % relative to $[M+H]^+_{calc.}$. The error between $[M+H]^+_{calc.}$ and $[M+H]^+_{exp.}$ for GLP-1_{7-36} and GLP-1_{9-36} was 0.05 and 0.06 % respectively.

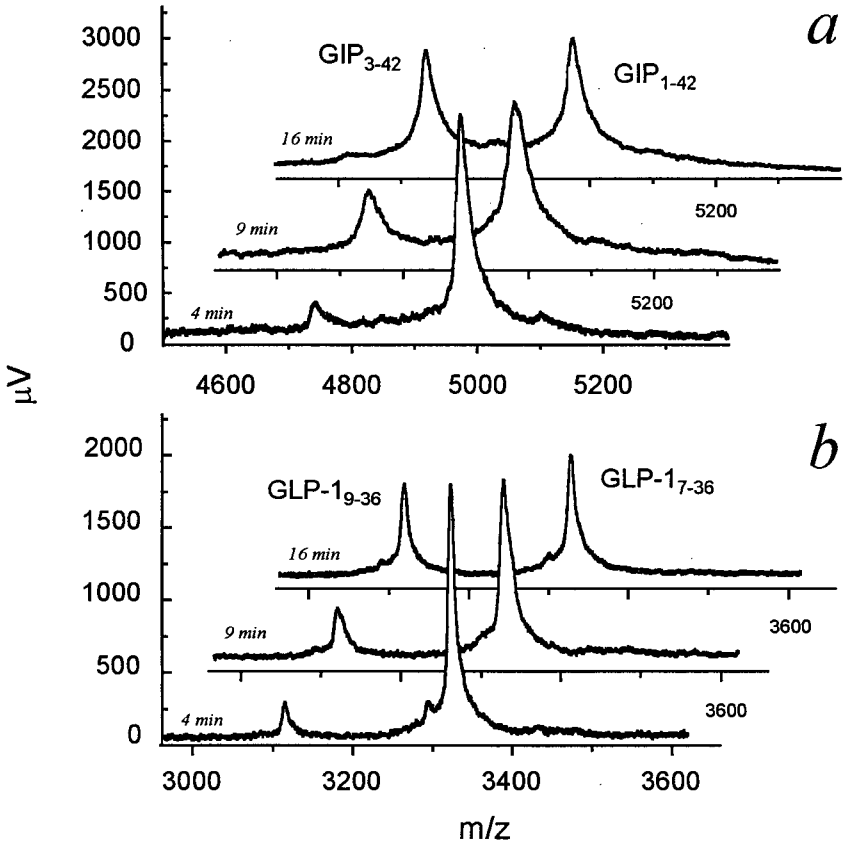

Fig. 5. MALDI-TOF MS analysis of DP IV-catalyzed GIP_{1-42} and GLP-1_{7-36} hydrolysis. GIP_{1-42} (5 μM) and GLP-1_{7-36} (15 μM) were incubated with purified porcine kidney DP IV (0.58 and 2.9 nM respectively). Samples of GIP_{1-42} metabolites (2.5 pmol) and GLP-1_{7-36} metabolites (7.5 pmol) were removed from the incubation mixture at 4, 9 and 16 min. Analyte was immediately crystallized and analyzed by MS. Samples were treated as per Experimental Procedures. (*a*) Signals in the range *m/z* 4980.1 ± 5.3 correspond to GIP_{1-42} (*M* 4975.6) while *m/z* 4745.2 ± 5.5 correspond to GIP_{3-42} (*M* 4740.4). (*b*) Peaks of *m/z* 3325.0 ± 1.2 correspond to GLP-1_{7-36} (*M* 3297.7) and *m/z* 3116.7 ± 1.3 correspond to GLP-1_{9-36} (*M* 3089.6). The mass differences between *m/z* and *M* are attributed to an estified glutamate residue in the GLP-1_{7-36} molecule, adding a mass of 29 Da (Table 1).

In order to gain insight into the identity of the major metabolites found in the circulation, GIP_{1-42} and GLP-1_{7-36} were incubated in human serum. The MS spectra generated at hourly intervals are shown in Fig. 6. Metabolites were identified on the basis of their *m/z* ratio. Table 1 summarizes the $[M+H]^+_{exp.}$ versus the $[M+H]^+_{calc.}$ of possible

metabolite sequences. Indistinct minor peaks were not considered for analysis nor were sequences where the errors between $[M+H]^+_{exp.}$ and $[M+H]^+_{calc.}$ were > 0.20 %.

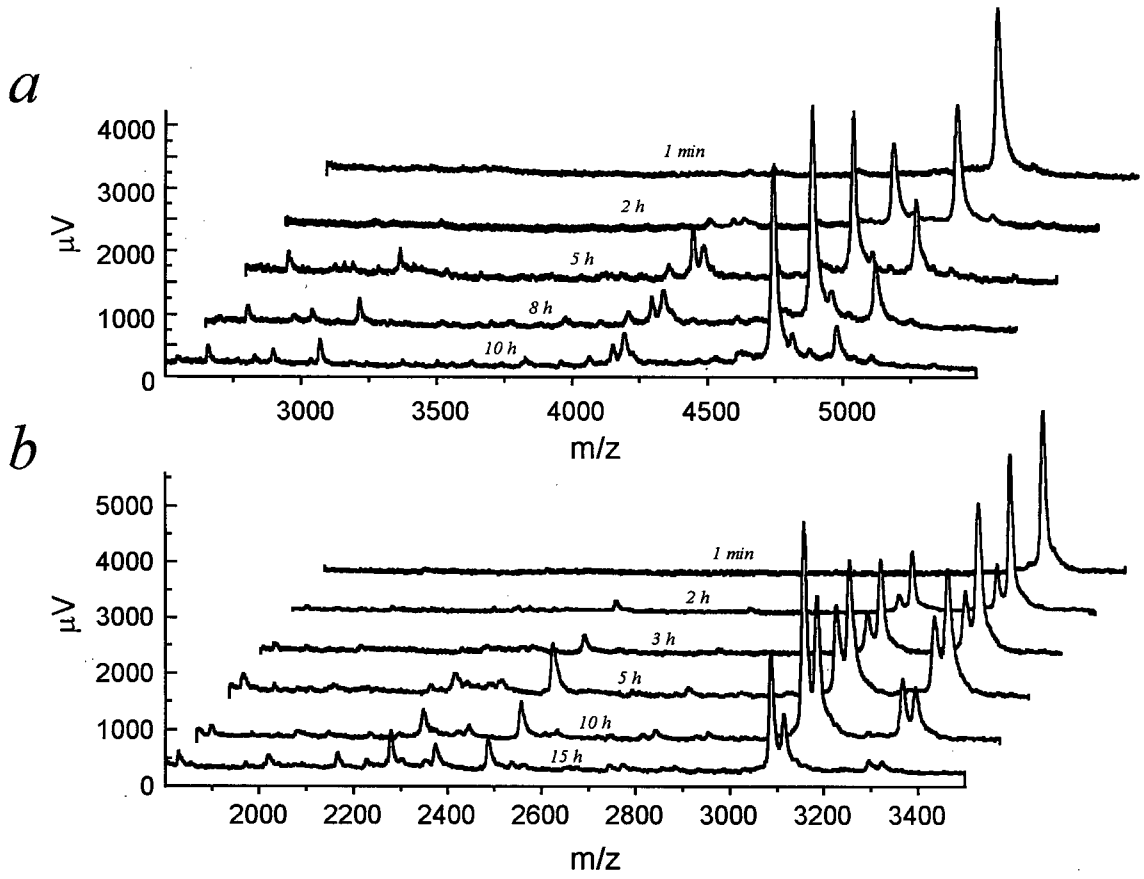

Fig. 6. MALDI-TOF MS analysis of GIP_{1-42} and GLP-1_{7-36} degradation in serum. (*a*) GIP_{1-42} (30 μM) and (*b*) GLP-1_{7-36} (30 μM) were incubated in 20 % human serum as described in the Experimental Procedures. Samples (15 pmol) were removed at hourly intervals and analyzed by MALDI-TOF MS as previously outlined.

Over 15 h, serum-incubated GIP_{1-42} showed a consistently gradual decrease in the relative peak height of the intact peptide with a complementary increase in the relative peak height of a degradation product having *m/z* corresponding to GIP_{3-42}. Only after approximately 3 h, by which time more that half of the GIP_{1-42} was already converted to GIP_{3-42}, were minor peaks due to secondary stepwise degradation by other serum proteases observed. These results support the hypothesis that DP IV is the primary serum protease acting on GIP.

Similarly, serum-incubated GLP-1_{7-36} was degraded by serum DP IV activity to GLP-1_{9-36}. The serum degradation spectra for GLP-1_{7-36} at different time periods are illustrated in Fig. 6b. and show doublet peaks for both GLP-1_{7-36} and GLP-1_{9-36}. The *m/z* difference between these doublets was consistently 29, a mass corresponding to an ethyl group most likely attached as a protecting group to a glutamate residue during peptide synthesis of the commercial product. As the incubation time increased, the heights of the $[M+H]^+$+*ethyl ester* decreased relative to the height of the corresponding $[M+H]^+$. This suggests that non-specific serum esterases remove the ethyl group over time. Parallel studies of GLP-1_{7-36}, using the same commercially available substance, with purified DP IV did not result in doublet peaks, but only $[M+H]^+$+*ethyl ester* peaks (Fig. 5). Presumably this occurs because the purified enzyme preparation is free of contaminating non-specific esterases.

Table 1. GIP$_{1-42}$ and GLP-1$_{7-36}$ degradation products of serum protease activity. GIP$_{1-42}$ (30 μM) and GLP-1$_{7-36}$ (30 μM) were incubated with 20 % human serum in 0.1 mM TRICINE buffer pH 7.6 at 30 °C for 10 and 15 h respectively. MALDI-TOF MS analysis after this incubation period showed serum degradation products identified on the basis of their *m/z*. The peptide sequence plus cation adducts are indicated where observed. Sequences with the additional -CH_2CH_3 on a glutamate residue (found in the commercial GLP-1$_{7-36}$) are also identified. Where more than one possible sequence of similar *m/z* is possible, alternatives are given.

GIP$_{1-42}$ degradation (10 h)				**GLP-1$_{7-36}$ degradation (15 h)**			
$[M+H]^+_{exp.}$ m/z	Sequence	$[M+H]^+_{calc.}$ m/z	Difference %	$[M+H]^+_{exp.}$ m/z	Sequence	$[M+H]^+_{calc.}$ m/z	Difference %
4975.3	1-42	4975.5	0.00	3323.7	(7-36) + CH_2CH_3	3326.7	-0.09
4872.4	(1-41) + Na	4869.4	0.06	3296.1	7-36	3297.7	-0.05
4809.8	2-42	4811.4	-0.03	3115.2	(9-36) + CH_2CH_3	3118.6	-0.11
4740.9	1-40	4745.4	-0.09	3087.9	9-36	3089.6	-0.06
	3-42	4740.4	0.01	2884.3	9-34	2879.4	0.17
4527.2	1-38	4518.3	0.20		(7-32) + CH_2CH_3	2886.4	-0.07
4462.8	2-39	4469.2	-0.14	2855.7	7-32	2857.4	-0.06
4192.4	8-42	4193.1	-0.02	2771.0	(7-31) + CH_2CH_3	2773.3	-0.08
4149.7	3-37	4147.1	0.06		(10-34) + CH_2CH_3	2776.4	-0.19
	4-38	4155.1	-0.13		(11-35) + CH_2CH_3	2776.4	-0.19
4062.0	1-35	4067.0	-0.12	2743.8	7-31	2744.3	-0.02
	8-41	4065.1	-0.08		9-33	2748.3	-0.16
3955.5	1-34	3952.0	0.09		10-34	2747.4	-0.13
	5-37	3961.0	-0.14		11-35	2747.4	-0.16

GIP_{1-42} degradation (10 h)				GLP-1_{7-36} degradation (15 h)			
$[M+H]^+_{exp.}$	Sequence	$[M+H]^+_{calc.}$	Difference	$[M+H]^+_{exp.}$	Sequence	$[M+H]^+_{calc.}$	Difference
3824.6	11-42	3828.0	-0.09	2562.3	7.30	2558.2	0.16
3740.6	1-32	3736.9	0.10		11-33	2562.3	0.00
	8-38	3736.9	0.10		(9-31) + CH_2CH_3	2565.2	-0.11
	12-42	3741.0	-0.01	2534.9	9-31	2536.2	-0.05
3629.6	3-33	3630.9	-0.04	2487.2	7-29	2487.2	0.00
	13-42	3627.9	0.05	2375.0	7-28	2374.1	0.04
3560.2	14-42	3556.9	0.09		(8-29) + CH_2CH_3	2379.1	-0.17
3502.9	3-32	3502.8	0.00		(9-30) + CH_2CH_3	2379.1	-0.17
	4-33	3501.8	0.03		(11-31) + CH_2CH_3	2379.1	-0.17
	13-41	3499.8	0.09	2353.4	8-29	2350.1	0.14
3421.2	1-29	3423.7	-0.07		9-30	2350.1	0.14
	15-42	3425.8	-0.13		11-31	2350.1	0.14
3373.2	3-31	3374.7	-0.04		16-36	2352.3	0.05
	4-32	3373.7	-0.01	2305.8	(9-29) + CH_2CH_3	2308.6	-0.12
	11-38	3371.8	0.04	2279.5	9-29	2279.6	0.00
3069.0	7-32	3067.6	0.05	2226.1	7-27	2227.0	-0.04
	18-42	3068.6	0.01	2166.3	9-28	2166.0	0.01
2896.1	6-29	2901.5	-0.19		11-30	2164.0	0.11
2827.5	8-31	2826.4	0.04		18-36	2166.2	0.00
	12-35	2832.5	-0.18	2019.2	9-27	2018.9	0.01
	15-37	2831.5	-0.14	1971.0	7-25	1969.9	0.06
2657.2	20-41	2656.4	0.03	1828.5	7-23	1826.8	0.09
	21-42	2656.4	0.03		8-25	1831.8	-0.18
2544.4	22-42	2441.1	0.12		11-27	1831.9	-0.19
					18-33	1825.0	0.19

Kinetic Analysis using MALDI-TOF MS

Under normal circumstances increasing the concentration of an enzyme while maintaining a constant substrate concentration results in an increased rate of product formation. Fig. 7 illustrates that MALDI-TOF MS analysis of DP IV-catalyzed GIP_{1-42} and GLP-1_{7-36} hydrolysis can be used to demonstrate this relationship. Peptide turnover varies linearly with increasing concentrations of DP IV (Fig. 7 inset; r^2 = 0.9986 and 0.9849 for GIP_{1-42} and GLP-1_{7-36} hydrolysis respectively).

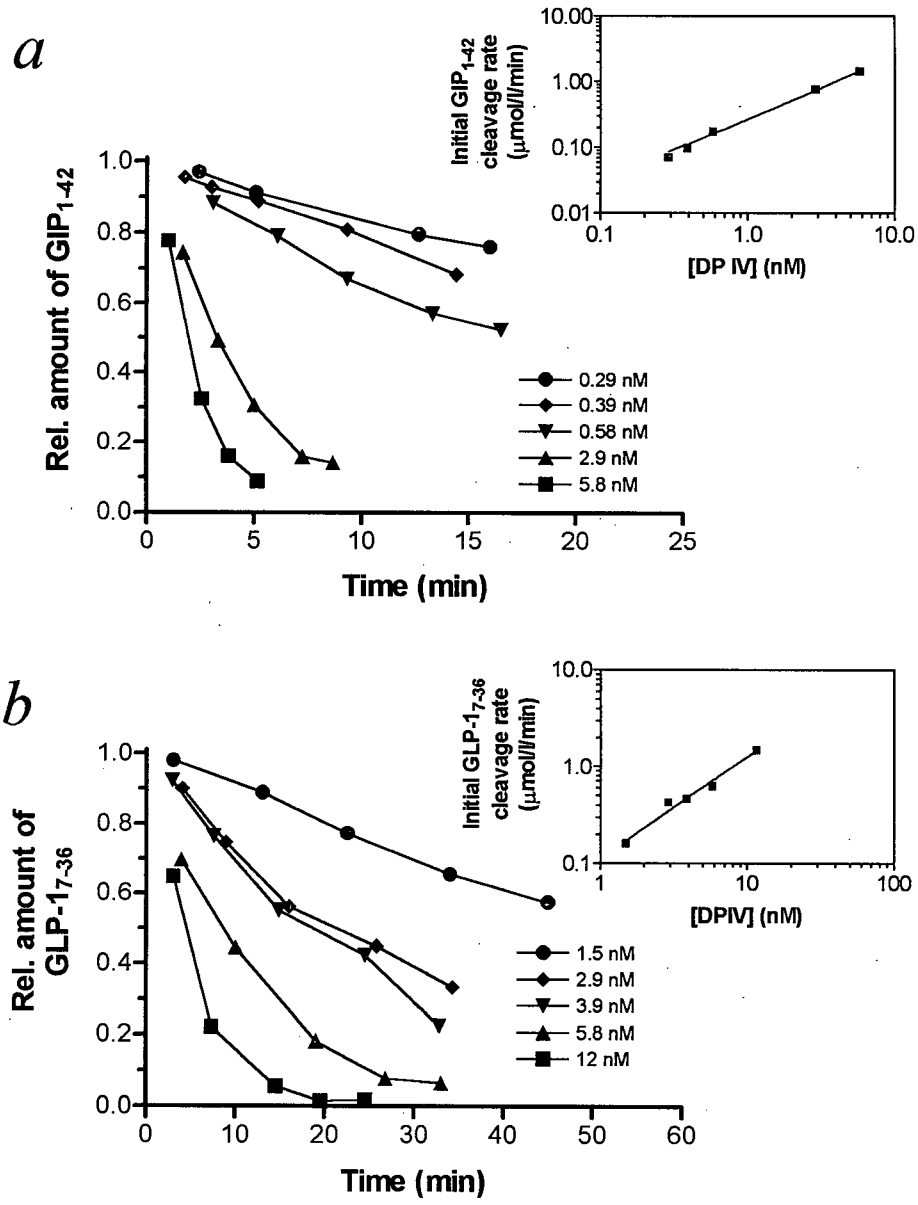

Fig. 7. Quantitative MALDI-TOF MS of DP IV-catalyzed GIP_{1-42} and GLP-1_{7-36} hydrolysis. (*a*) GIP_{1-42} (5 µM) and (*b*) GLP-1_{7-36} (15 µM) were incubated in various concentrations of purified porcine kidney DP IV. Samples of analyte (2.5 pmol GIP and 7.5 pmol GLP-1) were removed from the incubation mixture for MS analysis. Spectrum peaks were quantified as outlined in the Experimental Procedures and the relative amount of substrate determined as a fraction of remaining substrate plus product. The insets shows the linearity of the initial rate of hydrolysis for GIP_{1-42} ($y = 0.2468x + 0.0150$; $r^2 = 0.9986$) and GLP-1_{7-36} ($y = 0.1259x + 0.0196$; $r^2 = 0.9849$).

MALDI-TOF MS was used to demonstrate that GIP_{1-42} and GLP-1_{7-36} turnover was attenuated by Ala-thiazolidide and Ile-thiazolidide inhibition of purified DP IV and serum DP IV as predicted by the inhibitor binding constants (K_i) (Fig. 8). These results lend more credibility to MALDI-TOF MS as a feasible method for quantitative kinetic

analysis, as well as allowing the K_m values for purified porcine kidney-catalyzed GIP_{1-42} and GLP-1_{7-36} hydrolysis to be calculated. These results are summarized in Table 2 and where appropriate, expressed as a range derived from the two inhibitors.

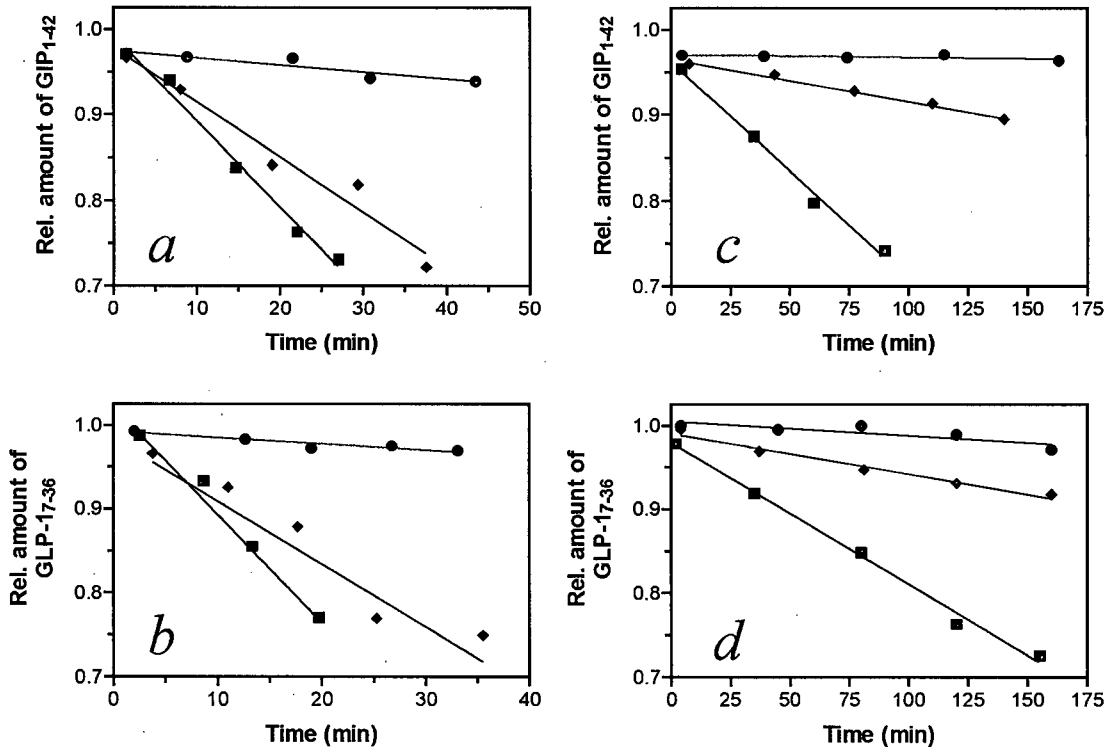

Fig. 8. Quantitative MALDI-TOF MS for kinetic analysis of DP IV-catalyzed GIP_{1-42} and GLP-1_{7-36} hydrolysis in the presence of specific DP IV inhibitors. (*a*, *b*) GIP_{1-42} (20 μM) and GLP-1_{7-36} (30 μM) were incubated with purified porcine kidney DP IV (0.59 and 2.9 nM respectively) in the presence or absence (▪) of alanine-thiazolidide (20 μM) (♦) or isoleucine-thiazolidide (20 μM) (•), two specific, reversible inhibitors of DP IV. (*c*, *d*) GIP_{1-42} and GLP-1_{7-36} (30 μM for both) were also incubated in 20 % human serum. Spectrum peaks were quantitatively analyzed as outlined in the Experimental Procedures.

Serum DP IV was determined to have the equivalent activity of $1.3 \cdot 10^{-5}$ mg·ml^{-1} of purified porcine kidney DP IV, as measured by the rate of H-Gly-Pro-4-nitroanalide hydrolysis using the standard curve in Fig. 9.

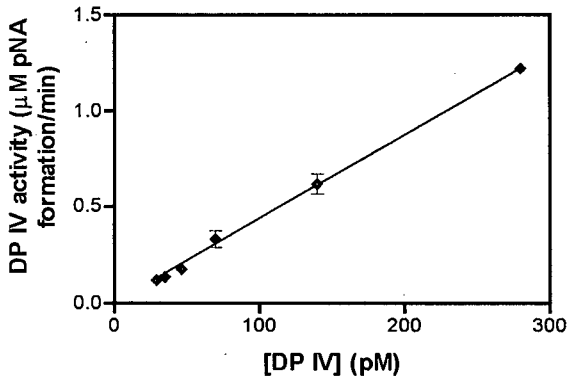

Fig. 9. Standard Curve for matching human serum DP IV activity with purified porcine kidney DP IV activity. The rate of hydrolysis of Gly-Pro-4-nitroanalide (0.4 mM) versus serially diluted purified porcine kidney DP IV was measured spectrophotometrically in 0.04 M HEPES buffer pH 7.6 at 30 °C ($y = 0.004376x$; $r^2 = 0.9979$).

The kinetic constants (k_{cat}) for GIP_{1-42} and $GLP\text{-}1_{7-36}$ hydrolysis by serum DP IV activity were calculated and compared in Table 2.

Table 2. Kinetic constants for the degradation of GIP_{1-42} and $GLP\text{-}1_{7-36}$ by DP IV as determined by quantitative MALDI-TOF MS. All assays were carried out in 0.1 mM TRICINE buffer pH 7.6 at 30 °C. K_m values were calculated using the Michaelis-Menten equation for competitive inhibition from the substrate/inhibitor/DP IV incubation experiments (Fig. 5.). Ala-thiazolidide has a K_i of 3.4 mM and Ile-thiazolidide has a K_i of 0.126 mM. Values for k_{cat} were calculated using M = 110 kDa as the molar mass of DP IV. Where appropriate, results are expressed as the range of the results obtained using the two inhibitors.

Peptide	DP IV source	K_m μM	V_{max} μmol·min^{-1}·mg^{-1}	k_{cat} s^{-1}	k_{cat}/K_m M^{-1}·s^{-1}	Reference
GIP_{1-42}	porcine kidney	1.8 ± 0.3*	13.6 ± 0.2	23	$13 \cdot 10^6$	this study
GIP_{1-42}	human serum	39 ± 29*	27 ± 12	22	$0.56 \cdot 10^6$	this study
GIP_{1-42}	human placenta	34 ± 3	3.8 ± 0.2	7.6	$0.22 \cdot 10^6$	Mentlein *et al.*, 1993b
$GLP\text{-}1_{7-36}$	porcine kidney	3.8 ± 0.3*	5.45 ± 0.05	9	$2.3 \cdot 10^6$	this study
$GLP\text{-}1_{7-36}$	human serum	13 ± 9*	11 ± 2	14	$1.1 \cdot 10^6$	this study
$GLP\text{-}1_{7-36}$	human placenta	4.5 ± 0.6	0.97 ± 0.05	1.9	$0.43 \cdot 10^6$	Mentlein *et al.*, 1993b

* K_m values were calculated from data of experiments using two DP IV inhibitors: Ala-thiazolidide and Ile-thiazolidide

The binding constant of GIP_{1-42} derived from the competitive inhibition of porcine kidney DP IV-catalyzed hydrolysis of H-Gly-Pro-4-nitroanilide was found to be 54 ± 8 μM (mean ± standard error).

Discussion

Mass spectrometry is being applied increasingly in biological research where classical techniques provide only limited information, and are extremely time consuming and expensive. MALDI-TOF MS, a particularly versatile and easily used method of mass spectrometry, was used to monitor the *in vitro* degradation of GIP and GLP-1 in human serum, as well as to investigate the kinetics of DP IV catalysis of these peptides.

It was observed by ourselves in this study and others (Siuzdak, 1994) that absolute quantification of MALDI signals is extremely difficult due to inconsistent shot to shot and sample to sample reproducibility. Although several studies have attempted to address this issue (Brown and Lennon, 1995; Gusev *et al.*, 1995; Schuerch *et al.*, 1994), laser beam heterogeneity and irradiance, as well as inconsistent sample preparation and crystallization are still cited as the most significant problems in obtaining consistent results. Fig. 4 illustrates the MALDI-TOF MS signal profile over a range of GIP and GLP-1 concentrations. As previously observed (Tang *et al.*, 1993), signal intensity does not continue to increase but rather plateaus or decreases as the relative amount of analyte increases with respect to matrix. The observation that diluting analyte results in a more intense signal is not uncommon. One explanation is that decreasing the amount of analyte relative to matrix results in a more optimum analyte:matrix ratio (Zaluzec *et al.*, 1995). Tang and colleagues (1993) suggest this nonlinearity is likely due to changes in the number of analyte layers that the laser can penetrate in order to produce intact ions which ultimately can reach the detector. This conclusion was based on their finding that

increasing the number of analyte molecules while maintaining a constant analyte:matrix does not improve the linearity of analyte concentration versus signal intensity. For GIP and GLP-1, the concentrations yielding signals of greatest intensity were 5 and 15 μM respectively, and subsequent incubations using higher peptide concentrations were diluted to these concentrations prior to MS analysis.

MALDI-TOF MS proved to be a highly sensitive technique to confirm the DP IV-catalyzed removal of N-terminal dipeptides from GIP_{1-42} and GLP-1_{7-36}, on the basis of the mass difference between substrate and product. Equally significant was the observation that monitoring the time course of peptide hydrolysis was an appropriate application of this analytical tool (Fig. 5). However, methodologically, the great advantage of MALDI-TOF MS over other approaches, and even over other types of mass spectrometry, is the tolerance of impurities in the analyte solution. An objective of this study was to analyze the metabolism of GIP and GLP-1 in serum, without prior purification, to test the hypothesis that DP IV is the principal protease responsible for serum inactivation of these hormones. This study clearly affirms that more than 50 % of GIP_{1-42} and GLP-1_{7-36} was converted to GIP_{3-42} and GLP-1_{9-36}, respectively, before significant secondary degradation was observed (Fig. 6). Addition of specific DP IV inhibitors (Fig. 8) reduced this conversion as predicted by inhibitor binding constants (K_i), suggesting that the serum protease responsible for the initial hydrolysis was in fact DP IV. The data presented in Table 1 suggests that secondary degradation of GIP and GLP-1 may include stepwise N-terminal removal of amino acids due to serum aminopeptidases.

In order to study the kinetics of DP IV-catalyzed GIP and GLP-1 hydrolysis, protocols were developed for the quantification of MS signals. This typically involves the

incorporation of an internal standard to the sample mixture, allowing an unknown quantity of analyte to be normalized relative to the standard (Nelson *et al.*, 1994, Duncan *et al.*, 1994; Harvey, 1993; Tang *et al.*, 1993). When measuring the activity of protein kinase and phosphatase, however, Craig *et al.* (1994) avoided the use of an internal standard by quantifying substrate and product peaks relative to each other. Essentially, these peaks served as their own internal standards. The approach of relative quantification was used in the present study. The feasibility of this method is demonstrated in Fig. 7 which shows the relationship between the rate of DP IV-catalyzed peptide hydrolysis and enzyme concentration. As expected, the initial reaction rates increased linearly as a function of DP IV concentration providing convincing evidence that our approach to MS quantification was valid.

Incubation of GIP and GLP-1 with purified porcine kidney DP IV or human serum in the presence and absence of two known specific DP IV inhibitors (Fig. 8) allowed the kinetic constants for peptide hydrolysis to be calculated. The K_m values calculated for purified DP IV correspond well to those previously reported for GIP and GLP-1 hydrolysis by purified human placental DP IV (Table 2) (Mentlein *et al.*, 1993b). The error in the MS-derived constants for GIP and GLP-1, as determined using only single trials of two DP IV inhibitors, was 17 and 7.9 % respectively. This compared to errors of 8.8 and 13 % as determined by seven HPLC-analyzed trials (Mentlein *et al.*, 1993b). Though MS and HPLC-generated kinetic analysis result in comparable variability, this study demonstrates that MS offers some considerable advantages. Significantly fewer trials means that MS is less time consuming and labour intensive, and since MALDI-TOF

MS can detect picomole amounts of analyte, complete kinetic analysis can occur with only minimal amounts of substance, making this approach much less expensive.

The fact that MALDI-TOF MS is tolerant of sample impurities also makes it an ideal tool to study the kinetics of serum proteases without prior purification. The rate specificity constants (k_{cat}/K_m) for GIP and GLP-1 hydrolysis by human serum DP IV were between 10^5 and 10^7, suggesting that DP IV-mediated peptide hydrolysis is significant at physiological concentrations of these hormones. The large variability in the K_m values of peptide hydrolysis by human serum DP IV is likely due to the presence of a distinct DP IV iso-enzyme in serum. In this regard, a novel 175 kDa soluble form of DP IV was recently identified and purified from human serum (Duke-Cohan *et al.*, 1995). Inhibitor binding constants (K_i) of Ala-thiazolidide and Ile-thiazolidide, the DP IV inhibitors used to estimate the kinetic constants of peptide hydrolysis in human serum, were evaluted using purified 105-110 kDa membrane-derived porcine kidney DP IV. Presumably, inhibitor interaction with the serum DP IV is not identical to that with the membrane-associated enzyme, resulting in the disparate K_m values of GIP and GLP-1 hydrolysis. Thus, these experiments support the findings of Duke-Cohan and colleagues (1995) that human serum contains a unique form of soluble DP IV, having similar, yet distinct kinetic properties as compared to the insoluble form.

The close correlation between MALDI-TOF MS-derived kinetic constants and those previously reported, or determined using a spectrophotometric competition assay, validate MS as a reliable method for kinetic analysis.

DP IV catalysis of GIP and GLP-1 renders these hormones biologically inactive and subsequent evidence has suggested that this hydrolysis represents the first step in

hormone metabolism. MALDI-TOF MS was used successfully in the present study to confirm this hypothesis and investigate the kinetics of DP IV-catalyzed incretin hydrolysis.

CHAPTER 2: EFFECT OF *in vivo* INHIBITION OF DP IV ON THE ENTEROINSULAR AXIS

Project Rationale

On the basis of HPLC and RIA analysis, it has been speculated that DP IV catalysis of GIP and GLP-1 is the primary mechanism of their degradation and inactivation (Kieffer *et al.*, 1995; Deacon *et al.*, 1995a; Mentlein *et al.*, 1993b). Mass spectrometry has proven to be a powerful tool to study directly the quantitative degradation of these hormones in serum. It has been shown in Project 1 of this thesis and by others (Kieffer *et al.*, 1995; Deacon *et al.*, 1995a; Mentlein *et al.*, 1993b) that competing substrates and specific DP IV inhibitors are able to suppress the hydrolysis of GIP and GLP-1 *in vitro*. Thus, mounting evidence suggests that targeting specific drugs at blocking DP IV activity may be a means of manipulating the concentrations of endogenously secreted, biologically active incretins *in vivo*. The aim of Project 2 was to develop a protocol for the inhibition of endogenous DP IV in order to study the effect of DP IV inhibition *in vivo* on the physiology of the enteroinsular axis. It is predicted that such inhibition prolongs the circulation time of biologically active incretins, allowing their insulinotropic effects to be exaggerated.

Experimental Procedures

Long Term Inhibition of Serum DP IV *in vitro*

Using the protocol described in Project 1, pooled human serum (20 %) was incubated with GIP_{1-42} (30 μM) and GLP-1_{7-36} (30 μM) in 0.1 mM TRICINE buffer pH 7.6 at 30 °C in the presence or absence of Ile-thiazolidide (20 μM). After a 21-24 h incubation, an equal volume of analyte and matrix (2',6'-dihydroxyacetophenone) was combined, crystallized, and analyzed by MALDI-TOF MS according to the previously outlined procedure. Signals were quantified as relative amounts of GIP_{1-42} or GLP-1_{7-36}: the net substrate peak height divided by the sum of the net substrate and product peak heights. Net peak heights were defined as peak height minus baseline.

Inhibition of Endogenous DP IV in the Rat

Overnight fasted male Wistar rats (200-225 g) were anaesthetized by an intraperitoneal injection of sodium pentobarbitol (65 mg/kg). A jugular vein cannula (heparin-filled PE-90 tubing) permitted the *i.v.* injection of a 1.5 μmol loading dose of Ile-thaizolidide in 0.9 % saline (200 μl), followed by a 0.75 μmol/min infusion of the compound for 30 min at a rate of 33.3 μl/min using a Syringe Infusion Pump 22 (Harvard Apparatus). The injection of the loading dose was taken as time 0 min and blood samples were collected from the tail vein at 0, 5, 10, 20, 30, 40, 50, 60, 75, 90, 120 and 135 min using 250 μl heparinized microcapillary tubes. Samples were stored on ice until

centrifuged at 10000 x g for 20 min at 4 °C. Plasma was collected and immediately assayed for DP IV activity.

DP IV Activity Assay

A colorimetric assay was used to assess rat plasma DP IV activity. Gly-Pro-4-nitroanilide, a chromogenic substrate of DP IV, is hydrolyzed into the dipeptide Gly-Pro and the yellow product 4-nitroaniline whose rate of appearance can be monitored spectrophotometrically. A 1.11 mM stock solution of Gly-Pro-4-nitroanilide (Sigma) was prepared in 0.1 mM TRIS buffer pH 7.4. The assay mixture consisted of 100 µl plasma, 450 µl stock Gly-Pro-4-nitroanilide solution and 450 µl 0.1 mM TRIS buffer pH 7.4, resulting in a final assay volume of 1 ml and a final Gly-Pro-4-nitroanilide concentration of 0.5 mM. The formation of the yellow product was monitored at 405 nm using an SP 8-100 *uv* spectrophotometer (Pye Unicam). Activity is expressed as a rate of 4-nitroaniline formation (µmol/min).

Radiolabeled GLP-1 Administration in the Presence of DP IV Inhibition *in vivo*

This experiment was designed to investigate whether inhibition of endogenous DP IV prevents the degradation of circulating incretins.

Inhibition of endogenous DP IV in overnight fasted, anaesthetized male Wistar rats (200-225 g) was established as outlined in a previous section. Control animals were administered 0.9 % saline in place of Ile-thiazolidide. At time 20 min, purified ^{125}I-GLP-

$1_{7\text{-}36}$ (specific activity of ~ 1 μCi/pmol; Novo Nordisk) calculated to achieve a circulating concentration of 50 - 100 pM, was injected into the animals via a jugular vein cannula. Blood was collected from the tail vein 2 and 5 min after the injection of radiolabeled hormone and immediately placed on ice. Blood was centrifuged at 10000 x g for 20 min at 4 °C, plasma removed, and immediately extracted.

Radiolabeled peptide was purified from plasma using C_{18} Sep Pak cartridges (Waters). These were primed with 5 ml acetonitrile (BDH) containing 0.1 % trifluoracetic acid (TFA; Pierce) (CH_3CN/TFA), 5 ml water containing 0.1 % TFA (H_2O/TFA) and dried by pushing 10 ml air through the column. Plasma was loaded onto the cartridge and washed with 10 ml H_2O/TFA, and 10 ml 20 % CH_3CN/TFA. Radiolabeled hormone was eluted with 2 ml 50 % CH_3CN/TFA into glass test tubes containing 30 μl 5 % RIA grade bovine serum albumin (BSA; Sigma). Extracted peptide was lyophilized and stored at -20 °C until analyzed by HPLC.

Samples were reconstituted in 100 μl water and injected onto a μBondapak C_{18} column (Waters). Elution solvents, CH_3CN/TFA and HPLC grade H_2O/TFA, were delivered to the column by one 110 B and one 114 M Solvent Delivery Module pump (Beckman). ^{125}I-Labeled peptides in the eluant were detected with a 170 Radioisotope Detector (Beckman). Following injection of peptide the column was washed with 40 % CH_3CN/TFA for 10 min, and ^{125}I-GLP-$1_{7\text{-}36}$ and its metabolites eluted using a 40-52 % CH_3CN/TFA gradient over 12 min at a flow rate of 1 ml/min. The column was then washed and re-equilibrated. Eluant fractions were collected during the gradient elution and the amount of radioactivity per fraction was measured in a Wallac 1277 Gammamaster γ-counter (LKB). The fraction of ^{125}I-GLP-$1_{7\text{-}36}$ and ^{125}I-GLP-$1_{9\text{-}36}$ present

in each sample were determined by calculating the relative area under the respective peptide peaks.

Glucose Administration in the Presence of DP IV Inhibition *in vivo*

In order to stimulate the endogenous release of incretins from the gut, glucose is typically administered orally. In the anaesthetized animal used in these experiments, direct administration of glucose intraduodenally serves the same purpose. This allowed the effect of endogenous DP IV inhibition on endogenously released incretin to be investigated. Glucose given intravenously, does not stimulate incretin secretion from the intestine and thus functions as a control to determine whether Ile-thiazolidide has any incretin-independent effects on insulin secretion and glucose absorption.

Intraduodenal Glucose Administration

Overnight fasted anaesthetized male Wistar rats (200-225 g) were surgically prepared with a jugular vein cannula and externalization of the proximal duodenum via a midline abdominal incision (< 2 cm). At time 0 a bolus intraduodenal (*i.d.*) injection of glucose (1 g/kg 50 % w/v dextrose) was delivered with a 1 ml syringe fitted with a 26 gauge needle. One group of rats was given *i.v.* Ile-thiazolidide according to the inhibition protocol outlined above, while control rats received an equal volume of 0.9 % saline without drug. Blood samples were collected from the tail vein at 0, 10, 20, 30, 45, 60, 75 and 90 min, and plasma prepared. Glucose measurements were made immediately on whole blood using a One Touch II Blood Glucose Meter (Lifescan). DP IV activity was

determined on the same day as the experiment by the assay method outlined above. The remaining plasma was stored at - 20 °C until assayed for insulin as described elsewhere (Pederson *et al.*, 1982).

Intravenous Glucose Administration

Overnight fasted, anaesthetized male Wistar rats (200-225 g) were treated with the DP IV inhibitor Ile-thiazolidide as previously described or received an equal volume of 0.9 % saline as a control. At 10 min, both groups of rats received an *i.v.* injection of glucose (0.5 g/kg 50 % w/v dextrose) via a jugular vein cannula. Blood samples were collected from the tail vein at 0, 10, 15, 30, 45, 60, 75 and 90 min as outlined above. Blood glucose, plasma insulin and plasma DP IV activity were assessed as described in the previous section.

Statistical Analysis

Comparisons between drug treated and control rats were assessed by unpaired, 2-tailed t-tests ($P < 0.05$ for significance).

Results

In vitro Inhibition of DP IV Activity by Ile-thiazolidide

Incubation of 30 μM GIP_{1-42} in 20 % human serum for 24 h resulted in the hydrolysis of 71.0 % of GIP_{1-42} into the DP IV reaction product GIP_{3-42} as assessed by MALDI-TOF mass spectrometry (Fig. 10a). Similarly, incubation of 30 μM GLP-1_{7-36} with 20 % human serum for 21 h resulted in hydrolysis of 89.3 % of the original GLP-1_{7-36} into the DP IV reaction product GLP-1_{9-36} (Fig. 10b). Neither GIP_{3-42} nor GLP-1_{9-36} were detected in parallel experiments conducted under identical conditions but in the presence of 20 μM Ile-thiazolidide.

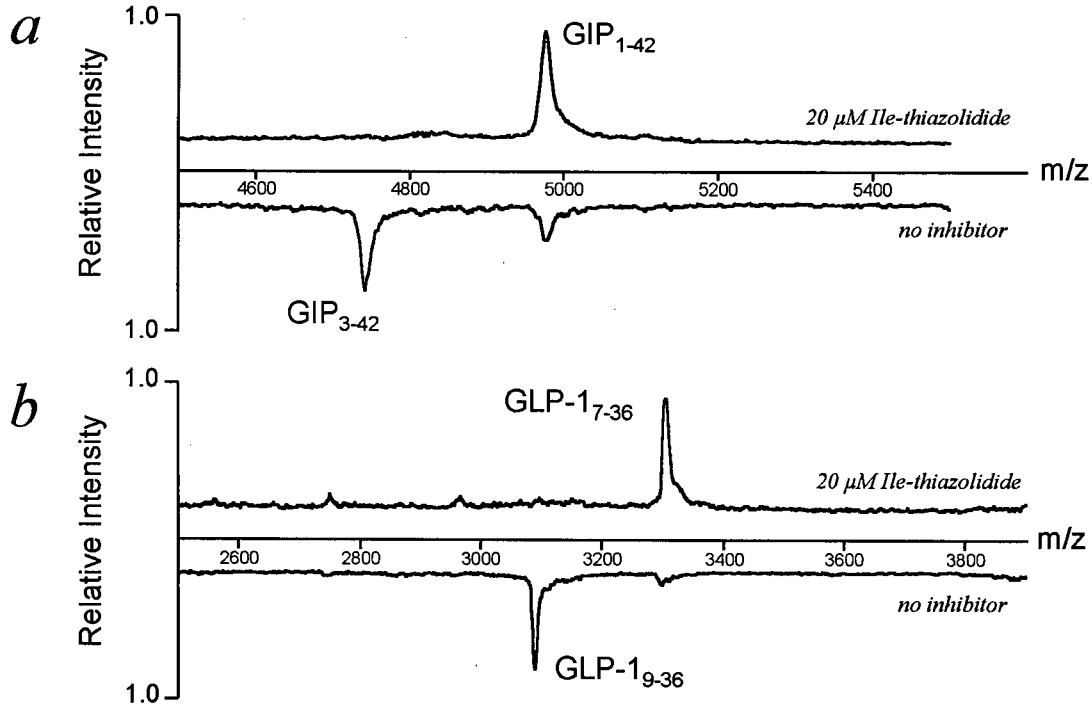

Fig. 10. Inhibition of human serum DP IV activity *in vitro* by Ile-thiazolidide. (*a*) GIP_{1-42} (30 μM) and (*b*) GLP-1_{7-36} (30 μM) were incubated in 20 % human serum for 21-24 h as described in the Experimental Procedures. Hydrolysis was demonstrated by MALDI-TOF MS as outlined in the Experimental Procedures. Signals of the intact hormone peaks (GIP_{1-42} and GLP-1_{7-36}) and the N-terminally truncated DP IV reaction products (GIP_{3-42} and GLP-1_{9-36}) are identified.

In vivo Inhibition of DP IV Activity by Ile-thiazolidide

The combination of a 1.5 μmol *i.v.* loading dose of Ile-thiazolidide followed by 0.75 μmol/min *i.v.* infusion of the drug for 30 min proved effective in suppressing plasma DP IV activity by 64.0 ± 4.2 % by 30 min. Inhibition of plasma DP IV activity was sustained for much longer than the 30 min infusion, resulting in only 51.7 ± 2.7 % (or 48.3 % suppression) of basal activity after 135 min (Fig. 11).

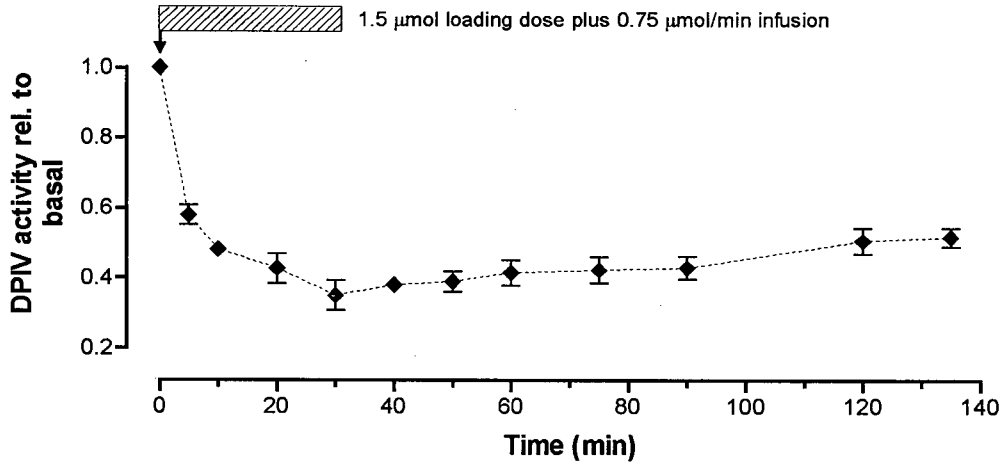

Fig. 11. Plasma DP IV activity profile in response to endogenous DP IV inhibition by Ile-thiazolidide. Drug was administered to anaesthetized rats, blood samples collected, and DP IV activity assess as outlined in the Experimental Procedures. Data are presented as mean DP IV activity relative to basal ± s.e.m. (n = 3).

GLP-1 Metabolism in the Presence of Ile-thiazolidide

In order to determine the effect of *in vivo* inhibition of DP IV on the metabolism of the incretins, *i.v.* ^{125}I-GLP-1$_{7-36}$ was administered to rats in the presence and absence of Ile-thiazolidide. Separation of Sep Pak extracted plasma by HPLC revealed that in the absence of Ile-thiazolidide only 13.4 % of ^{125}I-GLP-1$_{7-36}$ remained unhydrolyzed by endogenous DP IV by 2 min. However, 90.0 % of labeled hormone administered in the

presence of the DP IV-inhibitor was still present in its intact form 5 min after its injection (Fig. 12).

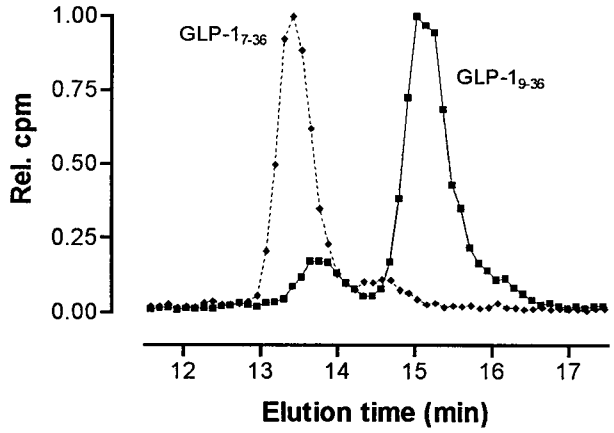

Fig. 12. HPLC of ^{125}I-GLP-1$_{7-36}$ following administration to rats in the presence and absence of Ile-thiazolidide. At time 20 min during the Ile-thiazolidide or saline control infusion purified ^{125}I-GLP-1$_{7-36}$, calculated to achieve a circulating concentration in the physiological range (50 - 100 pM), was injected intravenously. Blood samples were collected, extracted, lyophilized and HPLC analyzed. The elution profiles of the drug-treated rat (♦) and the control rat (▪) were analyzed from blood samples collected 5 and 2 min after the injection of radiolabeled hormone respectively.

Glucose Clearance in the Presence of Ile-thiazolidide

Intraduodenal glucose was administered to anaesthetized rats to stimulate the release of endogenous incretins in the presence and absence of Ile-thiazolidide in order to assess the effect of endogenous inhibition of DP IV on the enteroinsular axis. Fig. 13a,b,c summarizes the results from these experiments

Similar to the previous experiments, plasma DP IV activity was maximally suppressed by 71.4 ± 2.2 % at 30 min. By the termination of the experiment at 90 min, the effects of Ile-thiazolidide were still evident with 59.9 ± 4.4 % inhibition of plasma DP IV activity (Fig. 13a).

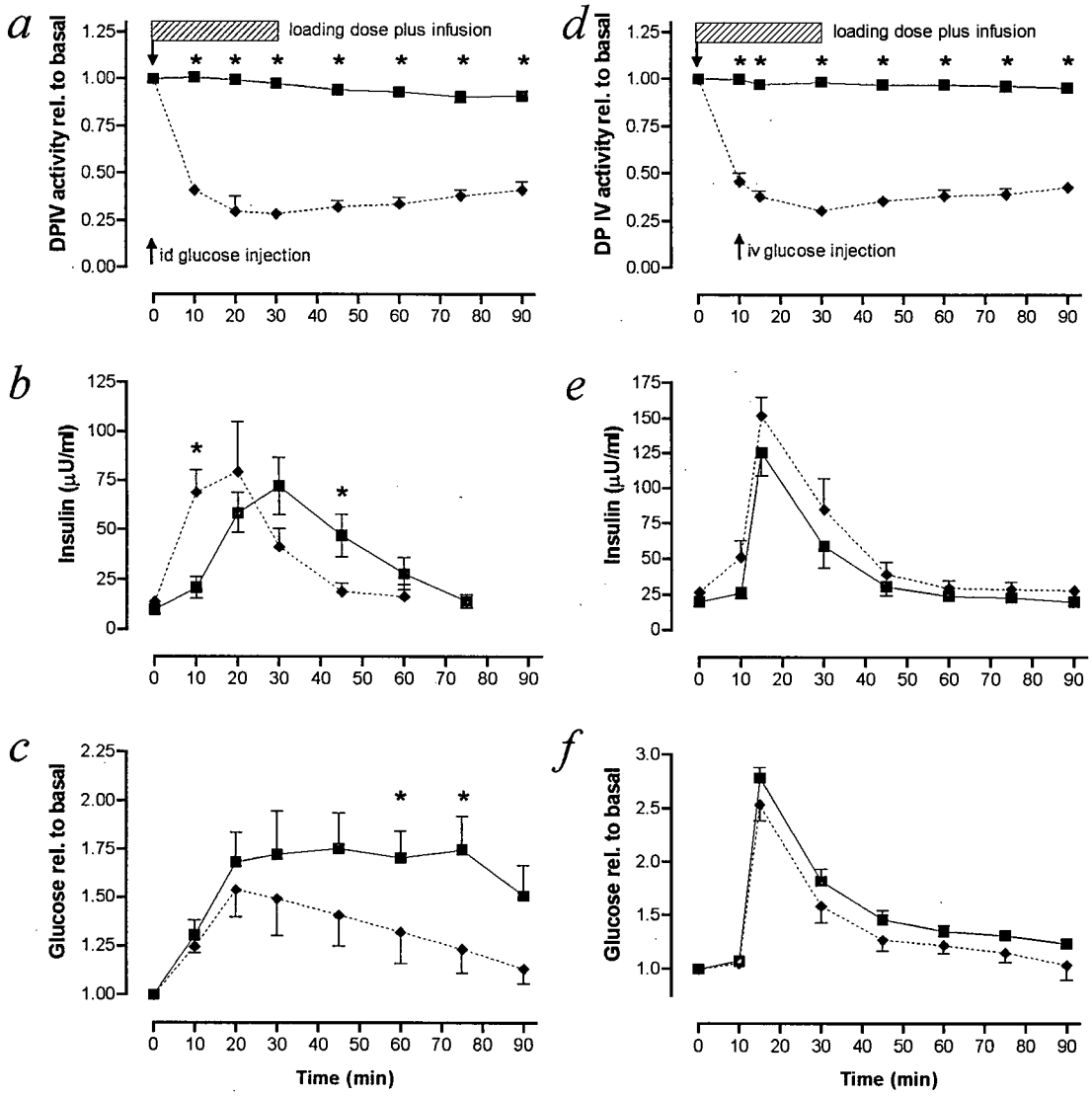

Fig. 13. Effect of endogenous DP IV inhibition on blood glucose and plasma insulin in response to a glucose challenge. Plasma insulin and blood glucose were assessed in response to *i.d.* (*a,b,c*) and *i.v.* (*d,e,f*) glucose in the presence (♦) and absence (▪) of Ile-thiazolidide as outlined in the Experimental Procedures. Data are presented as means ± s.e.m. for n = 5 or 6 rats for each drug-treated or control group. Significance was determined by unpaired, 2-tailed t-tests ($P < 0.05$ for significance (*)).

In animals which did not receive Ile-thiazolidide, plasma insulin concentrations reached a peak value of 72.2 ± 14.6 μU/ml by 30 min before returning to near basal levels by 75 min. Ile-thiazolidide-treated animals attained a similar plasma insulin peak of 79.5 ± 25.5 μU/ml, but reached this concentration by 20 min. Though the total integrated insulin response from the insulin secretory profiles for both groups of rats was similar (Fig. 14a),

the integrated insulin response during the 0-10 min time interval in the drug-treated rats was 2.68 x greater than for control animals (415 ± 61 versus 155 ± 28 μU).

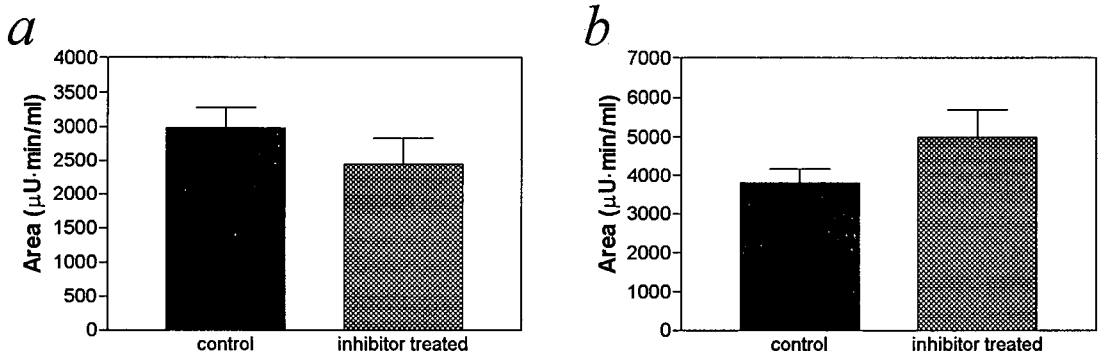

Fig. 14. Integrated insulin responses to (*a*) *i.d.* and (*b*) *i.v.* glucose challenges in the presence and absence of Ile-thiazolidide. Data are presented as mean areas under the insulin profiles from Fig. 4 ± s.e.m. of n = 5 or 6 profiles for each drug-treated or control group. Significance was determined on the basis of unpaired, 2-tailed t-tests.

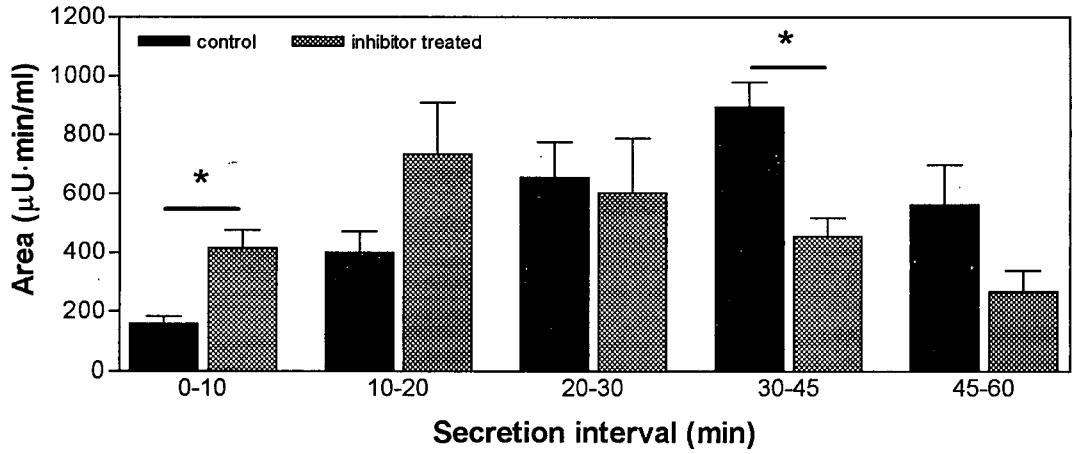

Fig. 15. Integrated insulin responses during distinct secretion intervals in response to an *i.d.* glucose challenge. Data are presented as mean areas ± s.e.m. of n = 5 profiles for each of the drug-treated or control groups. Significance was assessed on the basis of unpaired, 2-tailed t-tests (P < 0.05 for significance (*)).

Conversely, during the 30-45 time interval the integrated insulin response in the control animals was 2.07 x that of the Ile-thiazolidide-treated rats (894 ± 86 versus 432 ± 118 μU; Fig. 15). Though the peak insulin response to *i.d.* glucose was unaffected by Ile-thiazolidide, the peak occurred 10 min earlier in the drug-treated rats relative to control animals.

In control animals blood glucose levels rose and remained elevated for the majority of time of blood sampling (90 min). Rats treated with Ile-thiazolidide exhibited a comparable rise in blood glucose, reaching a peak by 20 min, followed by a steady rate of blood glucose clearance. Glucose levels at 60 and 75 min were significantly lower in the drug-treated group than in control animals.

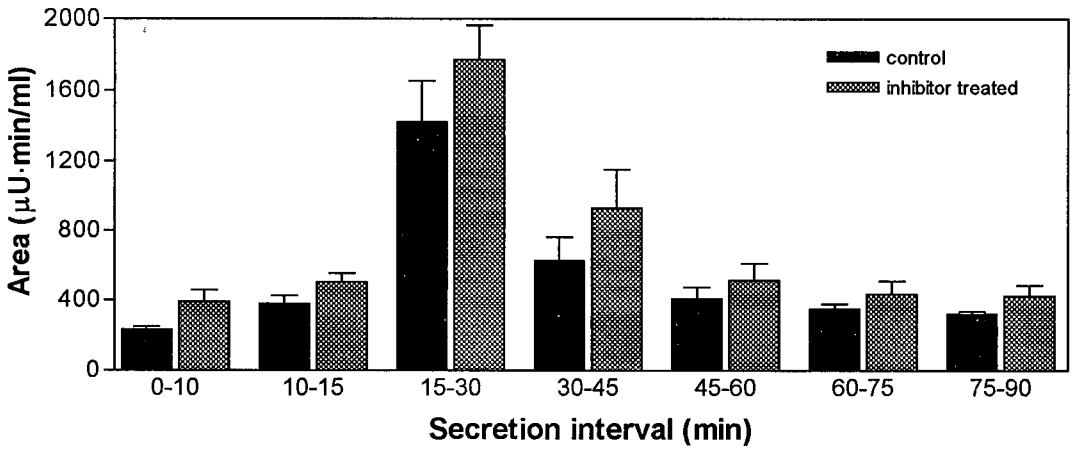

Fig. 16. Integrated insulin responses during distinct secretion intervals in response to an *i.v.* glucose challenge. Data are presented as mean areas ± s.e.m. of n = 6 profiles for each of the drug-treated or control groups. Significance was assessed on the basis of unpaired, 2-tailed t-tests.

In experiments designed to determine whether Ile-thiazolidide had any incretin-independent effects on insulin secretion and glucose clearance, *i.v.* glucose was administered to bypass the stimulation of endogenous incretin secretion from the gut. As in the previous experiments Ile-thiazolidide maximally suppressed plasma DP IV activity by 69.5 ± 2.5 % at 30 min, and it remained 57.2 ± 2.6 % inhibited at 90 min (Fig. 13d). Plasma insulin peaked at 15 min with 125 ± 17 and 152 ± 13 μU/ml for control and drug-treated rats respectively. The total integrated insulin response (Fig. 14b) and the integrated insulin-response during timed secretion intervals (Fig. 16) were not significantly

different between the two groups of rats nor was there a difference in blood glucose clearance.

Discussion

MALDI-TOF MS was used to investigate the *in vitro* degradation of GIP and GLP-1 after incubation in human serum. These results indicate that DP IV is the principal serum protease responsible for the degradation of GIP_{1-42} and GLP-1_{7-36} into the inactive polypeptides GIP_{3-42} and GLP-1_{9-36}, since the presence of Ile-thiazolidide was able to completely block the formation of the DP IV reaction products during the 21 - 24 h incubation. Because of the importance of both GIP and GLP-1 in the incretin response, it was of interest to determine the effect of blocking endogenous DP IV activity and characterizing the effect of this inhibition on the enteroinsular axis. Project 2 involved the development of an effective protocol for the *in vivo* inhibition of DP IV.

A colorimetric enzyme assay of DP IV plasma activity was used to monitor the degree of endogenous protease suppression. This was possible because Ile-thiazolidide is a true reversible inhibitor of DP IV. This represents a significant advantage over previous attempts to block endogenous DP IV activity, using the commercially available DP IV inhibitor Ile-Pro-Ile (Diprotin A), which serves as a competitive substrate of DP IV. Since Diprotin A is itself degraded by DP IV, it is not possible to reliably quantify DP IV activity over the course of an experiment using a simple colorimetric assay. The protocol for inhibiting DP IV activity *in vivo* as described in the present study consistently resulted in the suppression of plasma DP IV activity by 65 - 70 %.

To test the effectiveness of the inhibition protocol on incretin degradation, ^{125}I-GLP-1_{7-36} was administered to rats in the presence and absence of Ile-thiazolidide. Virtually all of the ^{125}I-GLP-1_{7-36} (calculated to achieve a physiological concentration of 50 - 100 pM), co-administered with Ile-thiazolidide, remained in its intact form 5 min after the administration of the radiolabeled hormone, while the majority of the peptide administered to animals not receiving Ile-thiazolidide was converted into the DP IV reaction product by 2 min after label administration. This suggests that the *in vivo* DP IV inhibition protocol described in the present study was effective in inhibiting the degradation of exogenously administered circulating incretin. Since the kinetics of DP IV-catalyzed GLP-1 and GIP hydrolysis are comparable (Mentlein *et al.*, 1993b), similar results would be expected with ^{125}I-GIP_{1-42}.

To assess the effect of DP IV inhibition on the gut-pancreas axis, secretion of endogenous GIP and GLP-1 was stimulated by the administration of *i.d.* glucose in the presence and absence of Ile-thiazolidide. Analysis of plasma insulin revealed that the peak insulin response to *i.d.* glucose was unchanged, but it occurred 10 min earlier than in rats not treated with Ile-thiazolidide. The integrated insulin responses during the 0 - 10 min time interval after glucose administration, were also greater in the drug-treated animals than controls, while during the 30 - 45 min interval, the integrated insulin levels for the control rats was greater. The finding that insulin levels do not remain elevated in the DP IV inhibited rats despite the prediction that the half-life of endogenously released incretins is prolonged, implies the existence of a mechanism which prevents the secretion of inappropriate amounts of insulin even in the presence of elevated incretin concentrations.

However, it appears that the earlier rise and peak in insulin levels contributes to the more rapid clearance of blood glucose in the inhibitor-treated rats.

In experiments designed to determine whether Ile-thiazolidide has a non-incretin dependent effect on insulin secretion and glucose clearance, glucose was administered intravenously in order to bypass the stimulation of endogenous GIP and GLP-1 from the gut. No difference in plasma insulin or blood glucose responses between inhibitor-treated and control rats was observed, implying that Ile-thiazolidide had no direct insulin-releasing action on islet beta cells, nor insulin-like effects on peripheral glucose uptake.

Based on these observations, it is concluded that inhibition of DP IV activity in the rat was able to improve glucose tolerance by an incretin-mediated mechanism. These incretins, particularly truncated forms of glucagon-like peptide-1 are receiving considerable attention because of their antidiabetogenic properties (Amiel, 1994). Interest in the potential clinical application of incretin therapy was generated by reports that exogenously administered GLP-1_{7-36} and GLP-1_{7-37} before and during a test meal in healthy and Type II diabetic subjects, produced a rapid normalization of postprandial hyperglycemia (Nathan *et al.*, 1992; Gutniak *et al.*, 1992). It was later demonstrated that exogenous GLP-1 retained its incretin activity in Type II diabetics whereas synthetic human GIP_{1-42} did not (Nauck *et al.*, 1993b). However, a subsequent report demonstrated that diabetics pre-treated with the sulphonylurea glyburide had an enhanced sensitivity to exogenous GIP (Meneilly *et al.*, 1993). Nauck *et al.* (1993c) also showed that exogenous GLP-1 was able to normalize diabetic hyperglycemia where diet and oral hypoglycemics were ineffective. Since both incretins demonstrate a glucose dependence for their insulinotropic effects (reviewed in Fehmann *et al.*, 1995), a significant advantage of

incretin therapy over tradition oral hypoglycemic or insulin therapy is that drug-induced hypoglycemia common to Type II diabetes (Gerich, 1989) may be avoided. This advantage was recently studied in the rat where unlike glyburide, the hypoglycemic effects of GLP-1 were self-limiting (Hargrove *et al.*, 1996). All of these studies, however, required the *i.v.* administration of exogenous hormone, whereas the present study provides a foundation for the development of a drug able to alter the effects of endogenous incretins. Inhibitors of various proteolytic enzymes are already in use as anti-hypertensive (Walkinshaw, 1992), and immunosupressive (Silverman, 1988) drugs, and antiviral agents (Kelleher *et al.*, 1996). It seems likely that the manipulation of plasma incretin concentrations by acute inhibition of DP IV could serve as a therapeutic approach for improving glucose tolerance, and provide an alternative therapy to currently prescribed drugs such as sulphonylureas and biguanides.

Future Directions

Ongoing research is directed at better understanding the relationship between the hormones GIP and GLP-1 and DP IV, an important protease responsible for their inactivation. Several research directions are outlined below, which arise from the work presented in this thesis and elsewhere.

Endogenous incretin levels

A significant barrier to our present knowledge of circulating incretin concentrations is the inability to distinguish between active and inactive GIP and GLP-1

using currently available RIAs. Since RIA represents an accurate and sensitive technique to determine plasma hormone levels, the development of N-terminally directed incretin RIAs is paramount for future research. However, before such assays are available, a combination of sample extraction, HPLC separation, followed by conventional RIA analysis of the HPLC fractions will allow the ratios of active versus inactive incretin concentrations for a given sample to be determined. Although this approach is significantly more labour-intensive than measuring hormone levels by RIA alone, the effects of DP IV inhibition on endogenous incretin concentrations can be clarified in this manner.

Zucker rat model of glucose intolerance

The fatty Zucker rat represents a hyperinsulinemic animal model for glucose intolerance found in diabetes and obesity (York *et al.*, 1972). The results of experiments described in this thesis may have significant implication for these disease states. Testing the effect of DP IV inhibiting drugs on glucose tolerance in Zucker rats, or in similar models, may provide greater justification for continuing research in this area and for further development of DP IV inhibitors.

Alternate routes of DP IV inhibitor administration

Studies are currently under way to investigate the effectiveness of oral Ile-thiazolidide administration in rats. Preliminary results indicate that doses identical to those used for *i.v.* administration as described in this thesis, are effective in inhibiting plasma DP

IV to a similar extent. Oral drug administration allows experiments to be conducted on conscious animals, thus more closely approximating a truer physiological state.

DP IV-resistant incretin analogues

As previously described, DP IV has a high degree of specificity in that it requires an unmodified protonated N-terminus where the N-terminal two amino acids must be in the D-conformation. Thus, DP IV-resistant substrate analogues which take advantage of this requirement can be synthesized. Such analogues of $GHRH_{1-44}$ have already been described in the literature as being resistant to DP IV catalysis (Frohman *et al.*, 1989). Preliminary experiments in our laboratory have demonstrated that [$desNH_2Tyr^1$]-GIP_{1-42} and [$D\text{-}Ala^2$]-GIP_{1-42} are resistant to hydrolysis by purified porcine kidney DP IV as well as hydrolysis by human serum DP IV activity *in vitro*. Further study is necessary to determine whether these analogues also retain their biological activity *in vivo*.

Localization of DP IV to Islets of Langerhans

Although DP IV is found in highest concentrations on renal and intestinal brush border membranes, and on the surface of immune cells, it has also been identified histochemically within the islets of Langerhans (Poulsen *et al.*, 1993; Mentzel *et al.*, 1996). The functional significance of this observation is not known. However, in light of recent research implicating DP IV in the metabolism of GIP and GLP-1, islet DP IV may be involved in regulating local incretin concentrations or even islet cell signal transduction. Research aimed at identifying which islet cells express DP IV and whether this protein is

associated with the incretin receptors is necessary to understand what effect islet DP IV exerts on incretin physiology.

Summary

The studies described in this thesis were designed to gain a better understanding of the physiological relevance of the interaction between the insulinotropic gut hormones GIP and GLP-1, and the circulating protease DP IV. Using mass spectrometric analysis of serum incubated GIP and GLP-1, it was concluded that DP IV plays an active role in the initial metabolism of these hormones *in vitro*. By administering a specific DP IV inhibitor, Ile-thiazolidide, to rats *in vivo*, in conjunction with an intraduodenal glucose challenge, it was determined that glucose tolerance improved in the presence of the DP IV inhibitor. These studies indicate that degradation of biologically active GIP and GLP-1 is slowed by DP IV inhibitors, allowing these hormones to exert their effect by altering the pattern of glucose-stimulated insulin secretion. The resulting insulin secretory profile is responsible for the more rapid clearance of glucose from the circulation.

REFERENCES

Ahrén, B. (1995) Insulinotropic action of truncated glucagon-like peptide-1 in mice, *Acta Physiol. Scand.* **153**, 205-206.

Amiel, S.A. (1994) Glucagon-like peptide: a therapeutic glimmer, *Lancet.* **343**, 4-5.

Andersen, D.A., Elahi, D., Brown, J.C., Tobin, J.D. & Andres, R. (1978) Oral glucose augmentation of insulin secretion, *J. Clin. Invest.* **62**, 152-161.

Andersen, D.K., Sun, Y.S., Brunicardi, F.C., Berlin, S.A., Lebovitz, H.E., Garsky, V. & Elahi, D. (1984) Regulation of hepatic glucose production by gastric inhibitory polypeptide (GIP), insulin (INS), and glucagon (GLUC), *Dig. Dis. Sci.* **29**, 6S.

Banting, F.G. & Best, C.H. (1921) The internal secretion of the pancreas, *J. Lab. Clin. Med.* 7, 251-264.

Bauvois, B. (1995) Modulation and functional diversity of dipeptidyl peptidase IV in murine and human systems *in* Dipeptidyl Peptidase IV (CD26) in Metabolism and the Immune Response, Fleischer, B., *ed.* R.G. Landes Biochemical Publishers, Georgetown. pp. 99-110.

Bayliss, W.M. & Starling, E.H. (1902) The mechanism of pancreatic secretion, *J. Physiol.* **28**, 325-353.

Bayliss, W.M. & Starling, E.H. (1903) On the uniformity of the pancreatic mechanism in vertebrata, *J. Physiol.* **29**, 175-180.

Beck, B. & Max, J.-P. (1983) Gastric inhibitory polypeptide enhancement of the insulin effect on fatty acid incorporation into adipose tissue in the rat, *Reg. Pept.* 7, 3-8.

Bell, G.I., Santerre, R.F. & Mullenbach, G.T. (1983) Hamster preproglucagon contains the sequence of glucagon and two related peptides, *Nature.* **302**, 716-718.

Bloom, S.R. (1975) GIP in diabetes, *Diabetologia.* **11**, 334.

Boduszek, B., Oleksyszyn, J., Kam, C.-M., Selzler, J., Smith, R.E., Powers, J.C. (1994) Dipeptide phosphonates as inhibitors of dipeptidyl peptidase IV, *J. Med. Chem.* **37**, 3969-3976.

Brandsch, M., Ganapathy, V. & Leibach, F.H. (1995) Role of dipeptidyl peptidase IV (DP IV) in intestinal and renal absorption of peptides *in* Dipeptidyl Peptidase IV (CD26) in Metabolism and the Immune Response, Fleischer, B., *ed.* R.G. Landes Biochemical Publishers, Georgetown. pp. 111-129.

Brandt, W., Ludwig, O., Thondorf, I. & Barth, A. (1996) A new mechanism in serine proteases catalysis exhibited by dipeptidyl peptidase IV (DP IV): results of PM3 semiempirical thermodynamic studies supported by experimental results, *Eur. J. Biochem.* **236**, 109-114.

Brown, J.C. (1974) Gastric inhibitory polypeptide (GIP) *in* Endocrinology 1973, Taylor, S. *ed.* Heinemann, London. pp. 276-274.

Brown, J.C., Dahl, M., Kwauk, S., McIntosh, C.H.S., Otte, S.C. & Pederson, R.A. (1981) Actions of GIP, *Pept.* **2**, 241-245.

Brown, J.C. & Pederson, R.A. (1970) A multiparameter study on the action of preparations containing cholecystokinin pancreozymin, *Scand. J. Gastroenterol.* **5**, 537-541.

Brown, J.C. & Pederson, R.A. (1976) GI hormones and insulin secretion, *Endocrinology (Proceedings of the Vth International Congress of Endocrinology).* 2: 568-570.

Brown, R.S. & Lennon, J.J. (1995) Mass resolution improvement by incorporation of pulsed ion extraction in a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, *Anal. Chem.* **67**, 1998-2003.

Brubaker, P.L. (1991) Regulation of intestinal proglucagon-derived peptide secretion by intestinal regulatory peptides, *Endocrinol.* **128**, 3175-3182.

Buchan, A.M.J., Polak, J.M., Capella, C., Solcia, E. & Pearse, A.G.E. (1978) Electron immunocytochemical evidence for the K cell localization of gastric inhibitory polypeptide (GIP) in man, *Histochem.* **546**, 37-44.

Buhl, T., Thim, L., Kofod, H., Ørskov, C., Harling, H., Holst, J.J. (1988) Naturally occurring products of proglucagon 111-160 in the porcine and human small intestine, *J. Biol. Chem.* **263**, 8621-8624.

Burhol, P.G., Jorde, R. & Waldum, H.L. (1980) Radioimmunoassay of plasma gastric inhibitory polypeptide (GIP), release of GIP after a test meal and duodenal infusion of bile, and immunoreactive plasma GIP components in man, *Digestion.* **20**, 336-345.

Callabaut, C., Krust, B., Jacotot, E., & Hovanessian, A.G. (1993) T cell activation antigen, CD26, as a cofactor for entry of HIV in $CD4^+$ cells, *Science.* **262**, 2045-2050.

Cataland, S., Crockett, S.E., Brown, J.C. & Mazzaferri, E.L. (1974) Gastric inhibitory polypeptide stimulation by oral glucose in man, *J. Clin. Endocrinol. Metab.* **39**, 223-228.

Chait, B.T., Wang, R., Beavis, R.C. & Kent, S.B.H. (1993) Protein ladder sequencing, *Science.* **262**, 89-92.

Cleator, I.G. & Gourlay, R.H. (1975) Release of immunoreactive gastric inhibitory polypeptide (IR-GIP) by oral ingestion of food substances, *Am. J. Surg.* **130**, 128-135.

Coxe, J.S., O'Dorisio, T.M., Cataland, S. & Crockett, S.E. (1981) Gastric inhibitory polypeptide hypersecretion in diabetes mellitus: effect of sulfonylurea treatment, *J. Clin. Endocrinol. Metab.* **52**, 1002-1005.

Craig, A.G., Hoeger, C.A., Miller, C.L., Goedken, T., Rivier, J.E. & Fischer, W.H. (1994) Monitoring protein kinase and phosphatase with matrix-assisted laser desorption/ionization mass spectrometry and capillary zone electrophoresis: comparison of the detection efficiency of peptide-phosphopeptide mixtures, *Biol. Mass Spectrom.* **23**, 519-528.

Crawford, C., Mason, R.W., Wikström, P. & Shaw, E. (1988) The design of peptidyl-diazomethane inhibitors to distinguish between the cysteine proteinases calpain II, cathepsin L and cathepsin B, *Biochem. J.* **253**, 751-758.

Creutzfeldt, W. (1979) The incretin concept today, *Diabetologia.* **16**, 75-85.

Creutzfeldt, W. & Ebert, R. (1985) New developments in the incretin concept, *Diabetologia.* **28**, 565-573.

Creutzfeld, W. & Ebert, R. (1993) The enteroinsular axis, *in* The Pancreas: Biology, Pathobiology, and Disease, Go, V.L.W., DiMagno, E.P., Gardner, J.D., Lebenthal, E., Reben, H.A. & Acheele, G.A. *eds.* Raven Press Ltd., New York. pp. 769-788.

Creutzfeldt, W., Ebert, R., Nauck, M. & Stöckmann, F. (1983) Disturbances of the entero-insular axis, *Scand. J. Gastroenterol. (Suppl.).* **82**, 111-119.

Creutzfeldt, W., Ebert, R., Willms, B., Frerichs, H. & Brown, J.C. (1978) Gastric inhibitory polypeptide (GIP) and insulin in obesity: Increased response to stimulation and defective feedback control of serum levels, *Diabetologia.* **14**, 15-24.

Creutzfeldt, W.O.C., Kleine, N., Willms, B., Ørskov, C., Holst, J.J. & Nauck, M.A. (1996) Glucagonostatic actions and reduction of fasting hyperglycemia by exogenous glucagon-like peptide 1(7-36) amide in type 1 diabetic patients, *Diabetes Care.* **19**, 580-586.

Creutzfeldt, W. & Nauck, M. (1992) Gut hormones and diabetes mellitus, *Diabetes/ Metab. Rev.* **8**, 149-177.

Crockett, S.E., Mazzaferri, E.L. & Cataland, S. (1976) Gastric inhibitory polypeptide (GIP) in maturity-onset diabeted mellitus, *Diabetes.* **25**, 931-935.

D'Alessio, D.A., Fujimoto, W.Y. & Ensinck, J.W. (1989) Effects of glucagonlike peptide 1-(7-36) on release of insulin, glucagon, and somatostatin by rat pancreatic islet monolayer cultures, *Diabetes.* **38**, 1534-1538.

D'Alessio, D.A., Kahn, S.E., Leusner, C.R. & Ensinck, J.W. (1994) Glucagon-like peptide 1 enhances glucose tolerance both by stimulation of insulin release and by increasing insulin-independent glucose disposal, *J. Clin. Invest.* **93**, 2263-2266.

D'Alessio, D., Thirlby, R., Laschansky, E., Zebroski, H. & Ensinck, J. (1993) Response of tGLP-1 to nutrients in humans, *Digestion.* **54**, 377-379.

David, F., Bernard, A.-M., Pierres, M. & Marguet, D. (1993) Identification of serine 624, aspartic acid 702, and histidine 734 as the catalytic triad residues of mouse dipeptidyl peptidase IV (CD26), *J. Biol. Chem.* **268**, 17247-17252.

Deacon, C.F., Johnsen, A.H. & Holst, J.J. (1995a) Degradation of glucagon-like peptide-1 by human plasma *in vitro* yields an N-terminally truncated peptide that is a major endogenous metabolite *in vivo*, *J. Clin. Endocrinol. Metab.* **80**, 952-957.

Deacon, C.F., Nauck, M.A., Toft-Nielsen, M., Pridal, L., Willms, B. & Holst, J.J. (1995b) Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly degraded from the NH_2-terminus in type II diabetic patients and in healthy subjects, *Diabetes.* **44**, 1126-1131.

Deacon, C., Johnsen, A.H. & Holst, J.J. (1995c) Human colon produces fully processed glucagon-like peptide-1 (7-36) amide, *FEBS Lett.* **372**, 269-272.

Demuth, H.-U. (1988) Studien zur entwicklung und zum funktionsmechanismus irreversibler inhibitoren von serinproteasen unter besonderer beruecksichtigung post-prolin-spaltender enzyme, PhD Dissertation, Halle-Wittenberg: Martin-Luther-University.

Demuth, H.-U. (1990) Recent developments in the irreversible inhibition of serine and cysteine proteases, *J. Enzyme Inhib.* **3**, 249-278.

Demuth, H.-U. & Heins, J. (1995) Catalytic mechanism of dipeptidyl peptidase IV *in* Dipeptidyl Peptidase IV (CD26) in Metabolism and the Immune Response, Fleischer, B. *ed.* R.G. Landes Biochemical Publishers, Georgetown. pp. 1-37.

Deschamps, I., Heptner, W., Desjeux, J.F., Baltakse, V., Machinot, S. & Lestradet, H. (1980) Effects of diet on insulin and gastric inhibitory polypeptide levels in obese children, *Pediatr. Res.* **14**, 300-303.

Dockray, J.G. (1989) Comparative neuroendocrinology of gut peptides *in* Handbook of Physiology, Schultz, S.G., Makhlouf, G.M. and Rauner, B.B. *eds.* American Physiology Society, Bethesda. pp. 133-170.

Drucker, D.J., Ehrlich, P., Asa, S.L. & Brubaker, P.L. (1996) Induction of intestinal epithelial proliferation by glucagon-like 2, *Proc. Natl. Acad. Sci. USA.* **93**, 7911-7916.

Duke-Cohan, J.S., Morimoto, C., Rocker, J.A. & Schlossmann, S.F. (1995) A novel form of dipeptidyl peptidase IV found in human serum, *J. Biol. Chem.* **270**, 14107-14114.

Duke-Cohan, J.S., Morimoto, C., Rocker, J.A. & Schlossmann, S.F. (1996) Serum high molecular weight dipeptidyl peptidase IV (CD26) is similar to a novel antigen DPPT-L released from activated T cells, *J. Immunol.* **156**, 1714-1721.

Duncan, M.W., Matanovic, G. & Cerpa-Poljak, A. (1993) Quantitative analysis of low molecular weight compounds of biological interest by matrix-assisted laser desorption ionization, *Rapid Commun. Mass Spectrom.* 7, 1090-1094.

Dupré, J., Ross, S.A., Watson, D. & Brown, J.C. (1973) Stimulation of insulin secretion by gastric inhibitory polypeptide, *J. Clin. Endocrinol. Metab.* **37**, 826-828.

Ebert, R. & Creutzfeldt, W. (1982) Influence of gastric inhibitory polypeptide antiserum on glucose-induced insulin secretion in rats, *Endocrinol.* **111**, 1601-1606.

Ebert, R., Frerichs, H. & Creutzfeldt, W. (1976a) Serum gastric inhibitory polypeptide (GIP) response in patients with maturity onset diabetes and in juvenile diabetics, *Diabetologia.* **12**, 388.

Ebert, R., Frerichs, H. & Creutzfeldt, W. (1979a) Impaired feedback control of fat induced gastric inhibitory polypeptide (GIP) secretion by insulin in obesity and glucose intolerance, *Eur. J. Clin. Invest.* **9**, 129-135.

Ebert, R., Illmer, K. & Creutzfeldt, W. (1979b) Release of gastric inhibitory polypeptide (GIP) by intraduodenal acidification in rats and humans and abolishment of the incretin effect of acid by GIP-antiserum in rats, *Gastroenterol.* **76**, 515-523.

Ebert, R., Nauck, M. & Creutzfeldt, W. (1991) Effect of exogenous or endogenous gastric inhibitory polypeptide (GIP) on plasma triglyceride responses in rats, *Horm. Metab. Res.* **23**, 517-521.

Ebert, R., Unger, H. & Creutzfeldt, W. (1983) Preservation of incretin activity after removal of gastric inhibitory polypeptide (GIP) from rat gut extracts by immunoadsorption, *Diabetologia.* **24**, 449-454.

Ebert, R., Willms, B., Brown, J.C. & Creutzfeldt, W. (1976b) Serum gastric inhibitory polypeptide (GIP) levels in obese subjects and after weight reduction, *Euro. J. Clin. Invest.* **6**, 327.

Eckel, R.H., Fujimoto, W.Y. & Brunzell, J.D. (1979) Gastric inhibitory polypeptide enhanced lipoprotein lipase activity in cultured preadipocytes, *Diabetes.* **28**, 1141-1142.

Elahi, D., Andersen, D.K., Brown, J.C., Debas, H.T., Hershcopf, R.J., Raizes, G.S., Tobin, J.D. & Andres, R. (1979) Pancreatic α- and β-cell responses to GIP infusion in normal man, *Am. J. Physiol.* **237**, E185-191.

Elahi, D., Andersen, D.K., Muller, D.C., Tobin, J.D., Brown, J.C. & Andres, R. (1984) The enteric enhancement of glucose-stimulated insulin release: the role of GIP in aging, obesity, and non-insulin-dependent diabetes mellitus, *Diabetes.* **33**, 950-957.

Elahi, D., McAloon-Dyke, M., Fukagawa, N.K., Meneilly, G.S., Sclater, A.L., Minaker, K.L., Habner, J.F. & Andersen, D.K. (1994) The insulinotropic action of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (7-37) in normal and diabetic subjects, *Reg. Pept.* **51**, 63-74.

Elahi, D., Meneilly, G.S., Minaker, K.L., Rowe, J.W. & Andersen, D.K. (1986) Regulation of glucose production by gastric inhibitory polypeptide in man, *Can. J. Physiol. Pharmacol.* **Suppl.**, 18.

Elliot, R.M., Morgan, L.M., Tredger, J.A., Deacon, S., Wright, J. & Marks, V. (1993) Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns, *J. Endocrinol.* **138**, 159-166.

Elrick, H., Stimmler, L., Hlad, C.J. & Arai, Y. (1964) Plasma insulin response to oral and intravenous glucose administration, *J. Clin. Endrinol.* **24**, 1076-1082.

Falko, J.M., Crockett, S.E., Cataland, S. & Mazzaferri, E.L. (1975) Gastric inhibitory polypeptide (GIP) stimulated by fat ingestion in man, *J. Clin, Endocrinol. Metab.* **41**, 260-265.

Falko, J.M., Crockett, S.E., Cataland, S., O'Dorisio, T.M., Kramer, W. & Mazzaferri, E.L. (1980) The effect of increasing doses of ingested glucose on insulin and gastric inhibitory polypeptide (GIP) concentrations in man, *Clin. Endocrinol.* **13**, 587-593.

Farrell, J.I. & Ivy, A.C. (1926) Studies on the motility of the transplanted gastric pouch, *Am. J. Physiol.* **46**, 227-228.

Fehmann, H.-C., Göke, R. & Göke, B. (1995a) Cell and molecular biology of the incretin hormones glucagon-like peptide-1 and glucose-dependent insulin releasing polypeptide, *Endocrine Rev.* **16**, 390-410.

Fehmann, H.-C., Hering, B.-J., Wolf, M.-J., Brandhorst, H., Brandhorst, D., Bretzel, R.G., Federlin, K. Göke, B. (1995b) The effects of glucagon-like peptide-I (GLP-I) on hormone secretion from isolated human pancreatic islets, *Pancreas.* **11**, 196-200.

Fischer, G., Heins, J. & Barth, A. (1983) The conformation around the peptide bond between P_1- and P_2-positions is important for catalytic activity of some proline-specific peptidases, *Biochim. Biophy. Acta.* **742**, 452-462.

Fridolf, T. & Ahrén, B. (1991) GLP-$1_{(7\text{-}36)}$ amide stimulates insulin secretion in rat islets: studies on the mode of action, *Diabetes Res.* **16**, 185-191.

Frohman, L.A., Downs, T.R., Heimer, E.P. & Felix, A.M. (1989) Dipeptidylpeptidase IV and trypsin-like enzymatic degradation of human growth hormones-releasing hormone in plasma, *J. Clin. Invest.* **83**, 1533-1540.

Frohman, L.A., Downs, T.R., Williams, T.C., Heimer, E.P., Pan, Y.-C.E. & Felix, A.M. (1986) Rapid enzymatic degradation of growth hormone-releasing hormone by plasma in vitro and in vivo to a biologically inactive product cleaved at the NH_2 terminus, *J. Clin. Invest.* **78**, 906-913.

Fukase, N., Takahashi, H., Hanaka, M., Idarashi, M., Yamatani, K., Daimon, M., Sugiyama, K., Tominaga, M. & Sasaki, H. (1992) Differences in glucagon-like peptide-1 and GIP responses following sucrose ingestion, *Diabetes Res. Clin. Prac.* **15**, 187-195.

Gefel, D., Hendrick, G.K., Mojsov, S., Habner, J. & Weir, G.C. (1990) Glucagon-like peptide-I analogs: effects on insulin secretion and adenosine 3',5'-monophosphate formation, *Endocrinol.* **126**, 2164-2168.

Gerich, J.E. (1989) Oral hypoglycemic agents, *N. Eng. J. Med.* **321**, 1231-1245.

Göke, R., Richter, G., Kolligs, F., Fehmann, H.C., Arnold, R. & Göke, B. (1993) How do individual meal constituents influence the release of GLP-1 in humans?, *Digestion.* **54**, 380.

Gros, L., Hollande, F., Thorens, B., Kervran, A. & Bataille, D. (1995) Comparative effects of GLP-1-(7-36) amide, oxyntomodulin and glucagon on rabbit gastric parietal cell function, *Eur. J. Pharmacol.* **288**, 319-327.

Gusev, A.I., Wilkinson, W.R., Proctor, A. & Hercules, D.M. (1995) Improvement of signal reproducibility and matrix/comatric effects in MALDI analysis, *Anal. Chem.* **67**, 1034-1041.

Gutheil, W.G., Subramanyan, M., Filentke, G.R., Sanford, D.G., Munoz, E., Huber, B.T. & Bachovchin W.W. (1994) Human immunodeficiency virus 1 Tat binds to dipeptidyl peptidase IV (CD26): a possible mechanism for Tat's immunosuppressive activity, *Proc. Natl. Acad. Sci. USA.* **91**, 6594-6598.

Gutniak, M., Ørskov, C., Holst, J.J., Ahrén, B. & Efendic S. (1992) Antidiabetogenic effect of glucagon-like peptide-1 (7-36)amide in normal subjects and patients with diabetes mellitus, *N. Eng. J. Med.* **326**, 1314-1322.

Hafler, D.A., Fox, D.A., Benjamin, D. & Weiner, H.L. (1990) Antigen reactive memory T cells are defined by Ta1, *J. Immunol.* **137**, 414-418.

Hanski, C., Huhle, T., Gossrau, R. & Reutter, W. (1988) Direct evidence for the binding of rat liver DP IV to collagen in vitro, *Exp. Cell Res.* **178**, 64-72.

Hargrove, D. M., Nardone, N.A., Persson, L.M., Parker, J.C. & Stevenson, R.W. (1995) Glucose-dependent action of glucagon-like peptide-1 (7-37) in vivo during short- or long-term administration, *Metabol.* 44, 1231-1237.

Hargrove, D.M., Nardone, N.A., Persson, L.M., Andrews, K.M., Shepherd, K.L., Stevenson, R.W. & Parker, J.C. (1996) Comparison of the glucose dependency of glucagon-like peptide-1(7-37) and glyburide in vitro and in vivo, *Metab.* **45**, 404-409.

Hartmann, H., Ebert, R. & Creutzfeldt, W. (1986) Insulin-dependent inhibition of hepatic glycogenolysis by gastric inhibitory polypeptide (GIP) in perfused rat liver, *Diabetologia.* **29**, 112-114.

Harvey, D.J. (1993) Quantitative aspects of the matrix-assisted laser desorption mass spectrometry of complex oligosaccharides, *Rapid Commun. Mass Spectrom.* 7, 614-619.

Hegen, M., Mittrücker, H.-W., Hug, R., Demuth, H.-U., Neubert, K., Barth, A. & Fleicher, B. (1993) Enzymatic activity of CD26 (dipeptidyl peptidase IV) is not required for its signaling function in T cells, *Immunobiol.* **189**, 483-493.

Heins, J., Welker, P., Schonlein, C., Born, J., Hartrodt, B., Neubert, K., Tsuru, D. & Barth, A. (1988) Mechanism of proline-specific proteinases: (I) substrate specificity of dipeptidyl peptidase IV from pig kidney and proline-specific endopeptidase from *Flavobacterium meningosepticum*, *Biol. Chem. Hoppe-Seyler.* **372**, 313-318.

Heller, H. (1929) Über den blutzuckerwirksamen Stoff in Sekretinextrakten, *Naunyn Schmiedebergs Arch. Pharmacol.* **145**, 343-358.

Heller, H. (1935) Über das insulinotrope Hormon der Darmschleimhaut (Duodenin), *Naunyn Schmiedebergs Arch. Pharmacol.* **177**, 127-133.

Heller, R.S. & Aponte, G.W. (1995) Intra-islet regulation of hormone secretion by glucagon-like peptide-1-(7-36) amide, *Am. J. Physiol.* 269, G852-860.

Herrmann, C., Göke, R., Richter, G., Fehmann, H.C., Arnold, R. & Göke, B. (1995) Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients, *Digestion.* **56**, 117-126.

Herrmann-Rinke, C., Vöge, A., Hess, M. & Göke, B. (1995) Regulation of glucagon-like peptide-1 secretion from rat ileum by neurotransmitters and peptides, *J. Endocrinol.* **147**, 25-31.

Higashimoto, Y., Opara, E.C. & Liddle, R.A. (1995) Dietary regulation of glucose-dependent insulinotropic polypeptide (GIP) gene expression in rat small intestine, *Comp. Biochem. Physiol.* **110C**, 207-214.

Hirota, M., Hashimoto, M., Hiratsuka, M., Ohboshi, C., Yoshimoto, S., Yano, M., Mizuno, A. & Shima, K. (1990) Alterations of plasma immunoreactive glucagon-like peptide-1 behaviour in non-insulin-dependent diabetics, *Diabetes Res. Clin. Prac.* **9**, 179-185.

Holst, J.J. & Ørskov, C. (1994) Glucagon and other glucagon-derived peptides *in* Gut Peptides, Walsh, J.H. and Dochray, G.J. *eds.* Raven Press, New York. pp. 305-340.

Holst, J.J. (1994) Glucagonlike peptide 1: a newly discovered gastrointestinal hormone, *Gastroenterol.* **107**, 1848-1855.

Holst, J.J., Ørskov, C., Vagn Nielsen, O., & Schwartz, T.W. (1987) Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut, FEBS Lett. **211**, 169-174.

Hopfer, U. (1987) Membrane transport mechanisms for hexoses and amino acids in the small intestine *in* Physiology of the Gastrointestinal Tract 2nd Ed., Johnson, L.R. *ed.* Raven Press, New York. pp. 1499-1527.

Hopsu-Havu, V.K. & Glenner, G.G. (1966) A new dipeptide naphthylamidase hydrolyzing glycl-prolyl-β-naphthylamide, *Histochemie.* 7, 197-201.

Hsieh, F.Y.L., Tong, X., Wachs, T., Ganem, B. & Henion, J. (1995) Kinetic monitoring of enzymatic reactions in real time by quantitative high-performance liquid chromatography-mass spectrometry, *Anal. Biochem.* **229**, 20-25.

James, P., Quandroni, M., Carafoli, E. & Gonnet, G. (1994) Protein identification by mass profile fingerprinting, *Biochem. Biophys. Res. Commun.* **195**, 58-64.

Jia, X., Brown, Ma, P., Pederson, R.A. & McIntosh, C.H.S. (1995) Effects of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-I-(7-36) on insulin secretion, *Am. J. Physiol.* **268**, E645-651.

Jones, I.R., Owens, D.R., Luzio, S. & Hayes, T.M. (1989) Glucose dependent insulinotropic polypeptide (GIP) infused intravenously is insulinotropic in the fasting state in type 2 (non-insulin dependent) diabetes mellitus, *Horm. Metab. Res.* **21**, 23-26.

Jones, I.R., Owens, D.R., Moody, A.J., Luzio, S.D., Morris, T. & Hayes, T.M. (1987) The effects of glucose-dependent insulinotropic polypeptide infused at physiological concentrations in normal subjects and type 2 (non-insulin-dependent) diabetic patients on glucose tolerance and B-cell secretion, *Diabetalogia.* **30**, 707-712.

Jorde, R., Amland, P.F., Burhol, P.G., Giercksky, K.E. & Ebert, R. (1983a) GIP and insulin responses to a test meal in healthy and obese subjects, *Scand. J. Gastroenterol.* **18**, 1115-1119.

Jorde, R., Burhol, P.G. & Schulz, T.B. (1983b) Fasting and postprandial GIP values in man measured with seven different antisera, *Reg. Pept.* 7, 87-94.

Jörnvall, H., Carlquist, M., Kwauk, S., Otte, S.C., McIntosh, C.H.S., Brown, J.C. & Mutt, V. (1981) Amino acid sequence and heterogeneity of gastric inhibitory polypeptide (GIP), *FEBS Lett.* **123**, 205-210.

Karas, M. & Hillenkamp, F. (1988) Laser desorption ionization of proteins with molecular mass exceeding 10000 daltons, *Anal. Chem.* **60**, 2299-2301.

Kauth, T. & Metz, J. (1987) Immunohistochemical localization of glucagon-like peptide 1: Use of poly- and monoclonal antibodies, *Histochem.* **86**, 509-515.

Kawai, K., Suzuki, S., Ohashi, S., Mukai, H., Murayma, Y. & Yamashita, K. (1990) Effects of truncated glucagon-like peptide-1 on pancreatic hormone release in normal conscious dogs, *Acta Endocrinologica.* **123**, 661-667.

Kawai, K., Suzuki, S., Ohashi, S., Mukai, H., Ohmori, H., Murayama, Y. & Yamashita, K. (1989) Comparison of the effects of glucagon-like peptide-1-(1-37) and -(7-37) and glucagon on islet hormone release from isolated perfused canine and rat pancreases, *Endocinol.* **124**, 1768-1773.

Kelleher, A.D., Carr, A., Zaunders, J. & Cooper D.A. (1996) Alterations in the immune response of human immunodeficency virus (HIV)-infected subjects treated with an HIV-specific protease inhibitor, ritanovir, *J. Infect. Dis.* **173**, 321-329.

Kieffer, T.J. (1995) Release and metabolism of gastric inhibitory polypeptide, PhD Dissertation, Vancouver: University of British Columbia.

Kieffer, T.J., McIntosh, C.H.S. & Pederson, R.A. (1995) Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide-1 *in vitro* and *in vivo* by dipeptidyl peptidase IV, *Endocrinol.* **136**, 3585-3596.

Kolligs, F., Fehmann, H.C., Göke, R. & Göke, B. (1995) Reduction of the incretin effect in rats by the glucagon-like peptide 1 receptor antagonist exendin (9-39) amide, *Diabetes.* **44**, 16-19.

Komatsu, R., Matsuyoshi, T., Namba, M., Watanabe, N., Itoh, H., Kono, N. & Tarui, S. (1989) Glucagonostatic and insulinotropic action of glucagonlike peptide 1-(7-36) amide, *Diabetes.* **38**, 902-905.

Kosaka, T. & Lim, R.K.S. (1930) Demonstration of the humoral agent in fat inhibition of gastric secretion, *Proc. Soc. Exp. Med.* **27**, 890-891.

Krarup, T., Saurbrey, N., Moody, A.J., Kühl, C. & Madsbad, S. (1987) Effect of porcine gastric inhibitory polypeptide on β-cell function in type I and type II diabetes mellitus, *Metab.* **36**, 677-682.

Kreymann, B., Ghatei, M.A., Williams, G. & Bloom, S.R. (1987) Glucagon-like peptide-1 7-36: a physiological incretin in man, *The Lancet.* **I**, 1300-1303.

Kuzio, M., Dryburgh, J.R., Malloy, K.M. & Brown, J.C. (1974) Radioimmunoassay for gastric inhibitory polypeptide, *Gastroenterol.* **66**, 357-364.

Kwasowski, P., Flatt, P.R., Bailey, C.J. & Marks, V. (1985) Effects of fatty acid chain length and saturation on gastric inhibitory polypeptide release in obese hyperglycemic (ob/ob) mice, *Biosci. Rep.* **5**, 701-705.

La Barre, J. & Still, E.V. (1930) Studies on the physiology of secretin. *Am. J. Physiol.* **91**, 649-653.

La Barre, J. (1932) Sur les possibilités d'un traitement due diabete par l'incretine, *Bull. Acad. Roy. Méd. Belg.* **12**, 620-634.

Lambeir, A.-M., Borloo, M., De Meester, I., Belyaev, A., Augustyns, K., Hendricks, D., Scharpé, S. & Haemers, A. (1996) Dipeptide-derived diphenyl phosphonate esters:

mechanism-based inhibitors of dipeptidyl peptidase IV, *Biochim. Biophys. Acta.* **1290**, 76-82.

Lauritsen, K.B., Cristensen, K.C. & Stokholm, K.H. (1980) Gastric inhibitory polypeptide (GIP) release and incretin effect after oral glucose in obesity and after jejunoileal bypass, *Scand. J. Gastroenterol.* **15**, 489-495.

Layer, P., Holst, J.J., Grandt, D. & Goebell, H. (1995) Ileal release of glucagon-like peptide-1 (GLP-1): association with inhibition of gastric acid secretion in humans, *Dig. Dis. Sci.* **40**, 1074-1082.

Li, E., Wilk, E. & Wilk, S. (1995) Aminoacylpyrroline-2-nitrile: potent and stable inhibitors of dipeptidyl peptidase IV (CD26), *Arch. Biochem. Biophys.* **323**, 148-154.

Loew, E.R., Gray, J.S. & Ivy, A.V. (1939) The effect of duodenal instillation of hydrochloric acid upon the fasting blood sugar of dogs, *Am. J. Physiol.* **126**, 270-276.

Loew, E.R., Gray, J.S. & Ivy, A.V. (1940a) The effect of acid stimulation of the duodenum upon experimental hyperglycemia and utilization of glucose, *Am. J. Physiol.* **128**, 298-308.

Loew, E.R., Gray, J.S. & Ivy, A.V. (1940b) Is a duodenal hormone involved in carbohydrate metabolism? *Am. J. Physiol.* **129**, 659-663.

Löster, K., Zeillinger, K., Schuppan, D. & Reutter, W. (1995) The cysteine-rich region of dipeptidyl peptidase IV (CD26) is the collagen-binding site, *Biochem. Biophys. Res. Comm.* **217**, 341-348.

Marguet, D., Bernard, A.-M., Vivier I., Darmoul, D., Naquet, P. & Pierres, M. (1992) cDNA cloning for mouse thymocyte-activating molecule: a multifunctional ecto-dipeptidyl peptidase IV (CD26) included in a subgroup of serine proteases *J. Biol. Chem.* **267**, 2200-2208.

Martin, E.W., O'Dorisio, T.M., Spaeth, J., Thomford, N.R. & Cataland, S. (1980) The association between endogenous gastric inhibitory polypeptide release and suppression of gastric acid output following intraduodenal fat stimulation, *J. Sur. Res.* **29**, 71-74.

Maxwell, V., Shulkes, A., Brown, J.C., Soloman, T.E., Walsh, J.H. & Grossman, M.I. (1980) Effect of gastric inhibitory polypeptide on pentagastrin-stimulated acid secretion in man, *Dig. Dis. Sci.* **25**, 113-116.

Mazzaferri, E.L., Starich, G.H., Lardinois, C.K. & Bowen, G.D. (1985) Gastric inhibitory polypeptide responses to nutrients in caucasians and american indians with obesity and noninsulin-dependent diabetes mellitus, *J. Clin. Endocrinol. Metab.* **61**, 313-321.

McIntosh, C.H.S., Pederson, R.A., Koop, H. & Brown, J.C. (1981) Gastric inhibitory polypeptide stimulated secretion of somatostatinlike immunoreactivity from the stomch: inhibition by acetylcholine or vagal stimulation, *Can. J. Physiol. Pharm.* **59**, 468-472.

McIntyre, N., Holdsworth, C.D. & Turner, D.S. (1964) New interpretation of oral glucose tolerance, *The Lancet.* **II**, 20-21.

McIntyre, N., Holdsworth, C.D. & Turner, D.S. (1965) Intestinal factors in the control of insulin secretion, *J. Clin. Endocrinol.* **25**, 1317-1324.

Meneilly, G.S., Bryer-Ash, M. & Elahi D. (1993) The effect of glyburide on β-cell sensitivity to glucose-dependent insulinotropic polypeptide, *Diabetes Care.* **16**, 110-114.

Mentlein, R. (1988) Proline residues in the maturation and degradation of peptide hormones and neuropeptides, *FEBS Lett.* **234**, 251-256.

Mentlein, R., Dahms, P., Grandt, D. & Krüger, R. (1993a) Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV, *Reg. Pept.* **49**, 133-144.

Mentlein, R., Gallwitz, B. & Schmidt, W.E. (1993b) Dipeptidyl-peptidase IV hydrolyzes gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum, *Eur. J. Biochem.* **214**, 829-835.

Mentzel, S., Dijkman, H.B.P.M, van Son, J.P.H.F., Koene, R.A.P. & Assmann, K.J.M. (1996) Organ distribution of aminopeptidase A and dipeptidyl peptidase IV in normal mice, *J. Histochem. Cytochem.* **44**, 445-461.

Moens, K., Heimberg, H., Flamez, D., Huypens, P., Quartier, E., Ling, Z., Pipeleers, D., Gremlich, S., Thorens, B. & Schuit, F. (1996) Expression and functional activity of glucagon, glucagon-like peptide I, and glucose-dependent insulinotropic polypeptide receptors in rat pancreatic islet cells, *Diabetes.* **45**, 257-261.

Mojsov, S., Heinrich, G., Wilson, I.B., Ravazzola, M., Orci, L. & Habener, J.F. (1986) Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing, *J. Biol. Chem.* **261**, 11880-11889.

Mojsov, S., Kopczynski, M.G. & Habener, J.F. (1990) Both amidated and nonamidated forms of glucagon-like peptide I are synthesized in the rat intestine and the pancreas, *J. Biol. Chem.* **265**, 8001-8008.

Mojsov, S., Weir, G.C. & Habner, J.F. (1987) Insulinotropin: glucagon-like peptide 1 (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas, *J. Clin. Invest.* **79**, 616-619.

Moore, B. Edie, E.S. & Abram, J.H. (1906) On the treatment of diabetes mellitus by acid extract of duodenal mucous membrane, *Biochem. J.* **1**, 28-38.

Morgan, L.M. (1979) Immunoassayable GIP; investigations into its role in carbohydrate metabolism, *Ann. Clin. Biochem.* **16**, 6-12.

Morgan, L., Elliott, R., Tredger, J., Nightingale, J. & Marks, V. (1993) GLP-1 secretion in response to nutrients in man, *Digestion.* **54**, 374-388.

Morgan, L.M. (1996) The metabolic role of GIP: physiology and pathology, *Biochem Soc. Trans.* **24**, 585-591.

Morgan, L.M., Morris, B.A. & Marks, V. (1978) Radioimmunoassay of gastric inhibitory polypeptide, *Ann. Clin. Biochem.* **15**, 172-177.

Morimoto, C. & Schlossman, S.F. (1995) CD26: a key costimulatory molecule involved in CD4 memory T cell function and activation *in* Dipeptidyl Peptidase IV (CD26) in Metabolism and the Immune Response, Fleischer, B., *ed.* R.G. Landes Biochemical Publishers, Georgetown. pp. 131-143.

Morimoto, C., Lord, C.I., Zhang, C., Duke-Cohan, J.S., Letvin, N.L. & Schlossmann, S.F. (1994) Role of CD26/dipeptidyl peptidase IV in human immunodeficiency virus type 1 infection and apoptosis, *Proc. Natl. Acad. Sci. USA.* **91**, 9960-9964.

Murayama, Y., Kawai, K., Suzuki, S., Ohashi, S. & Yamashita, K. (1990) Glucagon-like peptide-1 (7-37) does not stimulate either hepatic glycogenolysis or ketogenesis, *Endocrinol. Japon.* **37**, 293-297.

Nathan, D.M., Schreiber, E., Fogel, H., Mojsov, S. & Habener J.F. (1992) Insulinotropic action of glucagon-like peptide-1-(7-37) in diabetic and nondiabetic subjects, *Diabetes Care.* **15**, 270-276.

Nauck, M., Stöckmann, F., Ebert, R. & Creutzfeldt, W. (1986) Reduced incretin effect in type 2 (non-insulin-dependent) diabetes, *Diabetologia.* **29**, 46-52.

Nauck, M.A., Bartels, E., Ørskov, C., Ebert, R & Creutzfeldt, W. (1993a) Additive insulinotropic effects of exogenous synthetic human gastric inhibitory polypeptide and glucagon-like peptide-1-(7-36) amide infused at near-physiological insulinotropic hormone and glucose concentrations, *J. Clin. Endorinol. Metab.* **76**, 912-917.

Nauck, M.A., Heimesaat, M.M., Ørskov, C., Holst, J.J., Ebert, R. & Creutzfeld W. (1993b) Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus, *J. Clin. Invest.* **91**, 301-307.

Nauck, M.A., Kleine, N., Ørskov, C., Holst, J.J., Willms, B. & Creutzfeld W. (1993c) Normalization of fasting hyperglycemia by exogenous glucagon-like peptide 1 (7-36 amide) in type 2 (non-insulin dependent) diabetic patients, *Diabetologia.* **36**, 741-744.

Nausch, I., Mentlein, R. & Heymann, E. (1990) The degradation of bioactive peptides and proteins by dipeptidyl peptidase IV from human placenta, *Biol. Chem. Hoppe-Seyler.* **371**, 1113-1118.

Nelson, R.W., McLean, M.A. & Hutchens, T.W. (1994) Quantitative determination of proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Anal. Chem.* **66**, 1408-1415.

O'Dorisio, T.M., Cataland, S., Stevenson, M. & Mazzaferri, E.L. (1976) Gastric inhibitory polypeptide (GIP): intestinal distribution and stimulation by amino acids and medium-chain triglycerides, *Am. J. Dig. Dis.* **21**, 761-765.

O'Halloran, D.J., Nikou, G.C., Kreymann, B., Ghatei, M.A. & Bloom, S.R. (1990) Glucagon-like peptide-1 (7-36)-NH2: a physiological inhibitor of gastric acid secretion in man, *J. Endocrinol.* **126**, 169-173.

Oben, J., Morgan, L., Fletcher, J. & Marks, V. (1991) Effect of the entero-pancreatic hormones, gastric inhibitory polypeptide and glucagon-like polypeptide-1(7-36) amide, on fatty acid synthesis in explants of rat adipose tissue, *J. Endocrinol.* **130**, 267-272.

Ohneda, A., Kobayashi, T. & Nihei, J. (1984) Response of gastric inhibitory polypeptide to fat ingestion in normal dogs, *Reg. Pept.* **8**, 123-130.

Opara, E.C. & Go, V.L.W. (1991) Influence of gastric inhibitory polypeptide (GIP) and glucose on the regulation of glucagon secretion by pancreatic alpha cells, *Reg. Pept.* **32**, 65-73.

Ørskov, C., Bersani, M., Johnsen, A.H., Højrup, P. & Holst, J.J. (1989) Complete sequences of glucagon-like peptide-1 from human and pig small intestine, *J. Biol. Chem.* **264**, 12826-12829.

Ørskov, C., Holst, J.J. & Nielsen, O.V. (1988) Effect of truncated glucagon-like peptide-1 (proglucagon 78-107 amide) on endocrine secretion from pig pancreas, antrum and stomach, *Endocrinol.* **123**, 2009-2013.

Ørskov, C., Holst, J.J., Knuhtsen, S., Baldissera, F.G.A., Poulsen, S.S. & Nielsen, O.V. (1986) Glucagon-like peptides GLP-1 and GLP-2, predicted products of the glucagon gene, are secreted separately from the pig small intestine, but not the pancreas, *Endocrinol.* **119**, 1467-1475.

Ørskov, C., Holst, J.J., Poulsen, S.S. & Kirkegaard, P. (1987) Pancreatic and intestinal processing of proglucagon in man, *Diabetologia.* **30**, 874-881.

Ørskov, C., Jeppesen, J., Madsbad, S. & Holst, J. (1991) Proglucagon products in plasma of noninsulin-dependent diabetics and nondiabetic controls in the fasting state and after oral glucose and intraveonous arginine, *J. Clin. Invest.* **87**, 415-423.

Ørskov, C., Rabenhøj, L., Wettergren, A., Kofod, H., Holst, J.J. (1994) Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans, *Diabetes.* **43**, 535-539.

Ørskov, C., Wettergren, A. & Holst, J.J. (1993) Biological effects and metabolic rates of glucagonlike peptide-1 7-36 amide and glucagonlike peptide-1 7-37 in healthy subjects are indistinguishable, *Diabetes.* **42**, 658-661.

Osei, K., Falko, J.M., O'Dorisio, T.M., Tields, P.G. & Bossetti, B. (1986) Gastric inhibitory polypeptide responses and glucose turnover rates after natural meals in type II diabetic patients, *J. Clin. Endocrinol. Metab.* **62**, 325-330.

Pederson, R.A. (1971) The isolation and physiological actions of gastric inhibitory polypeptide, PhD Dissertation, Vancouver: University of British Columbia.

Pederson, R.A. (1994) Gastric Inhibitory Polypeptide *in* Gut Peptides, Walsh, J.H. and Dochray, G.J. *eds.* Raven Press, New York. pp. 217-259.

Pederson, R.A. & Brown, J.C. (1972) Inhibition of histamine-, pentagastrin-, and insulin-stimulated canine gastric secretion by pure "gastric inhibitory polypeptide", *Gastro-enterol.* **62**, 393-400.

Pederson, R.A. & Brown, J.C. (1976) The insulinotropic action of gastric inhibitory polypeptide in the perfused isolated rat pancreas, *Endocrinol.* **99**, 780-785.

Pederson, R.A. and Brown, J.C. (1978) Interaction of gastric inhibitory polyupeptide, glucose, and arginine on insulin and glucagon secretion from the perfused rat pancreas, *Endocrinol.* **103**, 610-615.

Pederson, R.A., Buchan, A.M.J., Zahedi-Asl, S., Chan, C.B. & Brown, J.C. (1982) Effect of jejunal bypass in the rat on the enteroinsular axis, *Reg. Pept.* **3**, 53-63.

Pederson, R.A., Schubert, H.E. & Brown, J.C. (1975a) Gastric inhibitory polypeptide, its physiological release and insulinotropic action in the dog, *Diabetes.* **24**, 1050-1056.

Pederson, R.A., Schubert, H.E. & Brown, J.C. (1975b) The insulinotropic action of gastric inhibitory polypeptide, *Can. J. Physiol. Pharmacol.* **53**, 217-223.

Perley, M.J. & Kipnis, D.M. (1967) Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic subjects, *J. Clin. Invest.* **46**, 1954-1962.

Piazza, G.A., Callanan, H.M., Mowery, J. & Hixson, D.C. (1989) Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix, *Biochem. J.* **262**, 327-334.

Plaisancie, P., Bernard, C., Chayvialle, J. & Cuber, J. (1994) Regulation of glucagon-like peptide-1-(7-36) amide secretion by intestinal neurotransmitters and hormones in the isolated vascularly perfused rat colon, *Endocrinol.* **135**, 2398-2403.

Plaisancié, P., Dumoulin, V., Chayvialle, J-A. & Cuber, J-C. (1995) Luminal glucagon-like peptide-1(7-36) amide-releasing factors in the isolated vascularly perfused rat colon, *J. Endocrinol.* **145**, 521-526.

Polak, J.M., Bloom, S.R., Kuzio, M., Brown, J.C. & Pearse, A.G.E. (1973) Cellular localization of gastric inhibitory polypeptide in the duodenum and jejunum, *Gut.* **15**, 284-288.

Poulsen, M.D., Hansen, G.H., Dabelsteen, E., Høyer, P.E., Norén, O. & Sjöström, H. (1993) Dipeptidyl peptidase IV is sorted to the secretory granules in pancreatic islet A-cells, *J. Histochem. Cytochem.* **41**, 81-88.

Qualmann, C., Nauck, M.A., Holst, J.J., Ørskov, C. & Creutzfeldt, W. (1995) Glucagon-like peptide 1 (7-36 amide) secretion in response to luminal sucrose from the upper and lower gut: a study using α-glucosidase inhibition (acarbose), *Scand. J. Gastroenterol.* **30**, 892-896.

Reutter, W., Baum, O., Löster, K., Fan, H., Bork, J.P., Bernt, K., Hanski, C. & Tauber, R. (1995) Functional aspects of the three extracellular domains of dipeptidyl peptidase IV: characterization of glycosylation events, of the collagen-binding site and of endopeptidase activity *in* Dipeptidyl Peptidase IV (CD26) in Metabolism and the Immune Response, Fleischer, B., *ed.* R.G. Landes Biochemical Publishers, George-town. pp. 55-78.

Roberge, J.N. & Brubaker, P.L. (1991) Secretion of proglucagon-derived peptides in response to intestinal luminal nutrients, *Endocrinol.* **128**, 3169-3174.

Roberge, J.N. & Brubaker, P.L. (1993) Regulation of intestinal proglucagon-derived peptide secretion by glucose-dependent insulinotropic peptide in a novel enteroendocrine loop, *Endocrinol.* **133**, 233-240.

Ross, S.A. & Dupré, J. (1978) Effects of ingestion of triglyceride or galactose on secretion of gastric inhibitory polypeptide and on responses to intravenous glucose in normal and diabetic subjects, *Diabetes.* **27**, 327-333.

Ross, S.A., Brown, J.C. & Dupré, J. (1977) Hypersecretion of gastric inhibitory polypeptide following oral glucose in diabetes mellitus, *Diabetes.* **26**, 525-529.

Salera, M., Giacomoni, R., Pironi, L., Cornia, G., Capelli, M., Marini, A., Benfenati, F., Miglioli, M. & Barbara, L. (1982) Gastric inhibitory polypeptide release after oral glucose: Relationship to glucose intolerance, diabetes mellitus, and obesity, *J. Clin. Endocrinol. Metab.* **55**, 329-336.

Sarson, D.L., Bryant, M.G. & Bloom, S.R. (1980) A radioimmunoassay of gastric inhibitory polypeptide in human plasma, *J. Endocrinol.* **85**, 487-496.

Schjoldager, B.T.G., Mortensen, P.E., Christiansen, J., Ørskov, C. & Holst. J.J. (1989) GLP-1 (glucagon-like peptide 1) and truncated GLP-1, fragments of human proglucagon, inhibit gastric acid secretion in humans, *Dig. Dis. Sci.* **34**, 703-708.

Schmid, R., Schusdziarra, V., Aulehner, R., Weigert, N. & Classen, M. (1990) Comparison of GLP-1(7-36amide) and GIP on release of somatostatin-like immunoreactivity and insulin from the isolated rat pancreas, *Z. Gastroenterol.* **28**, 280-284.

Schmidt, W.E., Siegel, E.G., Ebert, R. & Creutzfeldt, W. (1986) N-terminal tyrosine-alanine is required for the insulin releasing activity of glucose-dependent insulinotropic polypeptide (GIP), *Eur. J. Clin. Invest.* **16**, A9.

Schön, E., Born, I., Demuth, H.-U., Faust, J., Neubert, K., Steinmetzer, T., Barth, A. & Ansorge, S. (1991) Dipeptidyl peptidase IV in the immune system: effects of specific enzyme inhibitors on activity of dipeptidyl peptidease IV and proliferation of human lymphocytes, *Biol. Chem. Hoppe-Seyler.* **372**, 305-311.

Schön, E., Demuth, H.-U., Eichmann, E., Horst, H.-J., Korner, I.-J., Kopp, J., Mattern, T., Neubert, K., Noll, F., Ulmer, A.J., Barth, A. & Ansorge, S. (1989) Dipeptidyl peptidase IV in human T lymphocytes: impaired induction of interleukin 2 and gamma interferon due to specific inhibition of dipeptidyl peptidase IV, *Scand. J. Immunol.* **29**, 127-132.

Schuerch, S., Schaer, M., Boernsen, K.O. & Schlunegger, U.P. (1994) Enhanced mass resolution in matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometry, *Biol. Mass Spectrom.* **23**, 695-700.

Schulz, T.B., Jorde, R. & Burhol. (1982a) Gastric inhibitory polypeptide release into the portal vein in response to intraduodenal amino acid loads in anesthetized rats, *Scand. J. Gastroenterol.* **17**, 709-713.

Schulz, T.B., Jorde, R., Burhol, P.G. & Christensen, O. (1982b) Augmented release of gastric inhibitory polypeptide into the portal vein in response to intraduodenal glucose and amino acids in anesthetized rats treated with methylprednisolone or alloxan, *Scand. J. Gastroenterol.* **17**, 357-362.

Service, F.J., Rizza, R.A., Westland, R.E., Hall, L.D., Gerich, J.E. & Go, V.L.W. (1984) Gastric inhibitory polypeptide in obesity and diabetes mellitus, *J. Clin. Endocrinol. Metab.* **58**, 1133-1140.

Shima, K., Hirota, M. & Ohboshi, C. (1988) Effect of glucagon-like peptide-1 on insulin secretion, *Reg. Pept.* **22**, 245-252.

Shima, K., Suda, T., Nishimoto, K. & Yoshimoto, S. (1990) Relationship between molecular structures of sugars and their ability to stimulate the release of glucagon-like peptide-1 from canine ileal loops, *Acta Endocrinologica.* **123**, 464-470.

Siegel, E.G., Schulze, A., Schmidt, W.E. & Creutzfeldt, W. (1992) Comparison of the effect of GIP and GLP-1 (7-36amide) on insulin release from rat pancreatic islets, *Eur. J. Clin. Invest.* **22**, 154-157.

Silverman R.B. (1988) The potential use of mechanism-based enzyme inactivators in medicine, *J. Enzyme Inhib.* **2**, 73-97.

Siuzdak, G. (1994) The emergence of mass spectrometry in biochemical research, *Proc. Natl. Acad. Sci. USA.* **91**, 11290-11297.

Soon-Shiong, P., Debas, H.T. & Brown, J.C. (1979) The evaluation of gastric inhibitory polypeptide (GIP) as the enterogastrone, *J. Sur. Res.* **26**, 681-686.

Soon-Shiong, P., Debas, H.T. & Brown, J.C. (1984) Bethanechol prevents inhibition of gastric acid secretion by gastric inhibitory polypeptide, *Am. J. Physiol.* **247**, G171-175.

Starich, G.H.M., Bar, R.S. & Mazzaferri, E.L. (1985) GIP increases insulin receptor affinity and cellular sensitivity in adipocytes, *Am. J. Physiol.* **249**, E603-607.

Subramanyam, M., Bachovchin, W.B. & Huber, B. (1995) CD26, a T cell accessory molecule induction of antigen-specific immune-suppression by inactivation of CD26: a clue to the AIDS paradox? *in* Dipeptidyl Peptidase IV (CD26) in Metabolism and the Immune Response, Fleischer, B., *ed.* R.G. Landes Biochemical Publishers, Georgetown. pp. 155-162.

Suzuki, S., Kawai, K., Ohashi, S., Mukai, H. & Yamashita, K. (1989) Comparison of the effects of various C-terminal and N-terminal fragment peptides of glucagon-like peptide-1 on insulin and glucagon release from the isolated perfused rat pancreas, *Endocinol.* **125**, 3109-3114.

Suzuki, S., Kawai, K., Ohashi, S., Mukai, H., Murayama, Y. & Yamashita, K. (1990) Reduced insulinotropic effects of glucagonlike peptide I-(1-36)-amide and gastric inhibitory polypeptide in isolated perfused diabetic rat pancreas, *Diabetes.* **39**, 1320-1325.

Suzuki, S., Kawai, K., Ohashi, S., Watanabe, Y. & Yamashita, K. (1992) Comparison of the insulinotropic activity of glucagon-superfamily peptides in rat pancreas perfusion, *Horm. Metab. Res.* **24**, 458-461.

Sykes, S., Morgan, L.M., Hampton, S.M. & Marks, V. (1979) Gastric inhibitory polypeptide, C-peptide and insulin: secretory responses of healthy volunteers to standard oral carbohydrate loads, *Clin. Sci.* **52**, 21P.

Tanaka, K., Waki, H., Ido, Y., Akita, S, Yoshida, Y. & Yoshida, T. (1988) Protein and polymer analyses up to *m/z* 100 000 by laser ionization time-of-flight mass spectrometry, *Rapid Commun. Mass Spectrom.* **2**, 151-153.

Tang, K., Allman, S.L., Jones, R.B. & Chen, C.H. (1993) Quantitative analysis of biopolymers by matrix-assisted laser desorption, *Anal. Chem.* **65**, 2164-2166.

Tanizawa, Y., Riggs, A.C., Elbein, S.C., Whelan, A., Donis-Keller, H. & Permutt, M.A. (1994) Human glucagon-like peptide-1 receptor gene in NIDDM: identification and use of simple sequence repeat polymorphisms in genetic analysis, *Diabetes.* **43**, 752-757.

Thim, L. & Moody, A.J. (1981) The primary structure of glicentin (proglucagon), *Reg. Pept.* **2**, 139-151.

Thomas, F.B., Mazzaferri, E.L., Crockett, S.E., Mekhjian, H.S., Gruenmer, H.D. & Cataland, S. (1976) Stimulation of secretion of gastric inhibitory polypeptide and insulin by intraduodenal amino acid perfusion, *Gastroenterol.* **70**, 523-527.

Thomas, F.B., Sinar, D., Mazzaferri, E.L., Cataland, S., Mekhjian, H.S., Caldwell, J.H. & Fromkes, J.J. (1978) Selective release of gastric inhibitory polypeptide by intraduodenal amino acid perfusions in man, *Gastroenterol.* **74**, 1261-1266.

Toft-Nielsen, M., Madsbad, S. & Holst, J.J. (1996) The effect of glucagon-like peptide I (GLP-1) on glucose elimination in healthy subjects depends on the pancreatic glucoregulatory hormones, *Diabetes.* 45, 552-556.

Unger, R.H. & Eisentraut, A.M. (1969) Entero-insular axis, *Arch. Intern. Med.* **123**, 261-266.

Vaillant, C.R. & Lund, P.K. (1986) Distribution of glucagon-like peptide I in canine and feline pancreas and gastrointestinal tract, *J. Histochem. Cytochem.* **34**, 1117-1121.

Valverde, I., Merida, E., Delgado, E., Trapotes, M.A. & Villanueva-Penacarill. M.L. (1993) Presence and characterization of glucagon-like peptide-1 (7-36)amide receptors in solubilized membranes of rat adipose tissue, *Endocrinol.* **132**, 75-79.

van der Burg, M.P.M., Guicherit, O.R., Fröhlich, M. & Gooszen, H.G. (1996) Insulinotropic effects of cholecystokinin, gastric inhibitory polypeptide and glucagon-like peptide-1 during perifusion of short-term cultured canine isolated islets, *Reg. Pept.* **60**, 61-67.

Vanhoof, G., De Meester, I., van Sande, M., Scharpé, S. & Yaron, A. (1992) Distribution of proline-specific aminopeptidases in human tissues and body fluids, *Eur. J. Clin. Biochem.* **30**, 333-338.

Varndell, I.M., Bishop, A.E., Sikri, K.L., Uttenthan, L.O., Bloom, S.R. & Polak, J.M. (1985) Localization of glucagon-like peptide (GLP) immunoreactants in human gut and pancreas using light and electron microscopic immunocytochemistry, *J. Histochem. Cytochem.* **33**, 1080-1086.

Villanueva-Penacarrillo, M.L., Alcantara, A.L., Clemente, F., Delgardo, E. & Valverde, I. (1994) Potent glycogenic effect of GLP-1 (7-36) amide in rat skeletal muscle, *Diabetologia.* **37**, 1163-1166.

Vorm, O. & Roepstorff, P. (1993) Peptide sequence information derived by partial acid hydrolysis and matrix-assisted laser desprption/ionization mass spectrometry, *Biol. Mass Spectrom.* **23**, 734-740.

Walkinshaw, M.D. (1992) Protein targets for structure-based drug design, *Med. Res. Rev.* **12**, 317-372.

Wang, L., Ahmad, S., Benter, I.F., Chow, A., Mitutani, S. & Ward, P.E. (1991) Differential processing of substance P and neurokinin A by plasma dipeptidyl (amino) peptidase IV, aminopeptidase M and angiotensin converting enzyme, *Peptides.* **12**, 1357-1364.

Wang, Z., Wang, R.M., Owji, A.A., Smith, D.M., Ghatei, M.A. & Bloom, S.R. (1995) Glucagon-like peptide-1 is a physiological incretin in rat, *J. Clin. Invest.* **95**, 417-421.

Wasada, T., McCorkle, K., Harris, V. & Kawai, K. (1981) Effect of gastric inhibitory polypeptide on plasma levels of chylomicron triglycerides in dogs, *J. Clin. Invest.* **68**, 1106-1107.

Weir, G.C., Mojsov, S., Hendrik, G.K. & Habner, J.F. (1989) Glucagonlike peptide I (7-37) actions on endocrine pancreas, *Diabetes.* **38**, 338-342.

Wen, J., Phillips, S.F., Sarr, M.G., Kost, L.J. Holst, J.J. (1995) PYY and GLP-1 contribute to feedback inhibition from the canine ileum and colon, *Am. J. Physiol.* **269**, G945-952.

Williams, R.H., May, J.M. & Biesbroeck, J.B. (1981) Determinants of gastric inhibitory polypeptide and insulin secretion, *Metab.* **30**, 36-40.

Willms, B., Ebert, R. & Creutzfeldt, W. (1978) Gastric inhibitory polypeptide (GIP) and insulin in obesity: II. reversal of increased response to stimulation by starvation or food restriction, *Diabetologia.* **14**, 379-387.

Willms, B., Werner, J., Holst, J.J., Ørskov, C., Creutzfeldt, W. & Nauck, M.A. (1996) Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients, *J. Clin. Endocrinol. Metab.* **81**, 327-332.

Wolf, B., Fischer, G. & Barth, A. (1978) Kinetics of dipeptidyl peptidase IV, *Acta Biol. Med. Germ.* **37**, 409-420.

Wrenger, S., Reinhold, D., Hoffmann, T., Kraft, M., Frank R., Faust, J., Neubert, K. & Ansorge, S. (1996) The N-terminal X-X-Pro sequence of the HIV-1 Tat protein is important for the inhibition of dipeptidyl peptidase IV (DP IV/CD26) and the suppression of mitogen-induced proliferation of human T cell, *FEBS Lett.* **383**, 145-149.

Yallow, R.S. & Berson, S.A. (1960) Immunoassay of endogenous plasma insulin in man, *J. Clin. Invest.* **39**, 1157-1175.

Yamashita, M. & Fenn, J.B. (1984) Electrospray ion source. Another variation on the free-jet theme, *J. Phys. Chem.* **88**, 4451 and 4471.

Yamatani, K., Ikezawa, Y., Hama, K., Igarashi, M., Daimon, M. & Sasaki, H., (1996) GLP-1 inhibits glucagon-induced glycogenolysis in rat perivenous hepatocytes specifically, Proceedings of the 10th International Congress of Endocrinology, P2-22.

Yanaihara, C., Matsumoto, T., Kadowaki, M., Iguchi, K. & Yanaihara, N. (1985) Rat pancreas contains the proglucagon (64-69) fragment and arginine stimulates its release, *FEBS Lett.* **185**, 307-310.

Yaron, A. & Naider, F. (1993) Proline-dependent structural and biological properties of peptides and proteins, *Crit. Rev. Biochem. Mol. Biol.* **28**, 31-81.

York, D.A., Steinke, J. & Bray, G.A. (1972) Hyperinsulinemia and insulin resistance in genetically obese rats, *Metab.* **21**, 277-284.

York, J.L. (1992) Enzymes: Classification, kinetics, and control *in* Textbook of Biochemistry With Clinical Correlations, Devlin, T.M., *ed.* Wiley-Lis, Toronto. pp. 135-193.

Zaluzec, E.J., Gage, D.A. & Watson, J.T. (1995) Matrix-assisted laser desorption/ ionization mass spectrometry: applications in peptide and protein characterization, *Protein Express. Purification.* **6**, 109-123.

Zhang, Y., Cook, J.T.E., Hattersley, A.T., Firth, R., Saker, P.J., Warren-Perry, M., Stoffel, M. & Turner, R.C. (1994) Non-linkage of the glucagon-like peptide 1 receptor gene with maturity onset diabetes of the young, *Diabetologia.* **37**, 721-724.

Zunz, E. & La Barre, J. (1929) Contributions à l'étude der variations physiologiques de la sécrétion interne du pancreas: relations entre les sécrétions externe et interne du pancréas, *Arch. Int. Physiol. Biochem.* **31**, 20-44.