

INVESTIGATIONS ON THE MOLECULAR FORMS AND BIOLOGICAL ACTIONS OF THE
GLUCAGON-LIKE PEPTIDES; GLP-1 and ~~GLP-2~~.

SATHISH KURIEN GEORGE

Submitted for the degree of Doctor of Philosophy.

Royal Postgraduate Medical School,
University of London.

ABSTRACT

The structure of mammalian pre-proglucagon has been deduced from nucleotide sequencing of cDNA derived from mammalian glucagon mRNA and genomic DNA. It contains two further glucagon-like peptides, GLP-1 and GLP-2, delimited by pairs of basic amino acid residues, in the region C-terminal to glucagon. This leads to the questions 1) whether these two peptides are actually cleaved off and secreted as free peptides by the glucagon-producing cells of the pancreas and gastrointestinal mucosa, and 2) what their biological activities might be.

Studies with rabbit antisera against synthetic N-terminal fragments and to the subsequently available whole sequences of GLP-1 and GLP-2 showed that human pancreas contained some GLP-1-like immunoreactivity with the chromatographic properties of free GLP-1 whole molecule, but the molar quantity of this was small in relation to that of the total glucagon-like immunoreactivity. A more pronounced deficit was obtained with respect to free GLP-2-like immunoreactivity. These disparities were due in part to the presence of a predominant large molecular size cleavage product containing both GLP-1 and GLP-2 antigenic determinants which was poorly crossreactive with the N-terminal GLP-1 fragment antiserum. In contrast to the pancreas, human and porcine gastrointestinal tract contained mainly free GLP-1 and GLP-2-like immunoreactivities suggestive of a difference in the post-translational processing of proglucagon between the mammalian pancreas and gut. Two glucagonomas contained different

proportions of large molecular size and free GLP-1 immunoreactivities, but untreated glucagonoma patients had higher concentrations of free GLP-1-like immunoreactivity than glucagon in their circulation.

Synthetic GLP-1 did not raise the plasma glucose concentration in cortisone-pretreated rabbits, and only produced a minimal, non-significant elevation of plasma insulin. Furthermore, it did not bind specifically to liver membranes nor stimulate the production of cAMP in isolated rat hepatocytes. In these respects therefore, it has no glucagon-like bioactivity. Low doses of GLP-1 inhibited pancreatic secretion, whereas higher doses stimulated pancreatic juice output; responses not observed with GLP-2. The physiological relevance of these stimulatory effects may however be questioned due to the low tissue concentrations of GLP-1. The observation by others that synthetic GLP-1 and GLP-2 are potent stimulators of rat hypothalamic and pituitary adenylate cyclase suggested that these peptides might modulate the secretion of anterior pituitary hormones. However, neither GLP-1 nor GLP-2 had any effect on the release of growth hormone, prolactin or adrenocorticotrophic hormone from rat anterior pituitary tissue.

The high degree of structural conservation of these peptides in mammals suggests that they have important biological roles. On the basis of their modest biological actions described, it would appear that these, are however, yet to be determined.

DEDICATION

This thesis is dedicated to the memory of my grandfather

Simon Tholath Paul.

CONTENTS

	<u>Page Number</u>
<u>TITLE</u>	I
<u>ABSTRACT</u>	II
<u>DEDICATION</u>	IV
<u>CONTENTS</u>	V
<u>AKNOWLEDGEMENTS</u>	IX
 <u>CHAPTER 1 - GENERAL INTRODUCTION</u>	
Historical review	1
Molecular characterisation of mammalian glucagon-like immunoreactivity (GLI) in:	
a) Porcine gut and pancreas	4
b) Human pancreas	9
c) Human gut	9
d) Human plasma	10
Other sources of GLI	12
Studies on the glucagon precursor	
a) Pulse chase	13
b) Molecular biology	15
i) Anglerfish pre-proglucagon	15
ii) Mammalian pre-proglucagon	21

GLP-1 and GLP-2 - post-translational processing?	23
Physiological effects of glucagon and related peptides	24
GLP-1 and GLP-2 - Biological roles?.....	27

CHAPTER 2 - a) DEVELOPMENT OF RADIOIMMUNOASSAYS FOR THE
GLUCAGON-LIKE PEPTIDES; GLP-1 and GLP-2

General introduction	29
Production and characterisation of antisera	
Introduction	30
Methods	34
Results and Discussion	38
Preparation of I ¹²⁵ -radiolabelled tracer	
Introduction	50
Methods and Results	53
Discussion	62
Assay format	
Introduction	63
Methods	65
Results and Discussion	75

b) EXTRACTION OF GLUCAGON-LIKE PEPTIDE IMMUNO-
REACTIVITY FROM TISSUE

Introduction	77
Methods	78
Results and Discussion	81

CHAPTER 3 - POST-TRANSLATIONAL PROCESSING OF MAMMALIANPROGLUCAGON

Introduction	87
Methods	88
Results	89
Discussion	112

CHAPTER 4 - BIOLOGICAL STUDIES ON THE GLUCAGON-LIKEPEPTIDES

a) EFFECT OF GLP-1 ON CARBOHYDRATE METABOLISM

Introduction	125
Methods	125
Results	127
Discussion	128

b) EFFECT OF GLP-1 ON GUT TROPHISM

Introduction	131
Methods	133
Results	136
Discussion	139

c) EFFECT OF GLP-1 ON ISOLATED RAT HEPATOCYTES AND ITS

EFFICACY IN BINDING LIVER MEMBRANES

Introduction	142
Methods	143
Results	147
Discussion	149

d) EFFECTS OF GLP-1 AND GLP-2 ON PITUITARY HORMONE

SECRETION

Introduction	151
Methods	152
Results	155
Discussion	156

e) EFFECTS OF GLP-1 AND GLP-2 ON EXOCRINE PANCREATIC

SECRETION IN THE RAT

Introduction	167
Methods	170
Results	171
Discussion	179

CHAPTER 5 - GENERAL CONCLUSION 182REFERENCES 188APPENDICES

A) Peptides, chemicals and equipment.....	210
B) Publications	213

ACKNOWLEDGEMENTS

My thanks to Professor S R Bloom, my supervisor, and Drs Otto and Margarita Uttenthal for their help and constructive criticism throughout the course of these studies. Also to Dr Enrique Blazquez for assistance with the receptor work, Dr Eric Adams for help with the pituitary culture studies and my colleagues at the Royal Postgraduate Medical School for their enthusiasm and support.

I am indebted to Alicia Barker for her expert typing, Peter Mulderry and Gerry McGregor for their kindness in proof-reading the manuscript and Kay Davies and Prad Chohan for their help with the artwork.

I also gratefully acknowledge the support of the Medical Research Council of Great Britain in having awarded me a studentship.

Finally, a special thanks to my parents for their encouragement and support throughout my education.

CHAPTER 1

GENERAL INTRODUCTION

Historical Review

The administration of the first insulin preparations for the treatment of diabetic patients was observed to induce a transient hyperglycaemia before lowering blood glucose levels. This hyperglycaemic factor in crude insulin preparations was suggested to be a second pancreatic hormone and given the name "glucagon" (Murlin et al 1923). It was successfully purified by Staub et al (1955) (employing repeated precipitations culminating in its crystallisation), and the amino acid sequence of porcine glucagon was established by Bromer et al (1956). It was shown to be a single chain polypeptide composed of 29 amino acids with a molecular weight of 3,485 daltons (see fig 1:1). This sequence was also thought to be conserved in other mammalian glucagons due to their almost identical amino acid compositions (Sundby 1976a).

Localising the site of origin of glucagon to the pancreatic A cell was largely accomplished by the work of Bencosme and colleagues (1955). Using regional extracts of the pancreas, they were able to correlate hyperglycemia inducing activity with the regional A cell concentrations. Confirmatory evidence for their findings was provided by Unger and co-workers (1959), who applied the principles of radioimmunoassay to the measurement of pancreatic glucagon, and by Baum et al (1962), who used immunofluorescence techniques to provide the first direct evidence for the production of glucagon in pancreatic A cells.

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-
Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr.

Figure 1:1 The amino acid sequence of porcine glucagon
according to Bromer et al (1956).

Arg-Ser-Leu-Gln-Asn-Thr-Glu-Glu-Lys-Ser-Arg-Ser-Phe-Pro-Ala-Pro-
Gln-Thr-Asp-Pro-Leu-Asp-Asp-Pro-Asp-Gln-Met-Thr-Glu-Asp-Lys-Arg-
His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-
Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-Lys-Arg-Asn-
Lys-Asn-Asn-Ile-Ala.

Figure 1:2 The amino acid sequence of porcine glicentin
according to Thim and Moody (1981).

However, although it was now increasingly accepted that the A cell of the Islets of Langerhans of the pancreas was the sole site of origin of glucagon, this dogma was challenged by Unger and co-workers (1961) when they reported that intestinal extracts of dog and man contained immunoreactive material which crossreacted in their glucagon radioimmunoassay. Subsequently, it was demonstrated that this "gut-glucagon-like" immunoreactivity (GLI or enteroglucagon) consisted of at least two molecular species, GLI I (M.wt 7,000) and GLI II (M.wt 3,500), both of which differed immunochemically from pancreatic glucagon based on their varying degrees of cross-reactivity with certain glucagon antisera (Valverde et al 1968). Their localisation to intestinal L cells morphologically distinct from the pancreatic A cell was accomplished by Sasaki and colleagues (1975).

Immunisation with glucagon mainly evokes the formation of two classes of antibodies in animals. The most common type of glucagon antiserum reacts with both glucagon and materials in extracts of the intestinal tract, whereas a few antisera react exclusively with glucagon. Assan and Slusher (1972), using fragments of the glucagon sequence demonstrated that glucagon antisera which react with gut extracts are directed towards the N-terminus and mid-portion of the glucagon molecule, whilst the other class of antisera that react exclusively with glucagon are directed towards the C-terminal end. This resulted in their being classified as the "N-terminal specific" and "C-terminal specific" glucagon antisera. These findings were further extended by the work of Heding et al (1976) who showed that the C-terminal immunodeterminant of glucagon lies within residues 24-29 of its sequence, whilst Yanaihara et al

(1979) suggested the N-terminal immunodeterminant to include residues 11-16 of the glucagon sequence. However, the crossreaction of glucagon-like material in canine stomach (Orci et al 1968) and the plasma of pancreatectomised dogs (Matsuyama & Foá 1974) with C-terminal specific glucagon antisera, emphasised the inadequacy of differentiating between pancreatic and gut glucagon immunoreactivities, solely on the basis of the specificity of glucagon antisera.

Molecular characterisation of mammalian glucagon-like immunoreactivity

a) Porcine Gut and Pancreas

The first of the gut GLI's to be isolated in a highly purified form was the porcine peptide named "glicentin" (Sundby et al 1976b), equivalent in size and immunochemical character to the previously identified GLI-I (Valverde et al 1968). Its initial partial amino acid sequencing, identified a C-terminal decapeptide (Jacobsen et al 1977) closely resembling that of a putative fragment of porcine/bovine proglucagon, isolated by Tager & Steiner (1973). From this information, together with the finding that an anti-glicentin serum detected a glicentin-like component in the pancreatic A cell, it was proposed that glicentin could contain the full sequence of glucagon (Moody et al 1977) and fulfill the role of a biosynthetic precursor of glucagon in the porcine pancreas (Moody et al 1978).

Proof that glicentin contains the full sequence of glucagon has been

obtained by determining its amino acid sequence (Thim & Moody 1981). It is composed of 69 amino acid residues (see fig 1:2) where glucagon forms the sequence 33-61, delimited by pairs of basic amino acid residues known to be cleavage points in the post-translational processing of proinsulin and other newly synthesised hormones (Steiner et al 1980). The N-terminal portion (residues 1-30) comprises the sequence of porcine glicentin-related pancreatic polypeptide (GRPP - Thim & Moody 1982) and its synchronous and equimolar secretion with glucagon by the porcine pancreas has been shown (Moody et al 1981a).

This led to a hypothetical model for the post-translational processing of the proglucagon molecule being proposed, which equated glicentin to the common prohormone, or at least a fragment thereof, which gives rise to both the pancreatic peptides GRPP and glucagon, and to the other enteroglucagons apart from glicentin itself (Moody et al 1981b).

The second most prominent component of porcine enteroglucagon (GLI-II) has been isolated from extracts of the porcine small intestine. On the basis of its ability to stimulate cAMP production in rat fundic glands, it was named "Oxyntomodulin" (Bataille et al 1981a). Using finger printing techniques and partial sequence analysis this peptide was found to consist of glucagon extended at its C-terminus by the carboxy-terminal octapeptide of glicentin ie is glicentin 33-69 (Bataille et al 1982). It was identical to the proglucagon fragment previously isolated from a mixed bovine/porcine pancreatic glucagon preparation (Tager & Steiner 1973), except for

an inversion of two amino acids in its C-terminal octapeptide, easily explained as a misinterpretation of equivocal carboxypeptidase sequencing data.

In addition to the N- and C-terminal specific glucagon antisera, the use of the R64 antiserum to glicentin reacting with an immunodeterminant in the N-terminal non-glucagon part of the glicentin sequence (Thim & Moody 1982) and another antiserum (4804) reacting with the C-terminal octapeptide extension of glucagon (Yanaihara et al 1979), made it possible to characterise the porcine enteroglucagons by the presence or absence of these sequences. Gel-filtration of porcine intestinal extracts (see fig 1:3) allowed for the following observations to be made (review - Holst 1983a).

I: The predominating component A, ($K_{av} = 0.25$), contained both the N- and C-terminal glicentin immunodeterminants, in addition to the N-terminal glucagon determinant. This confirmed that this component corresponded to glicentin in molecular size and immunochemical character.

II: Component B, consisting of about a fifth of the immuno-reactivity of component A, also contained both glicentin immunodeterminants, and the N-terminal glucagon immunodeterminant. It was therefore thought to represent a fragment of glicentin, from which part of the N-terminal sequence had been cleaved. Possible cleavage sites are the basic amino acid residues at positions 9 and 11, which might leave behind a molecule of $K_{av} 0.36$, as observed.

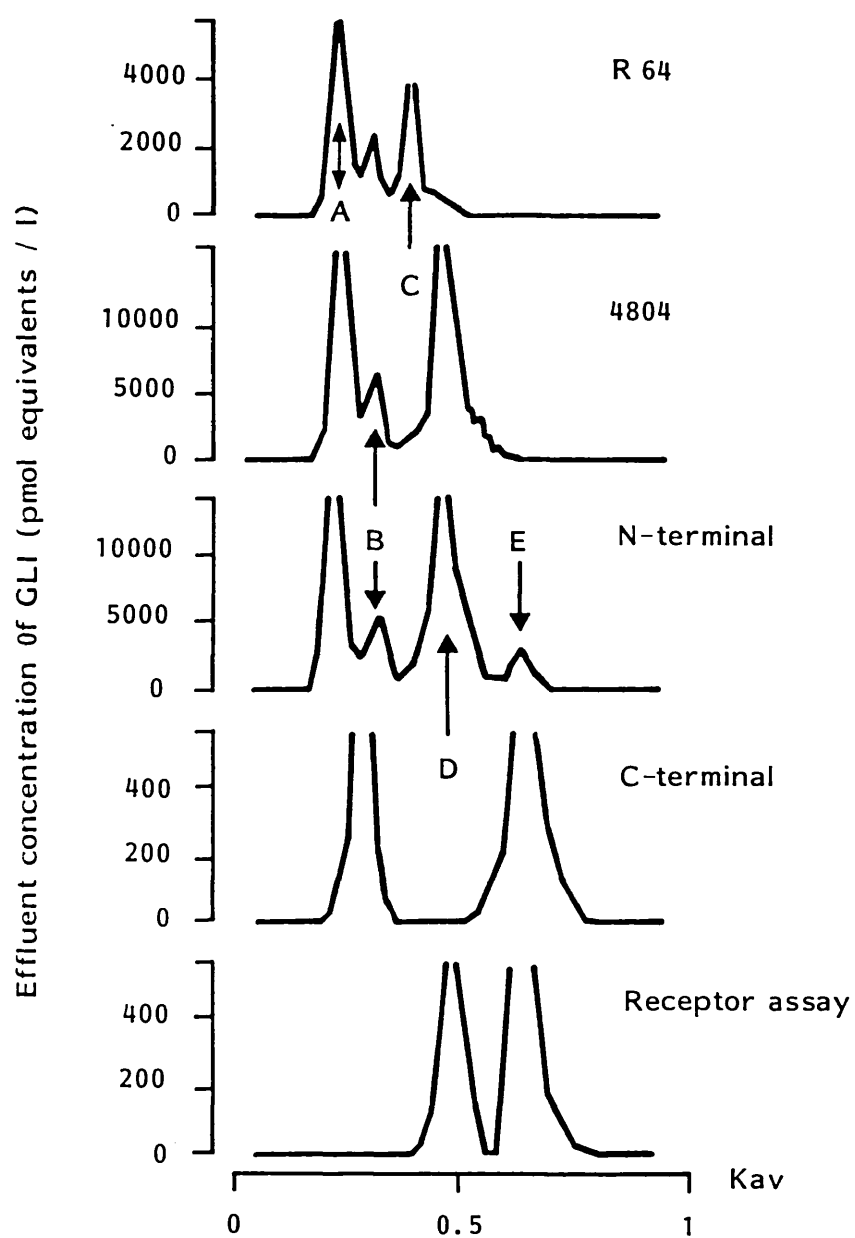


Figure 1:3 Molecular forms of glucagon-like immunoreactivity identified by combined chromatographic and immunochemical analysis of porcine intestinal extracts as per (Holst 1983a)

III: Component C, of K_{av} 0.40

glicentin immunodeterminant, and was therefore likely to correspond to GRPP.

IV: Component D, the second most prominent of these gel filtration peaks (K_{av} = 0.45), retained only the C-terminal glicentin and N-terminal glucagon immunodeterminants. This suggested that it might correspond to glicentin 33-69, the glucagon sequence with the C-terminal octapeptide extension, also known as oxyntomodulin.

V: Component E, the least prominent of these peaks, although containing both glucagon immunodeterminants was devoid of those for glicentin, thereby equating itself with glicentin 33-61, the glucagon sequence.

Further studies on component D using radioreceptor analysis and enzymatic digestion, confirmed that this peak II GLI was indeed identical to the glicentin sequence 33-69, oxyntomodulin (Holst 1982a).

The fact that the receptor assay, as well as the assay for the C-terminal immunodeterminant of glucagon both require an intact and free C-terminus for effective binding, and the co-elution of component E with glucagon on gel filtration, clearly indicate that this component corresponds to glicentin 33-61 or intact glucagon (Holst 1983b). However, it must be emphasized that its presence in mammalian gut is usually at very low concentrations (less than 5% of the total GLI - Holst 1977), and the only species having appreciable

amounts of glucagon in the gut are the dog and cat, where it is largely confined to the stomach (Srikant et al 1977).

b) Human Pancreas

The molecular heterogeneity of glucagon-like immunoreactivity (GLI) in humans, has not been as extensively documented as in the pig. Gel filtration of human pancreatic extracts revealed the presence of a major component co-eluting with glucagon, accompanied by two other larger molecular weight components. All of these possessed the C-terminal glucagon immunodeterminant confirming the presence of the free C-terminus of glucagon, and also the corresponding N-terminal immunodeterminant as recognised by the R59 antiserum (Ghatei et al 1983). This antiserum (R59) shows an equivalence in its crossreaction with both pancreatic glucagon and glicentin and therefore measures total GLI. The evidence therefore suggests that the human pancreas contains, other than glucagon, mainly variable N-terminal extensions of the glucagon sequence, and no component of a similar immunochemical and chromatographic character to glicentin.

c) Human gut

Similar analysis of human intestinal extracts using N- and C-terminally directed glucagon and glicentin antisera revealed very small amounts of glucagon related peptides in the gastric mucosa (in contrast to the dog and cat), whereas at least four different components could be identified in the distal intestine (Baldissera & Holst 1984). The major component is similar in size to porcine glicentin, but differs from it in the non-glucagon part of its N-terminal extension as it does not crossreact with the glicentin antiserum that recognises GRPP. The second corresponds closely to

oxyntomodulin, whilst the third component exhibited characteristics similar to those of glucagon; findings which have since been confirmed by other workers (Munck et al 1984). The fourth component contained the sequence of glucagon plus an N-terminal extension.

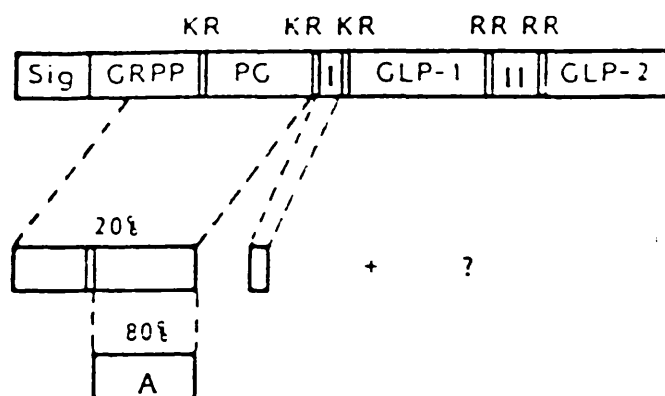
In summary, combined immunochemical and chromatographic studies clearly indicate that the post-translational processing of proglucagon differs quite markedly between the mammalian gut and pancreas (see fig 1:4). Glicentin and oxyntomodulin like components are prominent in the intestinal mucosa, whereas components resulting from cleavage at the C-terminus of the glucagon sequence are more characteristic of the pancreas.

d) Human plasma

Peripheral venous plasma from normal human subjects was shown on gel filtration to contain two principal molecular species of GLI (apart from void volume material), containing the N-terminal glucagon immunodeterminant. The major component had an elution position similar to that of porcine glicentin, whilst the second component was more characteristic of oxyntomodulin; neither component reacting with the C-terminal glucagon antiserum (Ghatei et al 1983).

The presence of a major glicentin-like plasma component corresponding in size to the major component found in human intestine but dissimilar to any component found in the human pancreas, is suggestive of the intestinal origins of this circulating glucagon-like immunoreactivity. These findings, along with the demonstration that increases in plasma glucagon-like immunoreactivity after the stimulus of a mixed meal, are entirely accounted for by a corresponding increase in this large molecular

PANCREAS



GUT

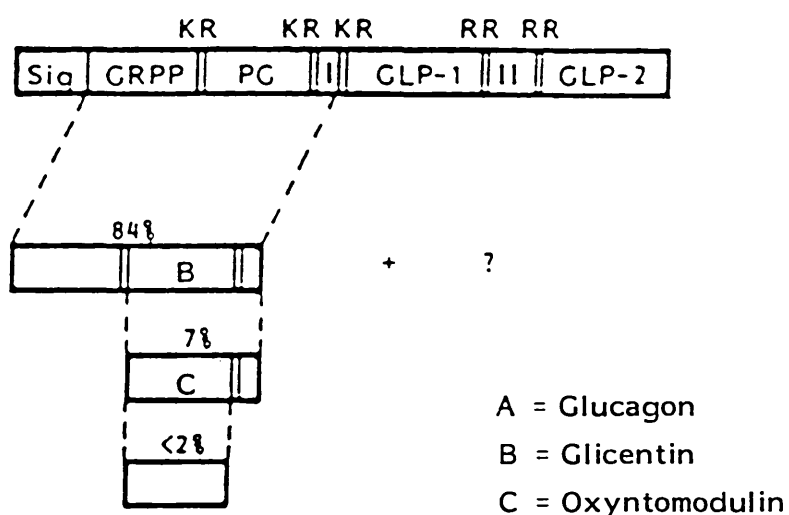


Figure 1:4 Schematic representation of the post-translational processing of the N-terminal portion of pre-proglucagon within the mammalian gut and pancreas. The quantity recovered of each component is expressed as a percentage of the total recovered GLI.

weight glicentin-like circulating component, is suggestive of its hormonal status (Ghatei et al 1983).

e) Other sources of glucagon-like immunoreactivity

The distribution of glucagon-like immunoreactivity in mammals, is however, not entirely restricted to the pancreas and gut. The presence of a glucagon-related peptide in rat submaxillary salivary glands was first claimed by Dunbar and colleagues (1976). Active glucagon biosynthesis within this tissue was demonstrated by the incorporation of L-tryptophan-³H into a 3,500 dalton polypeptide, which contained the C-terminal glucagon immunodeterminant (Perez Castillo & Blazquez 1980a). Extracts of rat thymus, adrenals, thyroid and hypophysis also contain glucagon related peptides (Perez Castillo & Blazquez 1980b).

Immunoreactive glucagon has also been found in the canine hypothalamus, amygdala and mesencephalon (Conlon et al 1979). Similarly, the presence of glucagon-related peptides in rat hypothalamus, cortex, thalamus and brain stem, crossreacting with glucagon antisera directed towards the central portion of the hormone but not with antisera specific for the free C-terminus of glucagon has been reported (Tager et al 1980). Digestion of these peptides with trypsin plus carboxypeptidase B released the immunoreactive C-terminal tryptic fragment of pancreatic glucagon from these larger forms. This suggests that rat brain may contain glucagon-like peptides which result from a type of post translational processing similar to that which occurs in the intestine (Tager et al 1980).

The Glucagon Precursor - Pre-proglucagon

a) Pulse-chase studies

When investigations on the mechanism of glucagon biosynthesis were first launched, they were directed towards a search for larger precursors and their conversion to the native hormone. This approach was inspired not only by the discovery of proinsulin some years previously, (Chance et al 1968), but also by the demonstration of a large molecular weight glucagon-like immunoreactive component in extracts of dog and human plasma and pancreas (Valverde et al 1970; Rigopoulou et al 1970).

Noe and Bauer (1971), employing the technique of pulse-chase labelling for studying the biosynthesis of glucagon in anglerfish islets, were the first to observe the incorporation of ^3H -labelled tryptophan into glucagon-related proteins with a considerably higher molecular weight than native glucagon. Tryptic digestion of this material resulted in the generation of a radioactive component with the molecular weight, immunochemical characteristics and electrophoretic mobility of glucagon, and this initial data strongly suggested that a larger peptide did serve as a precursor in the formation of glucagon. In further studies on newly synthesised anglerfish islet proteins, Noe and Bauer demonstrated that two glucagon immunoreactive molecules of approximate molecular weights of 12,000 and 9,000 daltons were being synthesised during a 20 minute pulse incubation with ^3H -tryptophan. During the following chase incubation with non-radioactive tryptophan, there was a gradual appearance of radioactivity in a glucagon immunoreactive peptide with the approximate size of glucagon, and a concomitant

disappearance of radioactivity in the 12,000 and 9,000 dalton peptides (Noe & Bauer 1975).

Using fluorography of labelled proteins separated by SDS slab-gel electrophoresis, immunoprecipitation and tryptic peptide analysis, Patzelt and co-workers (1979) studied the synthesis of glucagon-related proteins in pulse-chase experiments with isolated rat islets. Using these sensitive methods, they identified a family of peptides which were assigned the roles of precursors and intermediates in the formation of glucagon. The most rapidly formed of these glucagon immunoreactive peptides had a molecular weight of 18,000 daltons. Its rapid disappearance in pulse-chase experiments and its structural similarities to both glucagon and another peptide appearing during the chase period, were consistent with its proposed role as proglucagon. It was noteworthy that this 18,000 dalton protein underwent a small post-translational increase in molecular weight, prior to its disappearance after a chase period of 30-120 min. The nature of this modification was not identified and attempts to verify the involvement of a glycosylation process failed.

It was also observed in further pulse-chase studies, that following the post-translational increase in molecular weight of this 18,000 dalton protein, cleavage of a labelled component with a molecular weight of about 10,000 daltons takes place, followed by the appearance of label in native glucagon. This 10,000 dalton component lacked glucagon immunoreactivity, although its relationship to proglucagon was confirmed by tryptic peptide analyses. It was therefore believed to be a by product released

during the cleavage of the precursor. Small amounts of glucagon-related intermediates of 13,000 and 4,500 daltons were also identified, but it was not ascertained whether these were true intermediates or side products (Patzelt et al 1980). Therefore, although similarities in glucagon biosynthesis were shown to exist between anglerfish and mammalian islets, the mammalian precursor was concluded to be considerably larger.

b) Molecular biology studies

Recently, techniques of molecular biology have been applied to islets from anglerfish. These islets are separate from exocrine tissue and produce significant amounts of fish glucagon. Hence, the fraction of total islet mRNA which codes for glucagon is large. This has facilitated its use in translation studies and aided in the subsequent characterisation of glucagon encoding mRNAs.

i) Anglerfish Pre-proglucagon

The products of translation of total polyadenylated mRNA from anglerfish islets, as they were translated in a cell-free wheatgerm system were studied by Lund and colleagues (1980). They were able to identify, by immunoprecipitation with a glucagon antiserum, two proteins of 14,500 and 12,500 daltons. The addition of microsomal membranes to the translation products increased the quantity of the 12,500 dalton component, consistent with the cleavage of a leader sequence of a molecular weight of 2,000 daltons from the 14,500 dalton pre-proglucagon component (Blobel 1980).

In their further studies, Lund and colleagues prepared two separate complementary DNAs by reverse transcription, insertion into plasmids and cloning in bacteria. On partial analysis of the base sequence

of the two cDNAs, they concluded that two separate but partially homologous mRNAs encoded the two glucagon precursors they had previously identified (Lund et al 1981a). Their further efforts enabled them to determine the sequence of the cDNA that corresponded to the mRNA encoding the 14,500 dalton pre-proglucagon (anglerfish pre-proglucagon I), and thus its amino acid sequence (see fig 1:5) was deduced (Lund et al 1982a).

This polypeptide of 124 amino acids, starts with a sequence of predominantly hydrophobic amino acid residues, characteristic of the N-terminal leader or signal sequences of precursors of secreted proteins (Blobel 1980). This is followed by a 30 amino acid sequence and then -Lys-Arg-, which forms a potential cleavage site during the post-translational processing of hormone precursors (Steiner et al 1980). A sequence of 29 amino acids showing a 69% homology with mammalian glucagon (see table 1:1) and a close similarity to the previously reported sequence of anglerfish glucagon (Trakatellis et al 1975) follows, then a second -Lys-Arg- sequence, a pentapeptide, a third -Lys-Arg-, and finally a sequence of 34 amino acids, which shows remarkable homology with other members of the glucagon family, in particular, gastric inhibitory polypeptide (GIP).

Subsequent hybridisation experiments demonstrated that the two pre-proglucagons were encoded by at least two different genes (Lund et al 1982b), and the cDNA coding for the 12,500 dalton component (anglerfish islet pre-proglucagon II) has recently been sequenced (Lund et al 1983). These workers, were also able to identify anglerfish intestinal glucagon mRNA by hybridisation with the islet

	100	110	120
	→		← ISP-II
Anglerfish 1	D <u>Q</u> <u>A</u> <u>I</u> <u>K</u> D <u>F</u> V D R <u>L</u> K A <u>G</u> <u>Q</u> V	R R E	
Anglerfish 2	D <u>Q</u> <u>A</u> <u>A</u> <u>K</u> D <u>F</u> V S <u>W</u> <u>L</u> K A <u>G</u> <u>R</u> V	R R E	
Hamster	<u>G</u> <u>Q</u> <u>A</u> <u>A</u> <u>K</u> <u>E</u> <u>F</u> <u>I</u> <u>A</u> <u>W</u> <u>L</u> <u>V</u> <u>K</u> <u>G</u> <u>R</u> <u>G</u>	R R	<u>D</u> <u>F</u> <u>P</u> <u>E</u> <u>E</u> <u>V</u> <u>T</u> <u>I</u> <u>V</u> <u>E</u>
Human	<u>G</u> <u>Q</u> <u>A</u> <u>A</u> <u>K</u> <u>E</u> <u>F</u> <u>I</u> <u>A</u> <u>W</u> <u>L</u> <u>V</u> <u>K</u> <u>G</u> <u>R</u> <u>G</u>	R R	<u>D</u> <u>F</u> <u>P</u> <u>E</u> <u>E</u> <u>V</u> <u>A</u> <u>I</u> <u>V</u> <u>E</u>
Ox	<u>G</u> <u>Q</u> <u>A</u> <u>A</u> <u>K</u> <u>E</u> <u>F</u> <u>I</u> <u>A</u> <u>W</u> <u>L</u> <u>V</u> <u>K</u> <u>G</u> <u>R</u> <u>G</u>	R R	<u>D</u> <u>F</u> <u>P</u> <u>E</u> <u>E</u> <u>V</u> <u>N</u> <u>I</u> <u>V</u> <u>E</u>
Rat	<u>G</u> <u>Q</u> <u>A</u> <u>A</u> <u>K</u> <u>E</u> <u>F</u> <u>I</u> <u>A</u> <u>W</u> <u>L</u> <u>V</u> <u>K</u> <u>G</u> <u>R</u> <u>G</u>	R R	<u>D</u> <u>F</u> <u>P</u> <u>E</u> <u>E</u> <u>V</u> <u>A</u> <u>I</u> <u>A</u> <u>E</u>

	130	140
	→	← GLP-2
Hamster	<u>E</u> <u>L</u> <u>G</u> R R	<u>H</u> <u>A</u> <u>D</u> <u>G</u> <u>S</u> <u>F</u> <u>S</u> <u>D</u> <u>E</u> <u>M</u> <u>N</u> <u>T</u> <u>I</u> <u>L</u> <u>D</u> <u>S</u> <u>L</u> <u>A</u> <u>T</u> <u>R</u> <u>D</u> <u>F</u> <u>I</u>
Human	<u>E</u> <u>L</u> <u>G</u> R R	<u>H</u> <u>A</u> <u>D</u> <u>G</u> <u>S</u> <u>F</u> <u>S</u> <u>D</u> <u>E</u> <u>M</u> <u>N</u> <u>T</u> <u>I</u> <u>L</u> <u>D</u> <u>N</u> <u>L</u> <u>A</u> <u>A</u> <u>R</u> <u>D</u> <u>F</u> <u>I</u>
Ox	<u>E</u> <u>L</u> R R R	<u>H</u> <u>A</u> <u>D</u> <u>G</u> <u>S</u> <u>F</u> <u>S</u> <u>D</u> <u>E</u> <u>M</u> <u>N</u> <u>T</u> <u>V</u> <u>L</u> <u>D</u> <u>S</u> <u>L</u> <u>A</u> <u>T</u> <u>R</u> <u>D</u> <u>F</u> <u>I</u>
Rat	<u>E</u> <u>L</u> <u>G</u> R R	<u>H</u> <u>A</u> <u>D</u> <u>G</u> <u>S</u> <u>F</u> <u>S</u> <u>D</u> <u>E</u> <u>M</u> <u>N</u> <u>T</u> <u>I</u> <u>L</u> <u>D</u> <u>N</u> <u>L</u> <u>A</u> <u>T</u> <u>R</u> <u>D</u> <u>F</u> <u>I</u>

	150	160
		
Hamster	<u>N</u> <u>W</u> <u>L</u> <u>I</u> <u>Q</u> <u>T</u> <u>K</u> <u>I</u> <u>T</u> <u>D</u> <u>K</u> <u>K</u>	
Human	<u>N</u> <u>W</u> <u>L</u> <u>I</u> <u>Q</u> <u>T</u> <u>K</u> <u>I</u> <u>T</u> <u>D</u> <u>R</u>	
Ox	<u>N</u> <u>W</u> <u>L</u> <u>L</u> <u>Q</u> <u>T</u> <u>K</u> <u>I</u> <u>T</u> <u>D</u> <u>R</u> <u>K</u>	
Rat	<u>N</u> <u>W</u> <u>L</u> <u>I</u> <u>Q</u> <u>T</u> <u>K</u> <u>I</u> <u>T</u> <u>D</u> <u>K</u> <u>K</u>	

Amino acid sequence Homologies (%) between preproglucagons

	<u>Anglerfish-I</u>	<u>Anglerfish-II</u>	<u>Hamster</u>	<u>Ox</u>	<u>Rat</u>	<u>Pig</u>
<u>Human</u>						
Signal peptide	30	25	65	95	80	?
GRPP	10	13	83	77	67	77
Glucagon	69	76	100	100	97	100
ISP-I	0	17	100	83	100	83
GLP-1	61	71	100	100	100	100
ISP-II	-	-	92	85	85	?
GLP-2	-	-	91	85	97	?

Table 1:1 indicates the percentage homologies present between the constituent amino acid sequences of human preproglucagon and preproglucagons from other species.
ISP = intervening "spacer" peptide.

cDNA coding for anglerfish pre-proglucagon I (Lund et al 1981b), and deduced that its size was similar to that of islet mRNA (650 base pairs). It was therefore concluded that a gene encoding glucagon was expressed in anglerfish intestine, and that the respective intestinal and islet mRNAs were homologous.

ii) Mammalian pre-proglucagon

The application of similar genetic techniques has enabled the structure of mammalian preproglucagon to be deduced from the nucleotide sequencing of cDNA derived from hamster glucagon mRNA (Bell et al 1983a), the human preproglucagon gene (Bell et al 1983b) or glucagon mRNA (Lopez et al 1983) and rat glucagon mRNA (Heinrich et al 1984). Like the anglerfish proglucagon precursors, mammalian pre-proglucagon contains a signal peptide and a 30 amino acid GRPP-like peptide, linked to the glucagon sequence via -Lys-Arg-residues. In the region C-terminal to the glucagon sequence, separated from glucagon and each other by spacer peptides, and delimited by pairs of basic amino acid residues that form potential cleavage sites (Steiner et al 1980), are the sequences of two further peptides, glucagon-like peptide-I (GLP-1) and glucagon-like peptide-2 (GLP-2). ie mammalian pre-proglucagon contains an additional glucagon-like peptide (GLP-2) which is not present in the anglerfish (see fig 1:5)

The amino acid sequence of GLP-1 is completely conserved between the four mammalian species studied, whilst human GLP-2 exhibits homologies of 91%, 85% and 97% respectively with the GLP-2 sequences in hamster, ox and rat pre-proglucagon (see table 1:1).

The degree to which these peptides have been conserved in mammals, is therefore comparable with that of glucagon itself, which shows complete homology between these species except for a single amino acid substitution in the rat. In mammals therefore, it appears that the sequence of GLP-1 is even better conserved than that of glucagon (see table 1:1)

GLP-1 shows 61% and 71% homology with the comparable 31 amino acid residue peptide sequences in the two forms of anglerfish pre-proglucagon. This degree of conservation is only slightly less than that of human glucagon which shows 69% and 76% homology with the two anglerfish forms. It therefore seems likely, that GLP-1 fulfils a significant biological role, which has provided the evolutionary constraint to conserve its structure. The same argument applies with correspondingly less force to GLP-2, which is less well conserved and not present in the anglerfish. The question thus arises, of what the biological roles of GLP-1 and GLP-2 might be?

Although the post-translational processing of the N-terminal 69 amino acids of mammalian proglucagon corresponding to glicentin in the pig has been extensively studied, the fate of the C-terminal portion of the precursor containing the glucagon-like peptide 1 and 2 sequences is unknown. The deduced sequence of human pre-proglucagon cannot tell us where cleavage within the C-terminal half of the molecule actually takes place. GLP-1 is defined by -Lys-Arg- residues preceding its presumed N-terminus and by -Arg-Arg- residues following its presumed C-terminal glycyl

residue. Similarly, GLP-2 is defined by -Arg-Arg- residues preceding its N-terminus. Whereas the known cleavage points in the N-terminal region of proglucagon are -Lys-Arg- residues, it is not known whether cleavage takes place at -Arg-Arg- residues within the A or L cell golgi apparatus and secretory granules, and certainly the -Arg-Arg- residues at positions 17 and 18 of the glucagon sequence would not seem to form a cleavage site.

One of the primary aims of this thesis, is to establish how the C-terminal portion of mammalian pre-proglucagon is processed. From pulse-chase studies with rat islets (Patzelt et al 1980), ³H-tryptophan was observed to be incorporated into a component of a molecular size of approximately 10,000 daltons. However, this component was not reactive with glucagon antisera, and was at that time ascribed to a previously unidentified N-terminal extension of the glucagon sequence which was released as a by-product on the conversion of proglucagon to glucagon (Patzelt et al 1980). In the light of the deduced sequence of mammalian pre-proglucagon, it may now be postulated that this 10,000 dalton component might correspond to the combined sequences of GLP-1 and GLP-2, and points to the production of proglucagon 72-160 by the mammalian pancreas.

The high degree of sequence homology observed between glicentin and residues 1-69 of pancreatic proglucagon strongly suggests that the glucagon gene is expressed in the gut, and that pancreatic glucagon and the enteroglucagons are derived by tissue-specific processing of the same gene product. Furthermore, in support of this hypothesis, no evidence from the screening of human and rat genomic DNA libraries has been obtained to suggest that there is more than

a single pre-proglucagon gene in mammals (Heindrich et al 1984). Components of intestinal origin related to the GLP-1 and GLP-2 sequences may also therefore be produced. If so, it may be of physiological relevance to determine whether (as with the N-terminal portion), differences in processing of the C-terminal portion of proglucagon occur between the mammalian gut and pancreas.

Physiological Effects of Glucagon and the Glucagon-Related Peptides

a) Glucagon

The liver is the first organ encountered by glucagon after its secretion by the pancreas, and as is well documented, the action of glucagon on the liver results in increased glucose production (review - Park and Exton, 1972). This effect observed on isolated hepatocytes, isolated perfused liver and in in vivo animal experiments has also been demonstrated in man (Bomboy et al 1977). These authors showed that net splanchnic glucose production increased 2.3 fold after intravenous infusion of glucagon (5 ng/kg/min). Furthermore, it has also shown that the effect of exogenous hyperglucagonemia on splanchnic glucose output is evanescent, and despite ongoing infusion of glucagon, splanchnic glucose output returns to baseline in 30 minutes (Felig et al 1976).

The stimulation of hepatic glucose output by glucagon involves inhibition of liver glycogen synthesis, accompanied by both the stimulation of liver glycogenolysis and gluconeogenesis (Park &

Exton 1972). It is a receptor-mediated process involving the binding of glucagon to its specific receptors on hepatocyte membranes. As a result, adenylate cyclase, the enzyme responsible for the synthesis of cAMP, (the second messenger initiating the catabolism of glycogen), is activated (Rodbell et al 1971). Glucagon receptors on liver membranes have been used to develop a radioreceptor assay for glucagon. This assay has also been employed to assess the structural requirements for binding of glucagon analogues, and as an additional means of characterising chromatographically isolated glucagon-like immunoreactivity (Holst 1983b).

Glucagon stimulates the hydrolysis of adipose tissue triglycerides into glycerol and free-fatty acids. This lipolysis is also a receptor-mediated process; the binding of glucagon to specific adipocyte membrane receptors initiating the cAMP-dependent "lipolytic cascade" (review - Lefebvre 1975). Glucagon may also play a part in the promotion of ketogenesis by activating the carnitine acyltransferase system of enzymes, thereby accelerating the formation of acetoacetic and beta-hydroxybutyric acids (McGarry et al 1975). Enhancement of insulin secretion by the beta cells of the islets of Langerhans is effected by glucagon, and it has thus been proposed to be part of a sensitive "insulin-glucagon negative feedback system" (Unger 1981).

b) Oxyntomodulin

Some of the biological effects of the enteroglucagon group of peptides have also been determined. Peak II GLI corresponding to glicentin 33-69 (oxyntomodulin) was shown to bind to the glucagon

glucagon receptor of rat hepatic plasma membranes with a molar potency of some 10% relative to glucagon, and also exhibited a similar potency in activating adenylate cyclase (Kaneko et al 1979). It has also been shown to inhibit pentagastrin-stimulated gastric acid secretion in the rat with at least a 20 fold greater potency than glucagon (Dubrasquet et al 1982), and to stimulate adenylate cyclase activity in rat gastric glands (Bataille et al 1981).

c) Glicentin

Glicentin does not bind to the glucagon receptor of pig hepatic plasma membranes (Holst 1977), although the addition of glicentin to rat hepatocytes did cause the activation of adenylate cyclase and the release of glucose. However, these effects were small, (glicentin having only 1% of the molar potency of glucagon in this system) and may have been caused by the release of glucagon-like peptides from glicentin by enzymatic digestion (Thieden et al 1981). When infused at low doses ($370 \text{ pmol kg}^{-1}\text{hr}^{-1}$) into unanaesthetised rats with a permanent gastric fistula, glicentin also inhibits pentagastrin-stimulated gastric acid secretion with at least 8x the potency of glucagon (Kirkegaard et al 1982).

The presence of the entire sequence of glucagon in glicentin and glicentin 33-69 (oxyntomodulin), suggests that circulating glucagon-like immunoreactivities might function as an additional source of true glucagon, provided that plasma enzymes are capable of cleaving off the non-glucagon moieties. That this was feasible was demonstrated by Koranyi et al (1981), although the physiological significance of this finding is yet to be established.

The observation that a patient with a renal tumour producing a large amount of glucagon-like immunoreactivity of molecular size 6,000, as estimated by gel filtration, also had gross villous hypertrophy of the small intestine (Gleeson et al 1971; Bloom 1972); and the subsequent correlation of plasma GLI concentrations with accelerated small intestinal mucosal growth in a variety of experimental and pathological situations (review - Bloom & Polak 1982), suggests that the enteroglucagons may also have a direct trophic action on the small intestinal mucosa.

GLP-1 and GLP-2 - Biological roles ?

There now exists the interesting possibility, that the glucagon producing cells of the gut and pancreas may co-secrete a component(s) originating from the C-terminal portion of proglucagon (containing the GLP-1 and GLP-2 sequences), along with other peptides originating from the same precursor. The extent to which the sequences of both GLP-1 and GLP-2 have been conserved indicates involvement in an important biological role. This in theory, could be mediated via

- 1) a single entity consisting of proglucagon 72-160 and containing the sequences of both GLP-1 and GLP-2,
- 2) C-terminal extensions of the glucagon sequence, inclusive of GLP-I or both GLP-1 and GLP-2,
- 3) the individual peptide sequences of GLP-I and GLP-II, each with its own distinct biological role,

- 4) extensions of the GLP-1 or GLP-2 sequences inclusive of that of the intervening "spacer" peptide II.

Considering the wide spectrum of bioactivities exhibited by the glucagon-related peptides, it is conceivable that any peptide(s) co-secreted with them may be important modulators of their biological actions. Therefore, another fundamental aspect of the work described in this thesis involves an attempt to determine the biological roles of the glucagon-like peptides GLP-1 and GLP-2, using as a starting point the known biological actions of the glucagon-related peptides.

CHAPTER 2

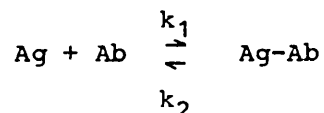
a) THE DEVELOPMENT OF RADIOIMMUNOASSAYS FOR THE GLUCAGON-LIKE

PEPTIDES GLP-1 AND GLP-2

INTRODUCTION

Radioimmunoassay was first introduced as an analytical technique by Yalow & Berson in 1960. It has many advantages when compared with previous methods. In the case of a peptide hormone, this is borne out by the gain in sensitivity (the ability to detect pico-molar quantities), specificity (to bind only a particular amino acid sequence) and the ease of performance over a classical bioassay. It can also be applied to substances where there were no previous assay methods and, thereby, offers a general system of measurement for an immensely wide range of compounds.

The fundamentals of this competitive binding assay involve exploiting the natural immune reaction between an antigen (Ag) and its specific antibody (Ab). This results in the formation of an antigen-antibody complex (Ag-Ab) which can be conveniently expressed by the following reversible reaction. The rate of the forward (k_1)



and reverse (k_2) reactions are a measure of the attraction between the antigen and the antibody and this is quantitatively expressed by the equilibrium "Affinity Constant", K ; where $K = k_1/k_2$. The higher the value of K the greater the affinity of the antibody for the antigen.

In radioimmunoassay, the specificity of the immune response is coupled with the sensitivity of radioisotopic techniques. Varying concentrations of unlabelled hormone (Ag) are allowed to compete with a finite concentration of radiolabelled hormone (Ag*) for a constant number of antibody (Ab) binding sites. Increasing the concentration of Ag will result in a concomitant decrease in the equilibrium concentration of Ag* bound to the Ab. This allows for the construction of a calibration curve from which the unknown concentration of Ag within a sample may be determined. The techniques involved in developing a sensitive, specific and precise radioimmunoassay are discussed below.

Production and Characterisation of Antisera

Introduction

Classically the immune response is divided into primary and secondary phases. Following immunisation with antigen, the primary response causes a small population of specifically programmed lymphocytes to proliferate to form a larger population. Amplification of this response on secondary immunisation results in the production of large quantities of antibody. The generation of antibodies is much a matter of chance, dependent on the variable responses of the immunised animals. In general, the immunogenicity of a material is directly related to its size and materials below a molecular weight of 5,000 are poor immunogens. This is overcome by their conjugation as haptens to large immunogenic molecules such as bovine serum albumin.

In the case of proteins, it would appear that the antigenic determinant (the specific sequence of amino acids bound by the

antibody) is usually conformational rather than sequential (Sela 1969). This implies that the determinant is composed of interspersed amino acids rather than a linear sequence, brought into an area of close proximity by the tertiary folding of the molecule. This has been confirmed by Atassi (1978) who has defined the antigenic determinant of egg white lysozyme to include six amino acid residues which are closely related in the three dimensional structure, though distant in sequence. However, for small peptides with little or no tertiary structure this is not usually the case. Their antigenic determinants tend to be composed of a linear sequence of up to six amino acid residues (Heding et al 1976; Yanaihara et al 1979).

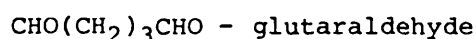
In radioimmunoassay, therefore, the recognition of a specific sequence of amino acids by the antibody renders it a structural assay. It is feasible that the antibody may recognise this determinant within other homologous molecules. This may be within extended sequences, fragments or other members of the same peptide hormone family. Assessing antibody crossreactivity is therefore vital for the proper interpretation of assay data.

Radioimmunoassay can be usefully allied to chromatographic techniques to investigate the existence of larger molecular weight forms (possible precursors) or smaller fragments of a polypeptide hormone which share the antigenic determinant. As already discussed, this approach has been successfully employed in similar investigations on the glucagon family of peptides (see chapter 1). However, the recent discovery that encoded within the mammalian pre-proglucagon gene are the sequences of two additional

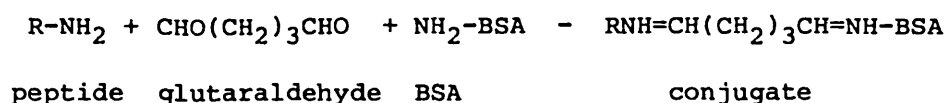
glucagon-like peptides, GLP-1 and GLP-2, which may be expressed as peptides of similar size to glucagon, has made the picture more complex. In order to study the occurrence and distribution of tissue molecular forms of GLP-1 and GLP-2 like immunoreactivity, the strategy applied was to attempt to develop separate radioimmunoassays for each of these peptides, using antisera recognising specific immunodeterminants within the amino acid sequences of each of these two peptides. For this purpose fragments of GLP-1 and GLP-2 corresponding to GLP-1-(1-19) and GLP-2-(1-9)+Y were obtained custom-synthesized, prior to the commercial availability of the respective synthetic whole molecules of GLP-1 and GLP-2.

Conjugation

The conjugation of these four peptide sequences as haptens to bovine serum albumin (BSA) was carried out using the bi-functional coupling reagent bis-diazobenzidine or glutaraldehyde. Glutaraldehyde has the structural formula shown below. It contains two reactive



aldehyde carbonyl groups capable of undergoing nucleophilic addition and is, therefore, able to link an electron-rich free amino group of a peptide with a similar group within the BSA molecule. Such a coupling would still allow for free rotation of the hapten and thus adequate exposure to the animals immune system. The site of linkage between the hapten and protein can have some degree of

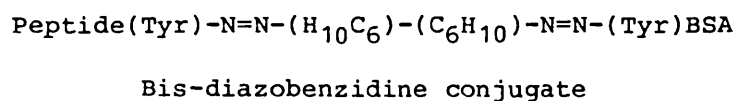


influence on the specificity of the antiserum as antibodies produced against the sterically unhindered part of the hapten are more likely to cross-react with the free antigen.

Bis-diazotised benzidine is another commonly used coupling agent. Being a somewhat larger molecule than glutaraldehyde, it also serves in providing a spacer arm that holds the peptide away from the carrier and thereby provides better exposure to the animals immune system.



Bis-diazonium salts react with certain aromatic compounds to yield azo compounds. The basis of this coupling reaction involves the electrophillic attack by the diazonium ion on an aromatic ring containing a powerful electron releasing group. Under these circumstances, the peptide is usually conjugated to the BSA carrier via either its tyrosyl or histidyl residues.



Rabbits were the animals chosen for immunisation. This was based on the immunogenicity of BSA-conjugates in this species, their low maintenance costs and the relative ease of repeatedly obtaining blood samples of a reasonable volume.

Methods

Conjugation of GLP-2-(1-9)+Y and Human GLP-2-(1-34) - Glutaraldehyde

400 nmol of either GLP-2-(1-9)+Y or h-GLP-2-(1-34) was dissolved in 0.5 ml of 0.1M phosphate buffer (pH 7.5), maintained in an ice bath at 4°C. To this solution was added 0.5 ml of BSA (200 nmol/ml) dissolved in the same buffer. 1.5 ml of a 0.2% solution of glutaraldehyde (30 μ mol) in 0.1M phosphate buffer was then added dropwise over 15 min whilst the mixture was thoroughly stirred. Further mixing was allowed to continue at 4°C for another 12 hours resulting in the conjugated product. The ratio of peptide: carrier: coupling agent was usually maintained at 4:1:300.

Conjugation of GLP-1-(1-19) and GLP-1-(1-37) - Bis-diazobenzidine

Bis-diazotised benzidine is prepared fresh for this type of conjugation reaction by dissolving 80 μ mol of benzidine dihydrochloride in 10 ml of 0.18M HCl, followed by the dropwise addition of 1 ml of 0.16M NaNO₂. All operations are carried out at 4°C and the reaction mixture is allowed to mix gently over-night (15 hr). It is then checked for oxidising activity with starch iodide paper, a negative result indicative of the completion of the diazotisation process. A final yield of approximately 80 μ mol/ml of bis-diazotised benzidine is expected.

To 400 nmol of either GLP-1-(1-19) or GLP-1-(1-37) is added 130 μ l (1.0 μ mol) of the bis-diazotised benzidine solution. This and all further additions being carried out at 4°C and under conditions ensuring thorough mixing of the reactants. 100 μ l (40 μ mol) of NaHCO₃ is immediately added, followed by a 100 μ l (100 nmol) of BSA,

and the pH adjusted to 9.8 with aqueous NaOH. Routinely, this type of conjugation employs a peptide to carrier to coupling agent ratio of 4:1:10.

Peptide Incorporation

In all the conjugation mixtures a tracer (2×10^4 cts/10s) of the corresponding I^{125} labelled peptide is added to assess the percentage of peptide coupled to the carrier. This is determined by subjecting an aliquot of the conjugate to gel filtration chromatography on a Sephadex G-50 column (30 x 1.5 cm) eluted with 0.1M acetic acid containing 0.5% BSA and at a flow rate of 12 ml/hr. The percentage distribution of radioactivity between the void volume (the elution position of conjugated peptides) and the elution position of the respective labelled peptide, conveniently quantifies the degree of incorporation. In the series of conjugates mentioned above peptide incorporations of less than 75% were never obtained.

Immunisation

The conjugate is injected in the form of an emulsion made with Freund's complete adjuvant; a mixture of one part detergent Arlacel A with four parts n-hexadecane. It permits slow release of the coupled hapten into the circulation and may serve to protect labile antigens against degradation. Freund's adjuvant alone (incomplete) will cause an inflammatory response which is thought to act as a stimulus for antibody formation. When made "complete" by the addition of 1 mg/ml of heat killed mycobacteria, this response is further enhanced.

The conjugate is reconstituted in water, mixed with an equal volume of Freund's complete adjuvant, and homogenised to produce an emulsion with oil in the continuous phase. Approximately 2 ml of emulsion containing the dose of conjugated peptide (100 nmol for primary, 50 nmol for secondary) was injected subcutaneously into the groin and axilla by multiple injection (0.5 ml/site) using a variation of the method of Vaitukaitis et al (1971).

Three months later, secondary immunisation (boosts) with lower doses (50 nmol) of conjugate in Freund's incomplete adjuvant is carried out and repeated at monthly intervals. After 7-10 days following each boost the rabbits were bled from a marginal ear vein and approximately 20 ml of blood was collected into a tube containing 40 units of heparin/10 ml blood. Following mixing and centrifugation, the plasma was decanted and stored at -20°C . Plasma was always collected in preference to serum owing to convenience and the irrelevance to radioimmunoassay with regards to which of these blood fractions is used. However, to avoid ambiguity, the word antiserum (owing to its familiarity) is used throughout this thesis.

Characterisation of Antibodies

Antisera were screened for antibodies to the respective peptides by incubating antibody dilutions of 1:80, 800, 8,000, 80,000 and 800,000 with 250 cp 10 sec of the corresponding radiolabelled peptide. The incubations were carried out at 4°C over 4-5 days in a total volume of 800 μl buffer (0.05M sodium barbitone, pH 8.0 containing the diluted antibody). Antisera showing a reasonable titre, and thus a high affinity for the radiolabelled peptide were

characterised further. Antigen-antibody interactions are markedly influenced by their environment and involve electrostatic and hydrophobic interactions which may be pH-dependent. Therefore, the effect of pH on the antibody affinity constant (K) was investigated by setting up standard curves in 0.05M sodium barbitone (pH 8.0), 0.06M sodium phosphate (pH 7.4), 0.05M sodium acetate (pH 5.5) and 0.2M glycine (pH 8.8) buffers containing 0.3% (v/v) BSA and 0.05% (w/v) sodium azide.

For each antiserum the ratio of bound:free peptide was plotted against the total amount of peptide bound, taking into account the quantity of radiolabelled peptide present (Scatchard 1949). For a single class of antibody binding sites reacting with the antigen according to the equation described on page 29, this form of graphical analysis will yield a straight line. Its slope is a measure of the antibody affinity constant (K); the intercept on the x axis giving the molar concentration of antibody binding sites (N) and the intercept on the y axis being equal to KN. In practice however, many antisera yield Scatchard plots which deviate from linearity at high concentrations of antigen; a phenomenon which is probably due to the combined influence of heterogeneity of antibody binding sites and "non-specific" binding of the labelled antigen.

Those antisera showing adequate titre and slope were assessed for crossreaction with other regulatory peptides. These tests were carried out under buffer conditions yielding the highest K value for each antiserum. Cross-reactivity was expressed as the "relative potency (%)" of the peptide standard to the crossreacting material, in terms of their individual concentrations which suppress the

binding of radiolabelled peptide to antibody by 50%. This method of assessing crossreaction has been in extensive use following Abraham (1969) and is a convenient expression of the relative potency of different ligands.

Results and Discussion

Antisera were screened for high affinity antibodies by incubating in serial dilution each antiserum with a fixed amount of the respective radiolabelled (^{125}I) peptide antigen (see fig 2:1). Following two boosts several high titre antisera were identified with titration curves which were displaced by significantly low concentrations of unlabelled antigen.

Antiserum UG3, the result of immunisation followed by two successive boosts with a glutaraldehyde conjugate was seen to have the highest affinity for GLP-2-(1-9)+Y. It is capable of binding 50% (5 fmol) of the added tracer (^{125}I -GLP-2-(1-9)+Y - 250 cp 10 sec) at a final dilution of 1:8000 (see fig 2:1). Scatchard analysis of standard curves in buffers ranging from pH 5.5 to 8.0 indicate the affinity constant of this antiserum to be unaffected by pH, with respective values of $1.9 \times 10^{10} \text{ l mol}^{-1}$ at both pH 5.5 and 8.0 (see fig 2:2). However, at pH 7.4 the K value falls to $8.7 \times 10^9 \text{ l mol}^{-1}$ and this is possibly attributed to the interference of phosphate ions in the antigen-antibody reaction. The UG3 assay was, therefore, routinely carried out in 0.05M sodium barbitone buffer (pH 8.0). Other features of this antiserum include non-linearity of Scatchard plots over the tested pH range which may indicate the heterogenous nature of this antiserum.

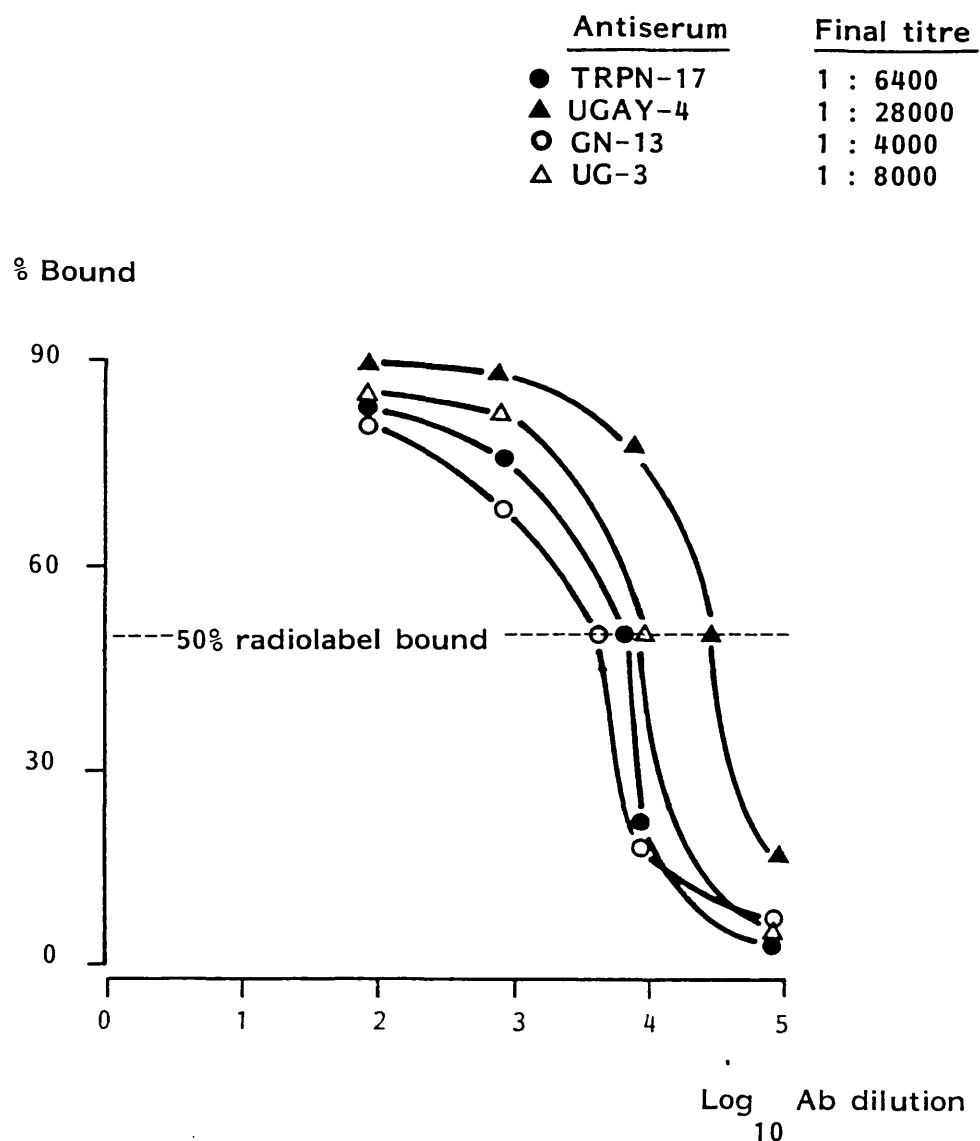


Figure 2:1 Antiserum titration curves obtained by serially diluting plasma from immunised rabbits. Also indicated are the antibody dilutions (final titre) which bind 50% of the respective radiolabel added.

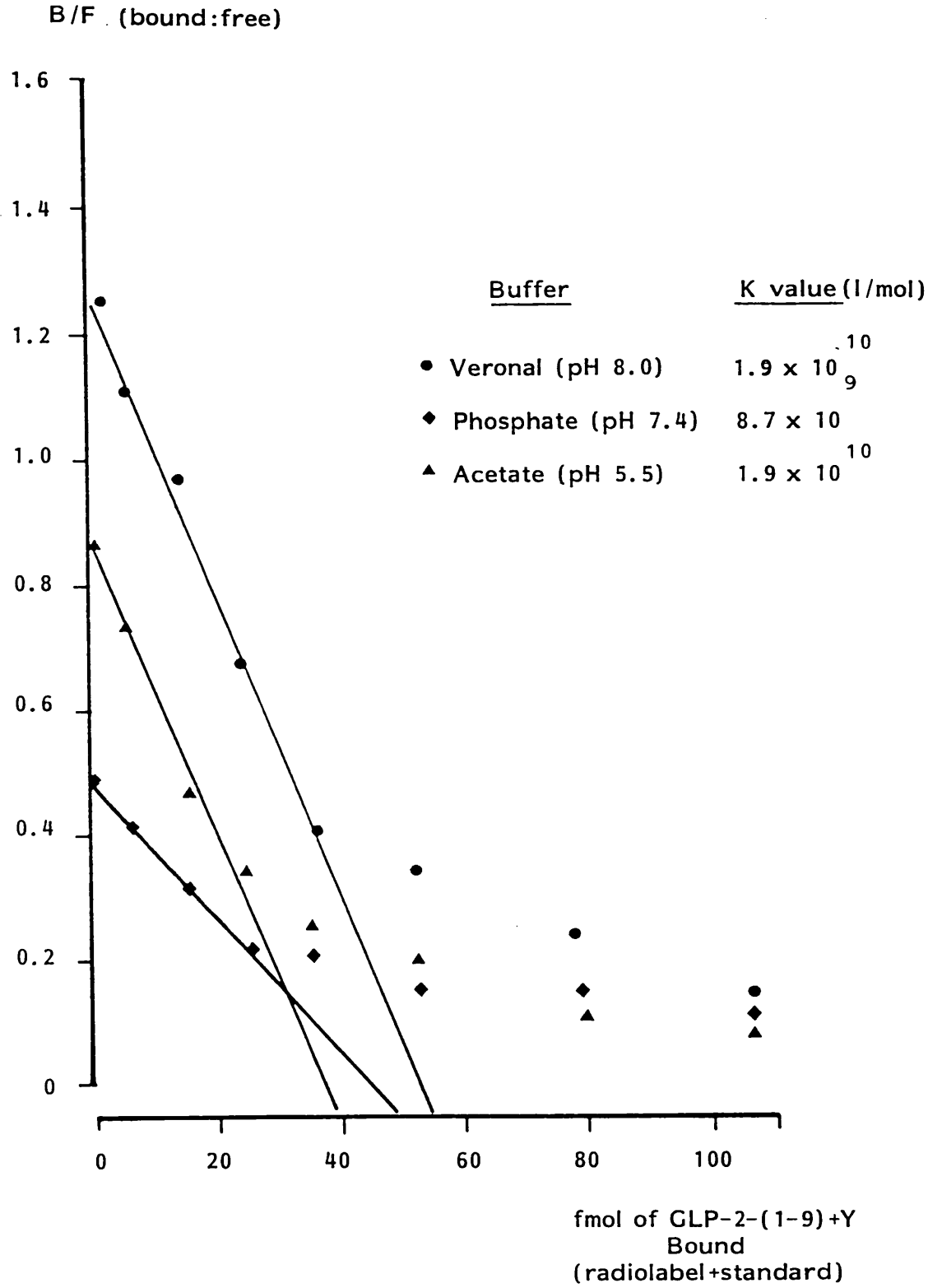


Figure 2:2 Representative Scatchard plots for antiserum UG3 at three different values of pH. The data was obtained from standard curves and the bound : free ratio (B/F) was plotted against the total amount of GLP-2-(1-9)+Y bound.

The crossreactivity of UG3 was measured against other regulatory peptides and these included glucagon (PG), vasoactive intestinal polypeptide (VIP), peptide histidine-isoleucine (PHI), secretin (Sec), somatostatin 1-14 and 1-28 (SS), pancreatic polypeptide (PP), neurotensin (NT), neuropeptide Y (NPY), peptide YY (PYY), peptide-histidine-methionine (PHM), growth hormone-releasing factor (GRF-1-44), cholecystokinin (CCK 1-8, 1-33), gastrin-34 (Gas), motilin (Mot), gastric inhibitory polypeptide (GIP), bombesin (BN), substance P (SubP), galanin (Gal), calcitonin gene-related peptide (CGRP), GLP-1-(1-19), GLP-1-(1-36), GLP-1-(1-37) and h-GLP-2-(1-34). No detectable crossreaction was observed. The inavailability of synthetic fragments of the peptide made it impossible to specify the antigenic determinant. However, one may speculate that UG3 would favour a C-terminal immunodeterminant as the likely coupling site for this conjugate would be the N-terminal histidine residue of GLP-2-(1-9)+Y, leaving the middle to C-terminal portion of the molecule well exposed to the rabbits immune system.

Immunisation with bis-diazobenzidene conjugates of GLP-1-(1-19) followed by three boosts produced the antiserum GN13. It was capable of binding 50% of the added radiolabel (4 fmol) at a final dilution of 1:4000 (see fig 2:1). Scatchard analysis for this antiserum yielded affinity constants of 4.6, 0.46 and 5.1 ($\times 10^9$ lmol^{-1}) respectively at pH values of 5.5, 7.4 and 8.0 (see fig 2:3). It appears that phosphate ions interfere in this assay and it was, therefore, routinely carried out in 0.05M sodium barbitone buffer (pH 8.0). The non-linearity of the Scatchard plots may indicate the heterogeneous nature of this antiserum.

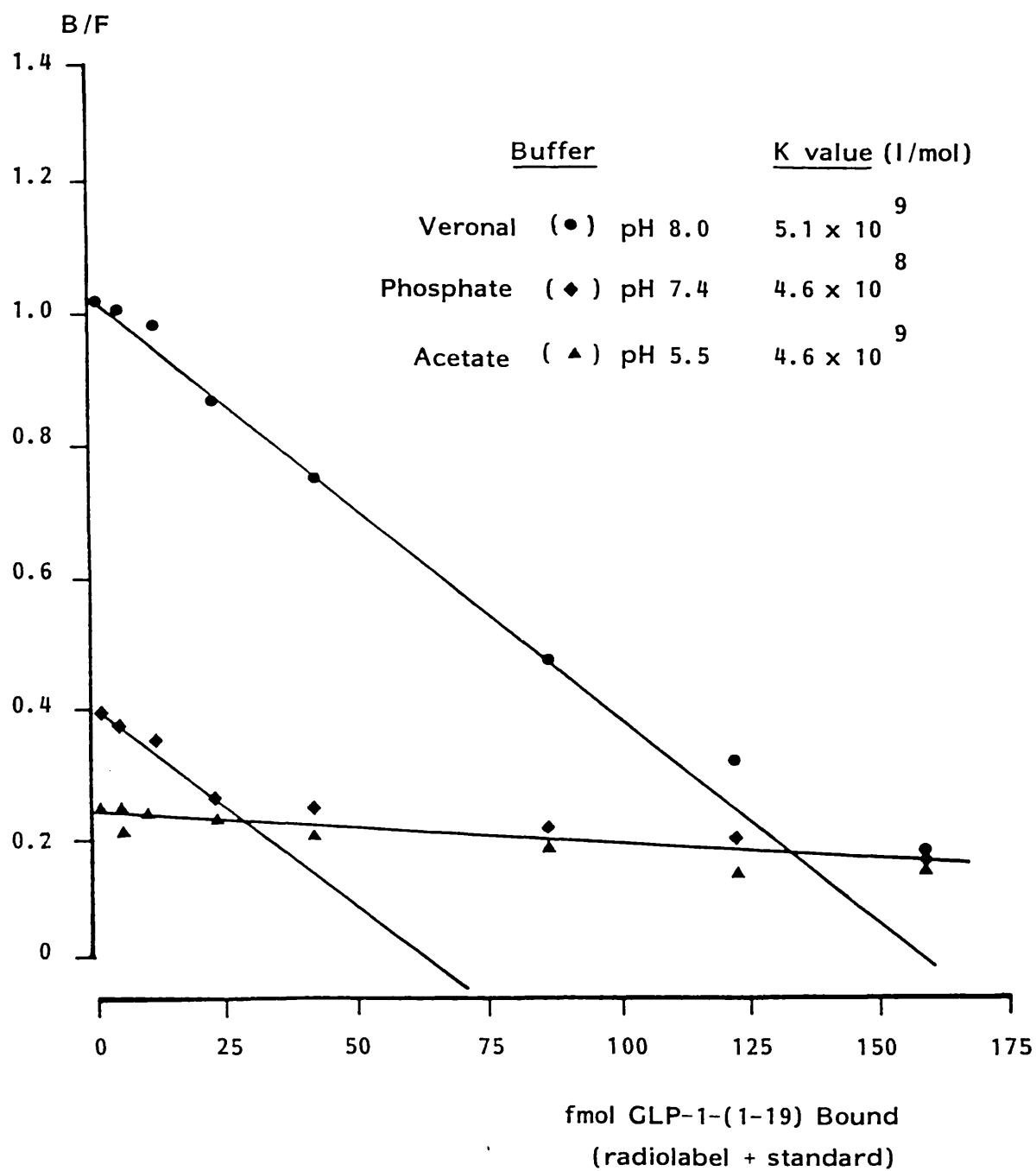


Figure 2:3 Representative Scatchard plots for antiserum GN13
at three different values of pH.

The crossreactivity of GN13 was assessed identically to that of UG3, with all the previously mentioned peptides tested for the presence of the GN13 antigenic determinant. No crossreactivity, other than that of 0.56% with PHM, and 20% with both GLP-1-(1-37) and GLP-1-(1-36)-amide was detected (see fig 2:4). The affinity constant for the interaction of GN13 with GLP-1-(1-36)-amide and GLP-1-(1-37) was low and of the order of $1 \times 10^5 \text{ l mol}^{-1}$ (see fig 2:5). GN13 is an antiserum raised to a bis-diazobenzidine conjugate of GLP-1-(1-19) where coupling via Tyr¹⁹ is more favourable than His¹. Under these circumstances the antigenic determinant is likely to be N-terminally directed and its crossreaction with GLP-1 whole molecule is suggestive of the presence of a similar determinant within this molecule.

Following two boosts with a glutaraldehyde conjugate of human GLP-2-(1-34), several rabbits produced antisera exhibiting a high titre in binding ¹²⁵I-labelled h-GLP-2-(1-34). Of these, UGAY-4 at a final titre of 1:28,000 (see fig 2:1) was found to have the highest affinity for this peptide, the binding constant (K) having a maximal value of $1.8 \times 10^{10} \text{ l mol}^{-1}$ (see fig 2:6) in 0.2M glycine buffer (pH 8.8). Examination of Scatchard plots over the pH range of 5.5 to 8.8 suggests that this antiserum may be heterogeneous. No crossreaction with the previously mentioned range of peptides was observed. Glutaraldehyde conjugation of h-GLP-2 is likely to favour coupling via the N-terminal histidine residue. The antigenic determinant is, therefore, more likely to be C-terminally directed. Consistent with this, there is no crossreaction of this antiserum with GLP-2-(1-9)+Y.

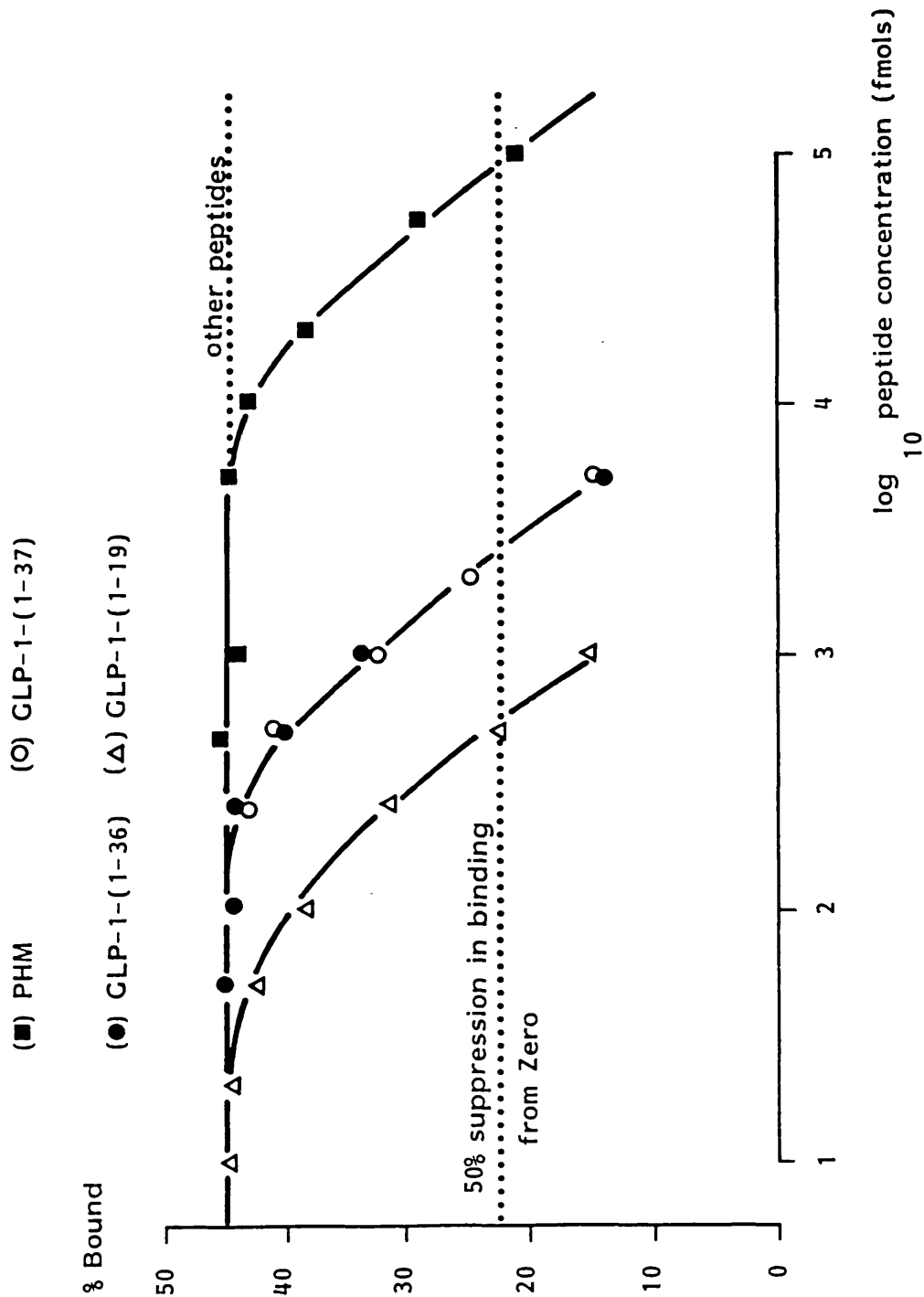


Figure 2:4 The crossreactivity of antiserum GN13 with GLP-1-(1-36)/(1-37) and PHM. Crossreactivity is expressed in terms of percent "relative potency" with respect to GLP-1-(1-19). It is calculated from the quantity of peptide producing a 50% decrease in the binding of GLP-1-(1-19) radiolabel to antibody divided into the amount of GLP-1-(1-19) standard producing an equivalent suppression of binding. Values of 20% for GLP-1-(1-36)/(1-37) and 0.6% for PHM were obtained.

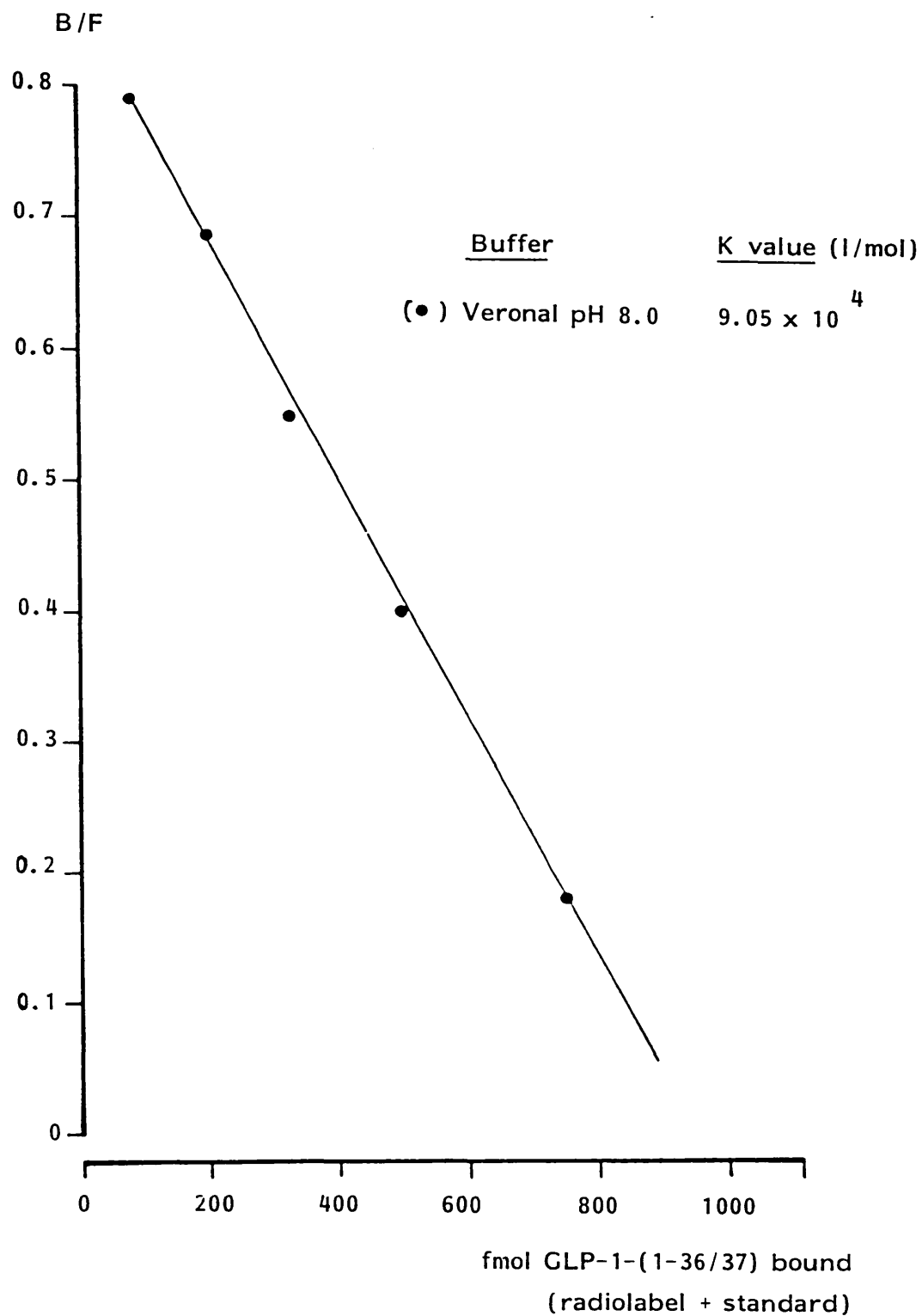


Figure 2:5 Representative Scatchard plot for the crossreacting assay of antiserum GN13 with GLP-1-(1-36)/(1-37) as standard.

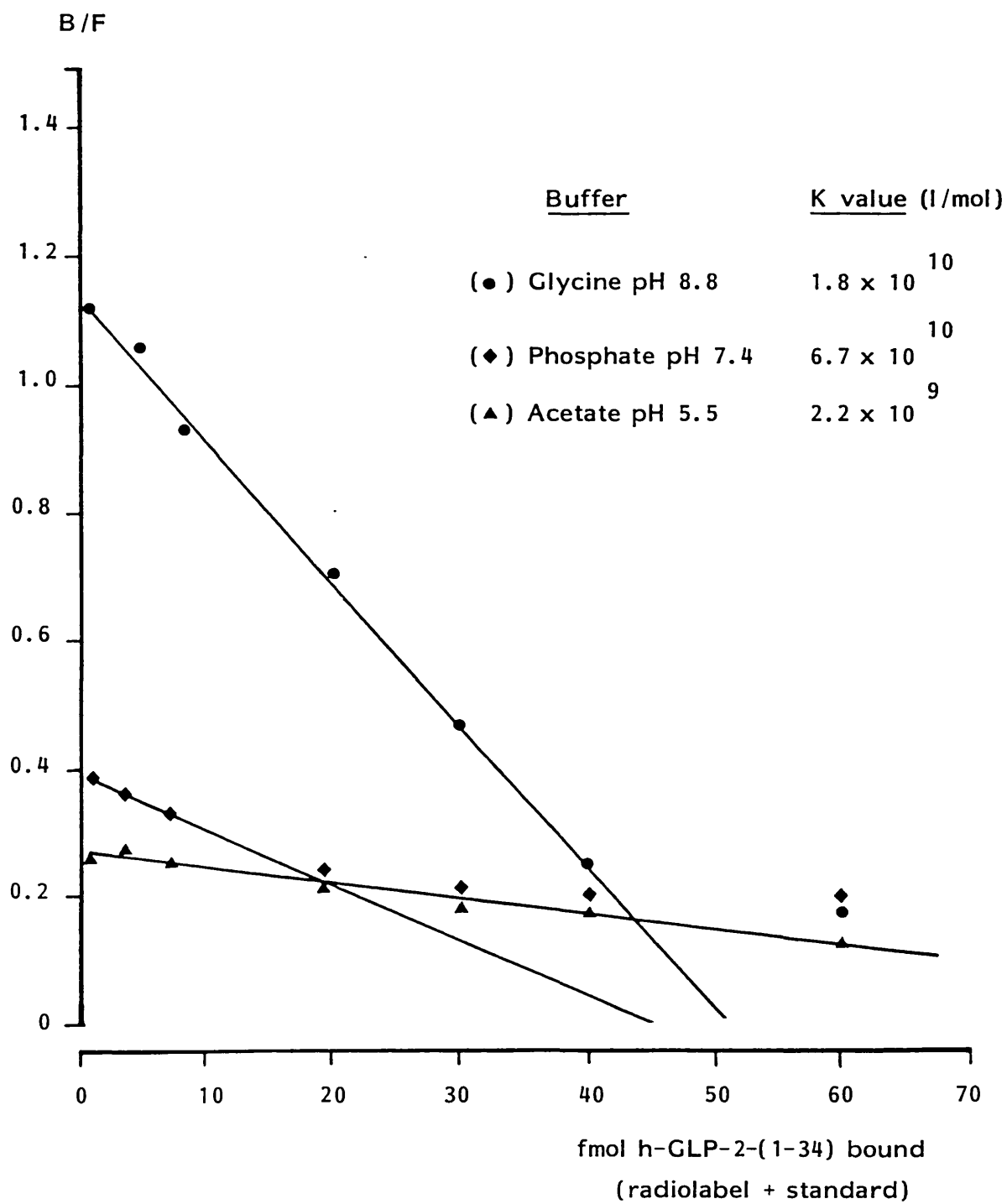


Figure 2:6 Representative Scatchard plot for the antiserum UGAY 4
at three different values of pH.

Immunisation of rabbits with a bis-diazobenzidine conjugate of GLP-1-(1-37) followed by three boosts produced several antisera capable of binding ^{125}I -GLP-1-(1-37). One of these, TRPN-17 was found to bind 50% of the radiolabel (2 fmol) at a final dilution of 1:6400 (see fig 2:1). It was also found to be the most avid exhibiting a maximal affinity constant for GLP-1-(1-36)/(1-37) of $7.1 \times 10^{10} \text{ l mol}^{-1}$ at pH 8.8 (see fig 2:7). The TRPN-17 assay was thus routinely carried out in 0.2M glycine buffer (pH 8.8). Scatchard analysis reveals non-linearity at higher antigen concentrations and may be indicative of the heterogeneous nature of this antiserum.

Assessment of crossreaction with the previously mentioned range of peptides resulted in the following observations. TRPN-17 crossreacts equally (100%) with both GLP-1-(1-36)-amide and GLP-1-(1-37). A crossreaction of 7.1% with GLP-1-(1-19) and 0.32% with pancreatic glucagon was also obtained (see fig 2:8). The ability of this antiserum to recognise the GLP-1-(1-19) fragment implies that the antigenic determinant lies within the mid to N-terminal region of the GLP-1-(1-37) molecule. This is a feasible argument provided conjugation via the Tyr¹⁹ residue had occurred. However, the availability of C-terminal sequences of GLP-1 whole molecule is a pre-requisite for validating this hypothesis.

In summary, although precise estimates of antiserum regional specificities were not obtained due to the inavailability of synthetic fragments of these peptides, these immunisation procedures were certainly successful in raising specific antisera to the GLP-2-(1-9)+Y, GLP-1-(1-19), h-GLP-2-(1-34) and GLP-1-(1-36/37)

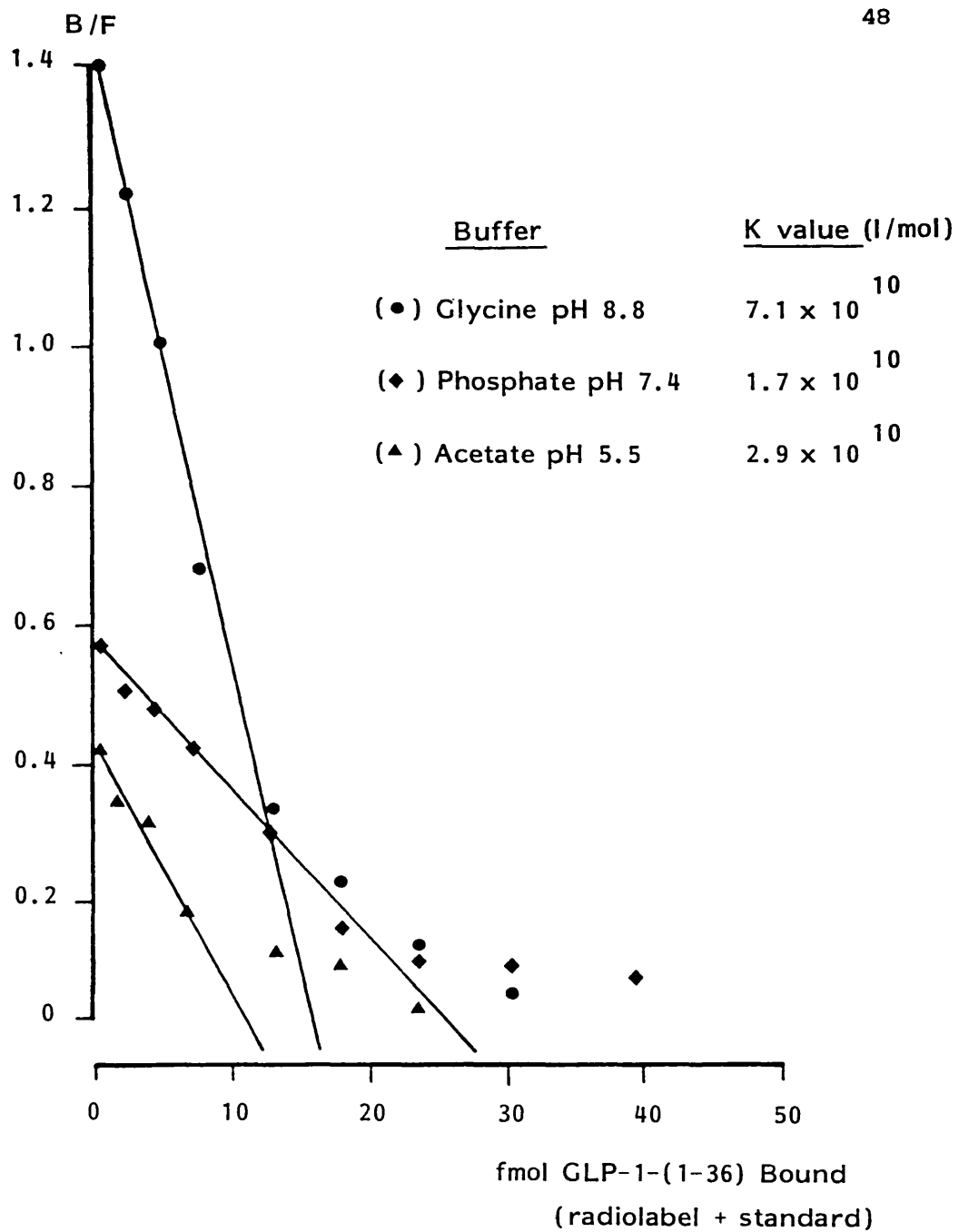


Figure 2:7 Representative Scatchard plots for the antiserum TRPN 17 at three different values of pH.

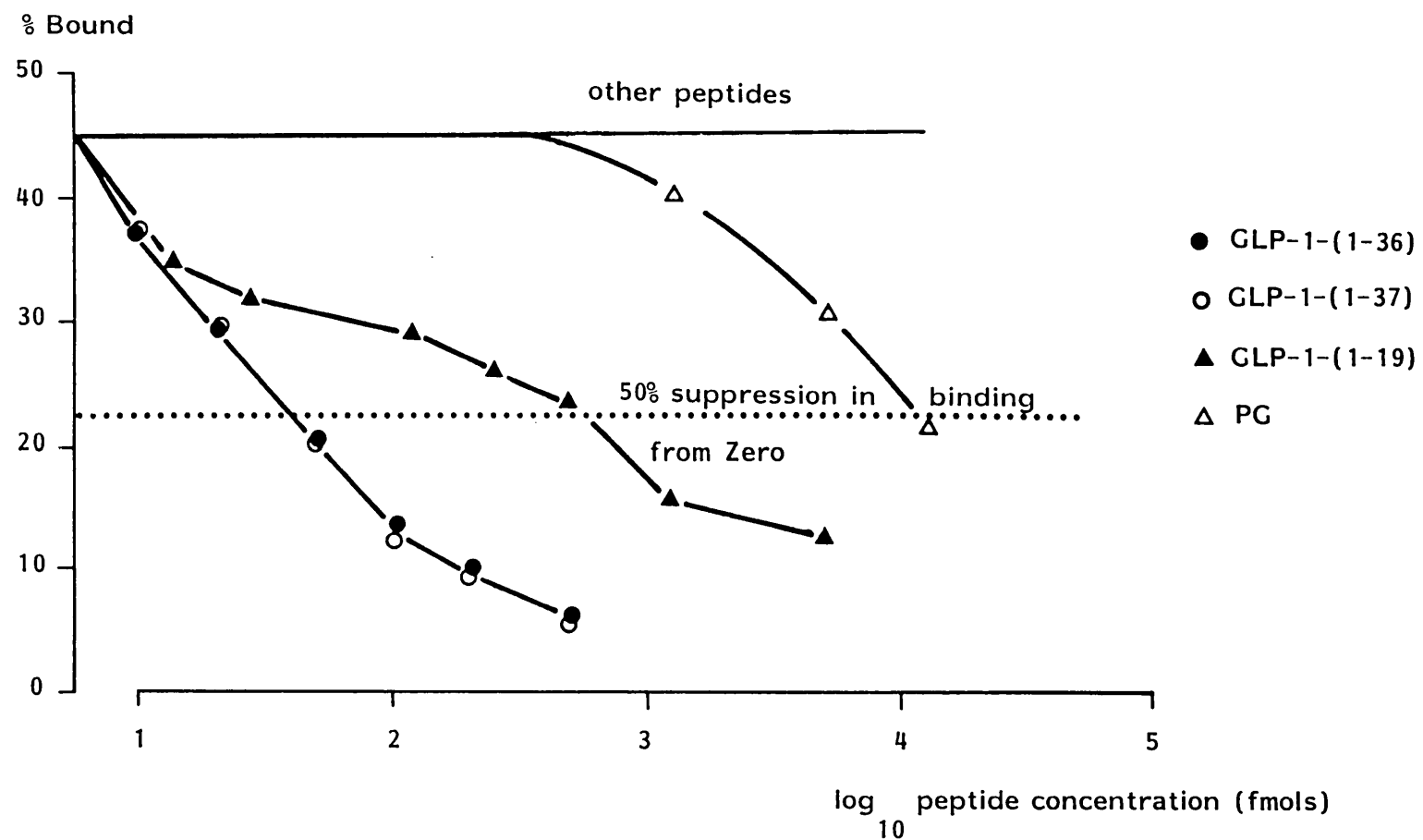


Figure 2:8 The crossreaction of antiserum TRPN 17 with GLP-1-(1-36)/(1-37) - 100%, GLP-1-(1-19) - 7% and PG - 0.3%. Relative potencies were calculated as described previously (see fig 2:4).

group of peptides. Those antisera exhibiting the highest affinity constants provided sensitive detection ranges of the pmol (10^{-12}M) order, and were used under optimised conditions in the following radioimmunological and chromatographic studies.

Preparation of ^{125}I -Radiolabelled Tracer

Introduction

With a high affinity antibody, the sensitivity of a radioimmunoassay may be increased by improving the specific activity of the radiolabelled antigen (tracer) used. Radioisotopes of iodine can be substituted on to the aromatic side chain of a tyrosine residue with relative ease, and similarly onto a histidine residue under somewhat more vigorous conditions. In this manner, it is possible to obtain stable preparations of radiolabelled peptides. ^{125}I is preferred to ^{131}I as a radiolabel because of its longer shelf life (half-life of 60 days compared to 8), its much greater isotopic abundance which provides a higher specific activity product and its gamma emission of lower energy which is more efficiently detected.

The iodination of a peptide is thought to occur via an electrophillic aromatic substitution reaction. The electrophile $^{125}\text{I}^+$ is generated from ^{125}I by the potent oxidising agent, Chloramine T (Hunter & Greenwood 1962). Under appropriate pH conditions (7.5 - 9.0), substitution of a hydrogen atom on tyrosine residues occurs at the ortho-position to the hydroxyl group. The reaction is terminated by the addition of a reducing agent (sodium metabisulphite) and dilution of the reactants in a potassium iodide solution so as to mop up any unreacted ^{125}I iodide.

The following considerations must be borne in mind when carrying out a "trace iodination" of this type. The concentration of the peptide is limiting and thus the reaction volume should be kept small (100 μ l) to conserve peptide. Chloramine T should be used at its lowest effective concentration and incubation times should be short (10 sec) so as to avoid potential oxidation of the peptide and the consequent loss of immunoreactivity. The optimal pH for iodination of tyrosine residues is 7.5 and at lower pH values other residues are substituted, although the efficiency of the reaction drastically declines.

Various types of damage can occur to a radiolabelled peptide to severely affect its immunoreactivity. The most commonly occurring chemical damage results from oxidation of methionine residues, although the disulphide bridges of cysteine may also be split by the reducing agent. The substitution of a large iodine atom in place of hydrogen may sterically affect immunoreactivity. Furthermore, if the peptide is di-iodinated, "decay-catastrophe" may produce a radioactive (and therefore detectable) non-immunoreactive fragment and, therefore, a concomitant loss in specific activity. Thus di-iodination should be avoided. With many labelled compounds there also appears to be a progressive release of free iodide on storage (Krohn et al 1977) which also, lowers the specific activity.

Although some of the resultant tracer damage is unavoidable, measures may be taken to minimise this. For example, internal radiation damage may be virtually eliminated by the "quenching effect" of the much more numerous storage solvent molecules.

Though this may generate highly chemically reactive free radicals

these in turn may be "soaked up" by inclusion of a large concentration of BSA (5%) in the storage solution. Furthermore, incubation damage may be reduced by the addition of antibacterial agents such as sodium azide and EDTA to the assay buffer.

On completion of a typical iodination reaction, the products may include damaged and undamaged unlabelled and labelled peptide, free iodide and salts inclusive of the oxidising and reducing agents. To obtain pure undamaged labelled peptide a high resolution separation procedure is required. In the case of these glucagon-like peptides, this initially employed molecular size exclusion chromatography. However, when high affinity antibodies were obtained, the requirement for radiolabels with greater specific activities (to increase assay sensitivity) necessitated employing reverse phase high pressure liquid chromatography (r.p. HPLC).

This separation technique exploits the differences in hydrophobicity inherent in the primary structures of peptides. The column composed of silica beads (particle size - 5 μm , coupled to C-18 aliphatic chains - octadecylsilysilica) is eluted with an organic solvent using a gradient of decreasing polarity. The mobile phase, therefore, usually consists of increasing concentrations of aqueous acetonitrile. Multi-component peptide mixtures are separated on the basis of their partitioning between the stationary and mobile phases.

The loading of a peptide mixture under initial elution conditions of high polarity (low aqueous acetonitrile concentration) promotes the adherence of these relatively hydrophobic molecules to the

stationary phase. Gradient elution with increasing concentrations of aqueous acetonitrile (decreasing polarity) is thought to induce alterations in the conformation of the bound peptides, such that their partitioning equilibria are displaced to favour increased interaction with the mobile phase and finally elution.

Trifluoroacetic acid (0.1% v/v) is used as an ion-pairing agent to block the residual negative charge on the silica matrix thereby reducing any interference ion exchange may induce to a high resolution separation procedure otherwise based exclusively on hydrophobicity.

Reverse phase HPLC has facilitated the resolution of iodination products to yield tracers of specific activities approaching that of pure Na¹²⁵I. It is assisted by the significant difference in hydrophobicity between the labelled and unlabelled peptide; the iodinated form being more hydrophobic and eluting later.

Radioiodination reactions are, therefore, often referred to as "trace iodinations". This is because the high specific activity tracer purified from the products of the reaction, usually accounts for only a small percentage of the peptide starting material.

Methods and Results

Iodination of GLP-2-(1-9)+Y

To 10 µg (9 nmol) of GLP-2-(1-9)+Y in 50 µl of 0.4M phosphate buffer (pH 7.4) was added 10 µl (1 mCi) of carrier free Na ¹²⁵I. This was rapidly followed by the addition of 10 µl of a 0.2% (w/v) solution of Chloramine T made up in the same buffer. The reactants were thoroughly mixed for precisely 10 sec and the reaction terminated by the addition of 20 µl of a 0.48% (w/v) solution of sodium metabisulphite. The contents were then diluted out by the addition

of 100 μ l of a 1% (w/v) solution of potassium iodide. Peptide was separated from unreacted iodide on a Sephadex G-15 column (60 x 0.9 cm) eluted with 0.06 M phosphate buffer containing 10 mmol/l EDTA, 7.7 mmol/l sodium azide and 0.3% (v/v) BSA (see fig 2:9). Peptide containing fractions at the void volume were mixed with aprotinin (50×10^3 kallikrein inhibitory units) and freeze-dried in 30 pmol aliquots. The specific activity of the radio-labelled peptide was determined by self-displacement from antibody and was estimated to be 25 Bq/fmol, and routinely between 60-80% of the radioactive iodide was incorporated into the peptide.

Iodination of GLP-1-(1-19)

The protocol is identical to that described above except for the amount of GLP-1-(1-19) used (3.0 nmol) and the chromatographic purification technique utilised (r.p. HPLC). The iodination products were loaded onto a Techsil 5 μ m particle size octadecyl silylsilica column equilibrated with 10% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Stepwise gradient elution was carried out from 10 to 30% acetonitrile over the course of 70 min (see fig 2:10). Unreacted iodide was eluted within the first 10 min followed by non-immunoreactive components between 15-40 min of elution. The major peak of immunoreactivity corresponding to the GLP-1-(1-19) tracer was eluted at 25% acetonitrile (51 min into the run) and was observed to contain 65% of the total radioactivity with a specific activity of 34 Bq/fmol.

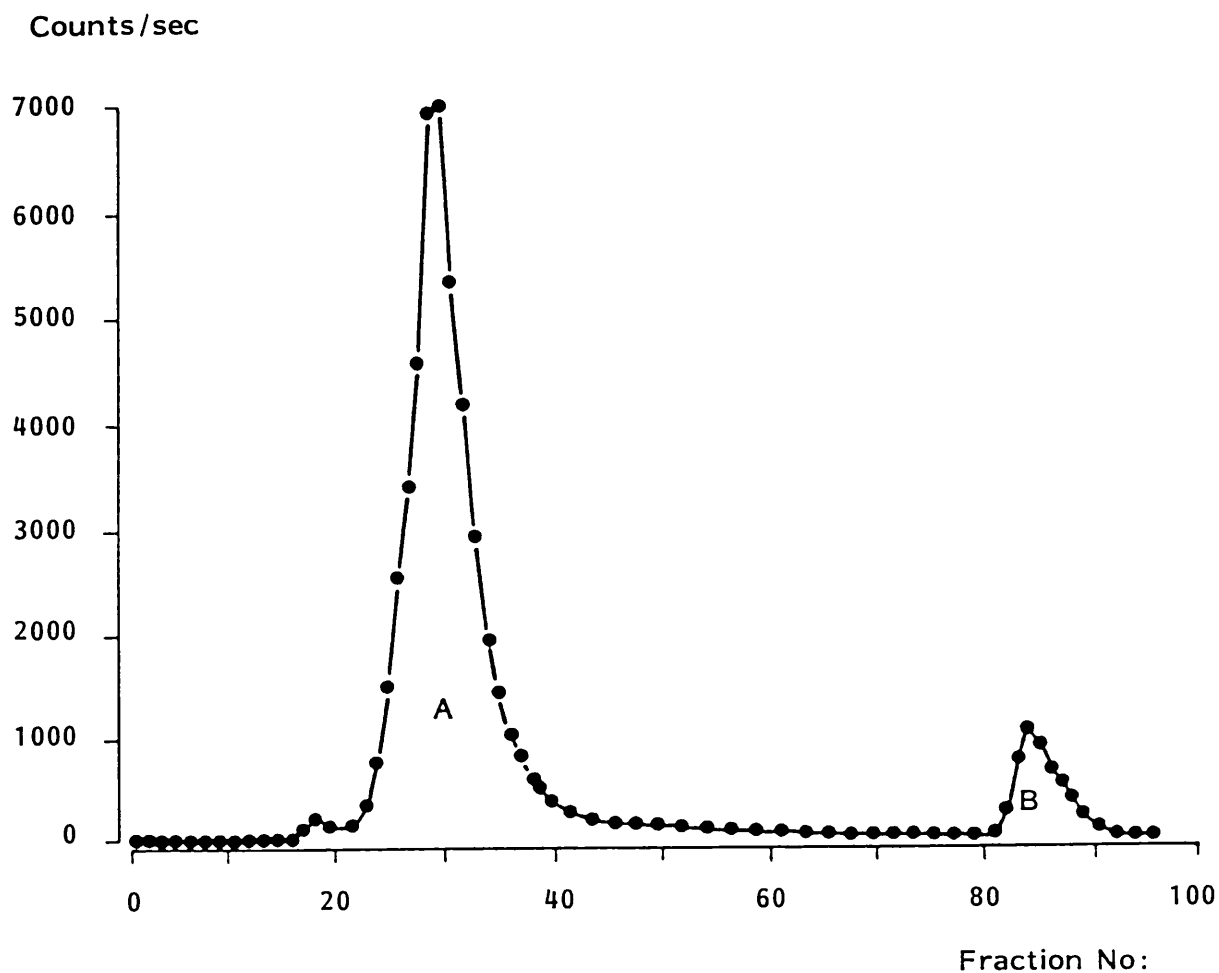


Figure 2:9 Chromatographic separation (Sephadex G15) of the radioactive components from a GLP-2-(1-9)+Y iodination mixture. Peak A corresponds to radiolabelled GLP-2-(1-9)+Y (Specific activity 25 Bq/fmol). Peak B corresponds to free radioactive iodide.

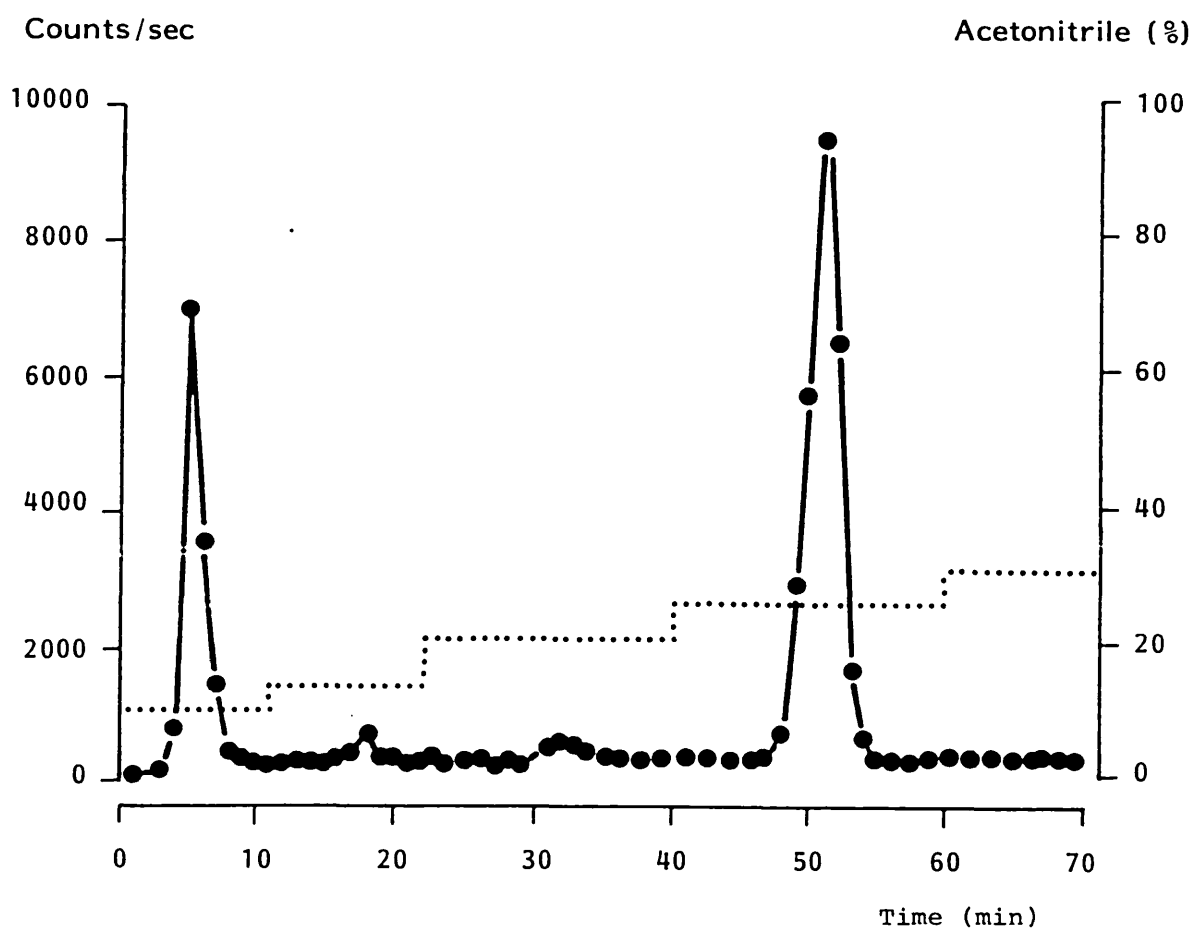


Figure 2:10 Reverse phase high pressure liquid chromatographic separation of the radioactive components from a GLP-1-(1-19) iodination mixture. The peak (# 51) corresponds to I^{125} -GLP-1-(1-19) which shows a specific activity of 34 bq/fmol.

Iodination of GLP-1-(1-37)

GLP-1-(1-37) (3 nmol) was radio-iodinated in a manner identical with that of GLP-1-(1-19). The purification employed r.p. HPLC, although the chromatographic characteristics of this peptide required the use of a different gradient. The iodination products were loaded onto a pre-equilibrated C18 column at 20% acetonitrile, and subjected to a gradient ranging from 20-60% acetonitrile over the duration of 90 min (see fig 2:11). Apart from the void volume peak consisting of unreacted iodide, two main peaks of immunoreactive tracer material were eluted at 45% acetonitrile.

The earlier eluting peak consists of 80% of the total radioactivity and exhibits a specific activity of 76 Bq/fmol. The later peak consists of less than 10% of the total radioactivity and has a specific activity greater than that of pure iodide (I^{125} - 81 Bq/fmol). This was thought to represent the di-iodinated product and characteristically showed a rapid decline in specific activity accompanied by an increase in non-specific binding. The material with a specific activity of 76 Bq/fmol, showed a value of 40 Bq/fmol after three months of use, and no significant increase in non-specific binding. In this chromatographic system, unlabelled peptide is thought to elute a few minutes earlier than the tracer as witnessed by the immunoreactivity but very low specific activities obtained for the 4-5 fractions preceding the main peak.

Iodination of h-GLP-2-(1-34)

Human GLP-2-(1-34) does not contain a tyrosine residue and therefore conditions are altered so as to facilitate the incorporation of I^{125}

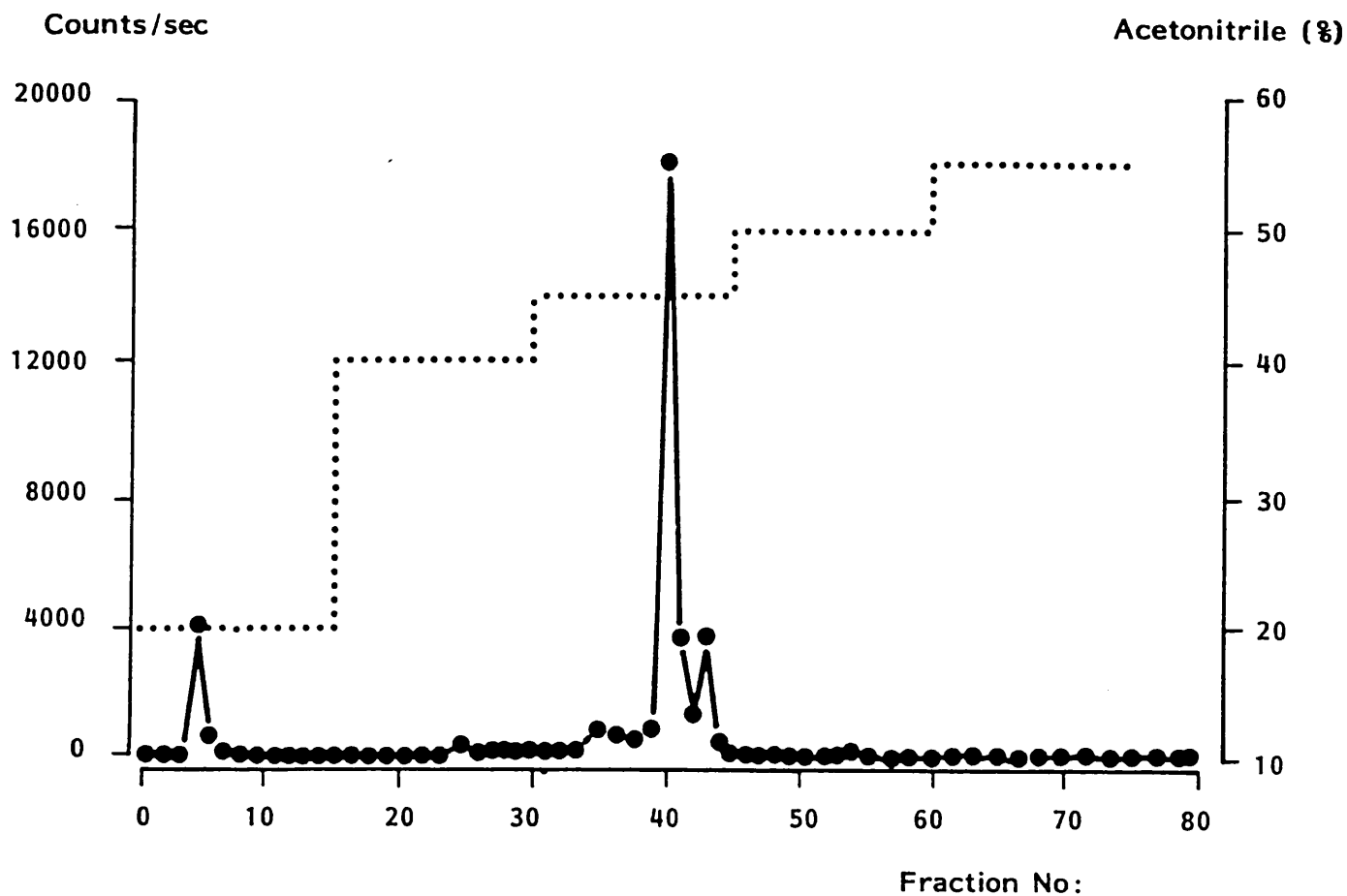


Figure 2:11 Reverse phase high pressure liquid chromatographic separation of the radioactive components from a GLP-1-(1-37) iodination mixture. The peak (# 40) corresponds to I^{125} -GLP-1-(1-37) which shows a specific activity of 76 Bq/fmol.

into the N-terminal histidine residue. This requires the maintenance of pH at 8.5, the reaction being carried out in 0.5M borate buffer. The rate of reaction compared to the iodination of a tyrosine residue is at least an order of magnitude less, thus requiring a more prolonged reaction time. However, exposure of methionine residues to Chloramine T for this duration may result in oxidation and a corresponding loss in immunoreactivity of the labelled material. A compromise is thus arrived at, where the concentration of the oxidising agent is halved, whilst allowing for longer reaction times. All reactants are made up in 0.5M borate buffer (pH 8.5).

To 5 μ g (1.3 nmol) of GLP-2-(1-34) dissolved in 50 μ l of borate buffer is added 10 μ l (1 mCi) of carrier-free NaI¹²⁵, followed by 5 μ l of a 0.2% (w/v) solution of Chloramine T. The reactants were thoroughly mixed for 180 sec and the reaction terminated by the addition of 20 μ l of a 0.48% (w/v) solution of sodium metabisulphite followed by dilution in 100 μ l of a 1% (w/v) solution of potassium iodide. The products of this reaction were then loaded onto a reverse phase HPLC system equilibrated at 20% acetonitrile.

Elution is carried out in a stepwise fashion from 20-60% acetonitrile over the course of 80 min (see fig 2:12). Three main peaks are obtained. The first eluting at 20% acetonitrile, containing 80% of the total radioactivity and showing no immunoreactivity, corresponds to free iodide. The second peak elutes broadly over a 40-45% acetonitrile gradient. It was found to contain 4% of the total radioactivity and exhibited a very high non-specific binding capacity, along with poor antibody binding

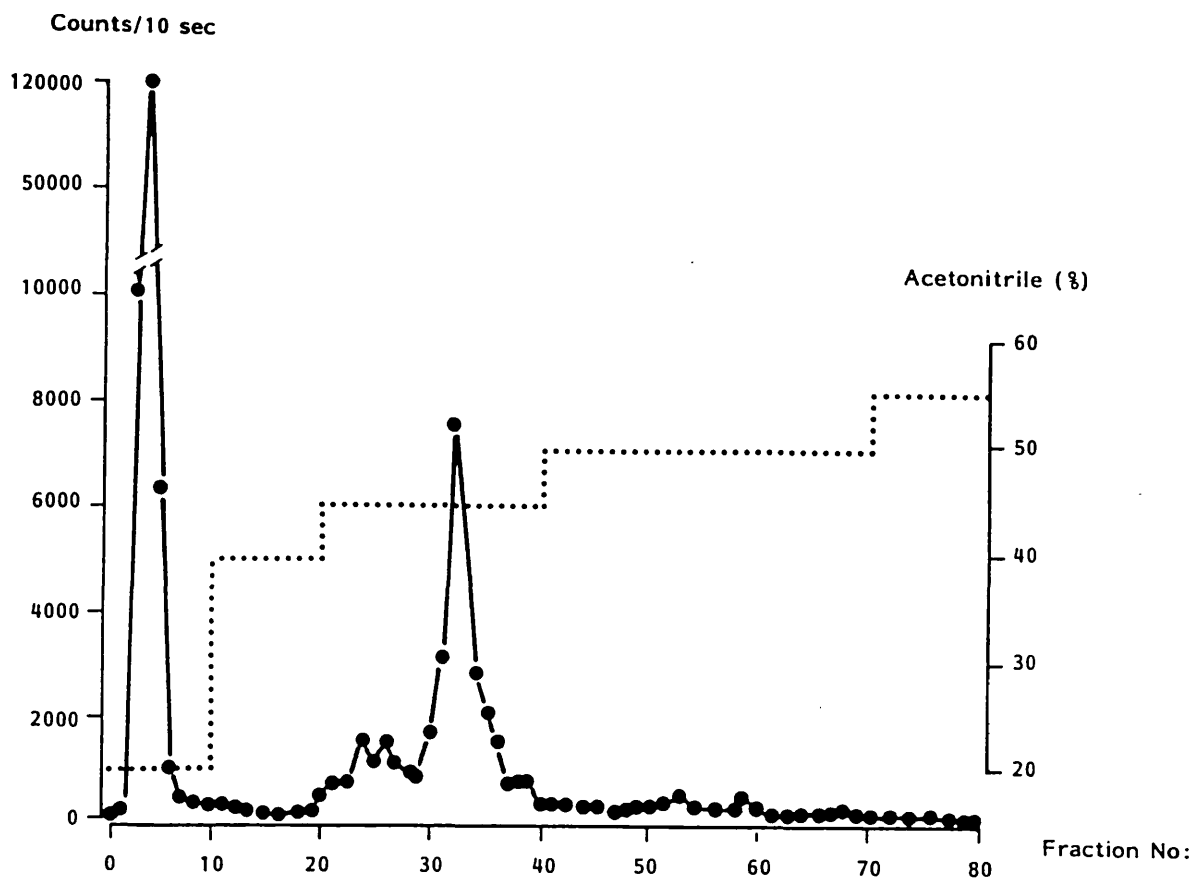


Figure 2:12 Reverse phase high pressure liquid chromatographic separation of the radioactive components from a h-GLP-2-(1-34) iodination mixture. The peak (# 32) corresponds to I^{125} -h-GLP-2-(1-34) which shows a specific activity of 22 Bq/fmol.

characteristics. This may be a result of chemical damage such as oxidation, rendering the peptide poorly immunoreactive. It may also explain earlier elution due to its modified polarity. The third peak eluting at 45% acetonitrile (32 min) contains 11.3% of the total radioactivity and corresponds to the tracer with an accompanying specific activity of 22 Bq fmol⁻¹.

Immunochemical Assessment of Tracers

The specific activities of the purified tracers were assessed using self-displacement curves (Roulston 1979). Aliquots of the purified tracer from component fractions of each peak were diluted in assay buffer, such that a 100 µl aliquot represented 300 cp 10 sec. A series of standard curves was constructed consisting of the normal range of assay standards (1-200 fmol). Included in each series is a set of tubes containing 1/2x, 2x, 4x and 8x the content of tracer fraction under test. Also included is a blank tube containing no antibody to assess non-specific binding. The assay is incubated for 4 days at 4°C and bound tracer separated from free by charcoal adsorption.

The percentage of tracer bound is determined for each series and the respective standard curves plotted. From this data the non-specific binding, degree of immunoreactivity and the steepness of the standard curve for each tracer fraction under test may be compared. Moreover, the specific activity of each test fraction may be determined from % bound values obtained for its self-displacement.

$$\text{Specificity activity} = \frac{\text{[Additional counts per sec in 2x tracer tube]}}{\text{[Counter Efficiency]}} \times \frac{\text{[Hormone equivalent (fmol/tube) of \% Bound value produced by 2x tracer tube]}}{\text{(Bq/fmol)}}$$

On the basis of the above sets of data, tracer fractions showing low non-specific binding, high specific activity and steep slopes were diluted in assay buffer containing 5% (v/v) BSA, aliquoted, lyophilised, sealed in vacuo and stored at -20°C.

Discussion

Using variations of the Chloramine T method of radioiodination (Hunter & Greenwood 1962) radiolabelled glucagon-like peptides varying in specific activity from 22 Bq fmol⁻¹ [h⁻GLP-2-(1-34)] to 76 Bq fmol⁻¹ [(GLP-1-(1-37))] were produced. The resolving power of reverse phase HPLC has made a significant contribution to the preparation of radiolabels of high specific activity such as the GLP-1-(1-37) tracer, and this has in turn improved the sensitivity of the GLP-1 assay. However, there is room for improvement in the specific activities of the other tracers produced, and undoubtedly subtle manipulation of the elution conditions will allow for better separation between the respective labelled and unlabelled peptides.

Parallelism and thus the relative immunoreactivity of the tracer may be assessed by comparison of the logit-log plots of the "tracer self-displacement curve" with that of the "standard curve" (Rodbard 1974). In the ideal case they should both yield straight lines of equal slope (-1.0). The "self-displacement" curves for the tracers produced are parallel to their standard curves and indicate an equivalence in immunoreactivity. This is illustrated in figure 2:13, where the logit-log plots of the "self-displacement" curves for the GLP-1-(1-37) tracer and standard are compared. Both plots are straight lines (as determined by regression analysis, $R = 0.98$) of identical slope (-0.92) which compares well with that for the ideal case (slope = -1.0).

The immunoreactivity of the radiolabelled glucagon-like peptides was monitored regularly and showed no significant decline over 2-3 months, although a progressive increase in non-specific binding was observed. Pre-assay purification of tracer aliquots on Sep-pak C18 cartridges, allowed for the selective removal of any released free iodide (Krohn et al 1977) and thus aided in lowering the assay non-specific binding. However, most of these labelled preparations were of sufficiently low specific activity, 6-7 months after iodination, to grossly affect the sensitivity of the assay and thus warrant the preparation of a new batch of tracer.

Assay Format

Introduction

The "sensitivity" of an assay may be defined as the smallest difference in concentration which can be reliably detected with 95%

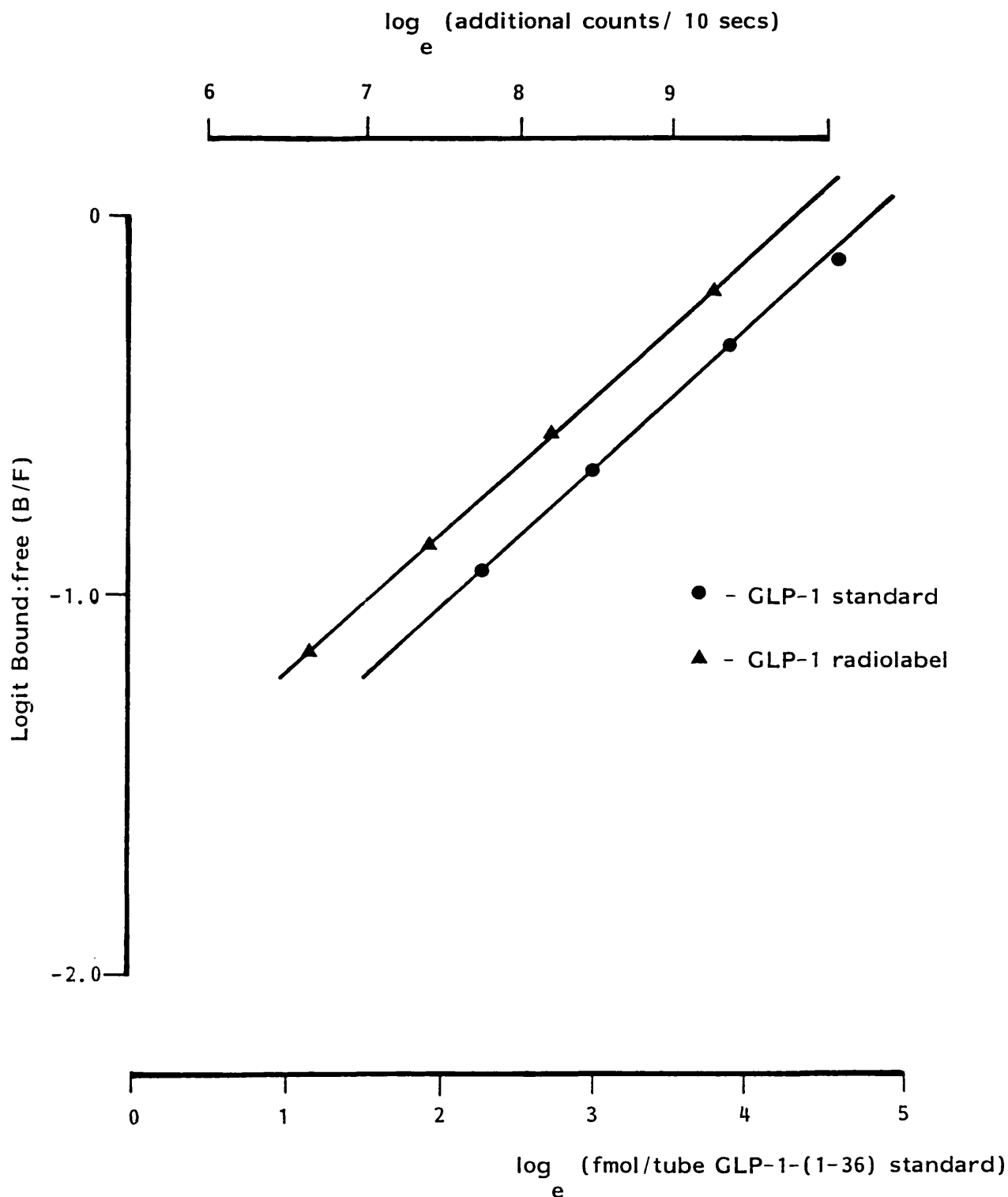


Figure 2:13 The logit-log plots of the self displacement curve of the radiolabel and the GLP-1-(1-37) standard curve are both straight lines ($R=0.98$) of identical slope (-0.92). Logit B/F is the natural log of $[(1-B/F \text{ ratio}) - (1-B/F \text{ ratio at zero})]$. When plotted against the natural log of the dose (ie concentration of standard or the number of additional counts), will in the ideal case produce a straight line of slope equal to -1.0 . This provides a convenient means of assessing parallelism and thus the relative immunoreactivity of different ligands.

confidence. In numerical terms this may be expressed as that point on the standard scale corresponding to a displacement of two standard deviations from the mean zero % bound value (ie mean Z%B - 2SD). It is dependent on the slope (antibody affinity) and the precision (error) at the zero % bound value.

Assay optimisation, resulting in increased precision and sensitivity must take into account the factors affecting an antigen-antibody reaction and the errors accumulated in the performance of a radioimmunoassay. Antibody affinities are pH-dependent and thus a buffer yielding the highest affinity for the respective antibody is used. Furthermore, all the assays mentioned within this thesis employ buffers containing 1% (v/v) BSA. It saturates likely peptide adsorption sites availing more unlabelled peptide to compete for the antibody binding sites. This has the effect of increasing the slope of the standard curve and thus the affinity of the system. Assay incubation times and temperatures were also chosen to maximise this effect.

Methods

Error Estimation

Potential sources of error may include those accumulated in the preparation of standards, gamma counting, pipetting and the separation of "bound" from "free" antigen. The use of quality control preparations to monitor consistency is, therefore, essential and allows the intra- and inter-assay variation to be calculated. The concentration of tracer used in these assays was sufficient to minimise the error in counting. Approximately 250 counts/10 sec of tracer was added to each assay tube. Both the "bound" and "free"

fractions were counted for 200 sec and corrected for background. Counting error is expressed as the inverse of the square root of the total count. In this case, by counting both separated fractions (ie the total count) the error is of the order of 1.4%. This would, however, increase if only the bound fraction were counted as at 20% bound the error would be 3.2% and at 10% bound this would be 4.5%.

The cumulative effect of experimental factors on the precision of the assay may be assessed in terms of the relative error in estimates of peptide concentration. This may be determined by analysing the data from six replicate standard curves contained within the same assay. The standard deviation of the % bound value (the response parameter) was calculated for each of the eight points on the curve (see fig 2:14). For each of these points, the error in the estimates of concentration (the dose parameter) was determined by dividing the standard deviation of the % bound value by the mean % bound value. The relative error (coefficient of variation) of peptide estimates was plotted against concentration and an example of such a "precision profile" is illustrated in figure 2:14 (inset).

Separation of "Bound" from "Free" Antigen

The physical separation of "bound" from free antigen is achieved by the addition of activated charcoal to each assay tube. Charcoal binds small peptides avidly and at low concentrations will rapidly remove unbound antigen from the solution. It also allows for the rapid processing of large numbers of assay tubes. To help prevent binding of antigen-antibody complexes to charcoal, clinical grade dextran (average mol.wt. 70,000) was also included. The dextran

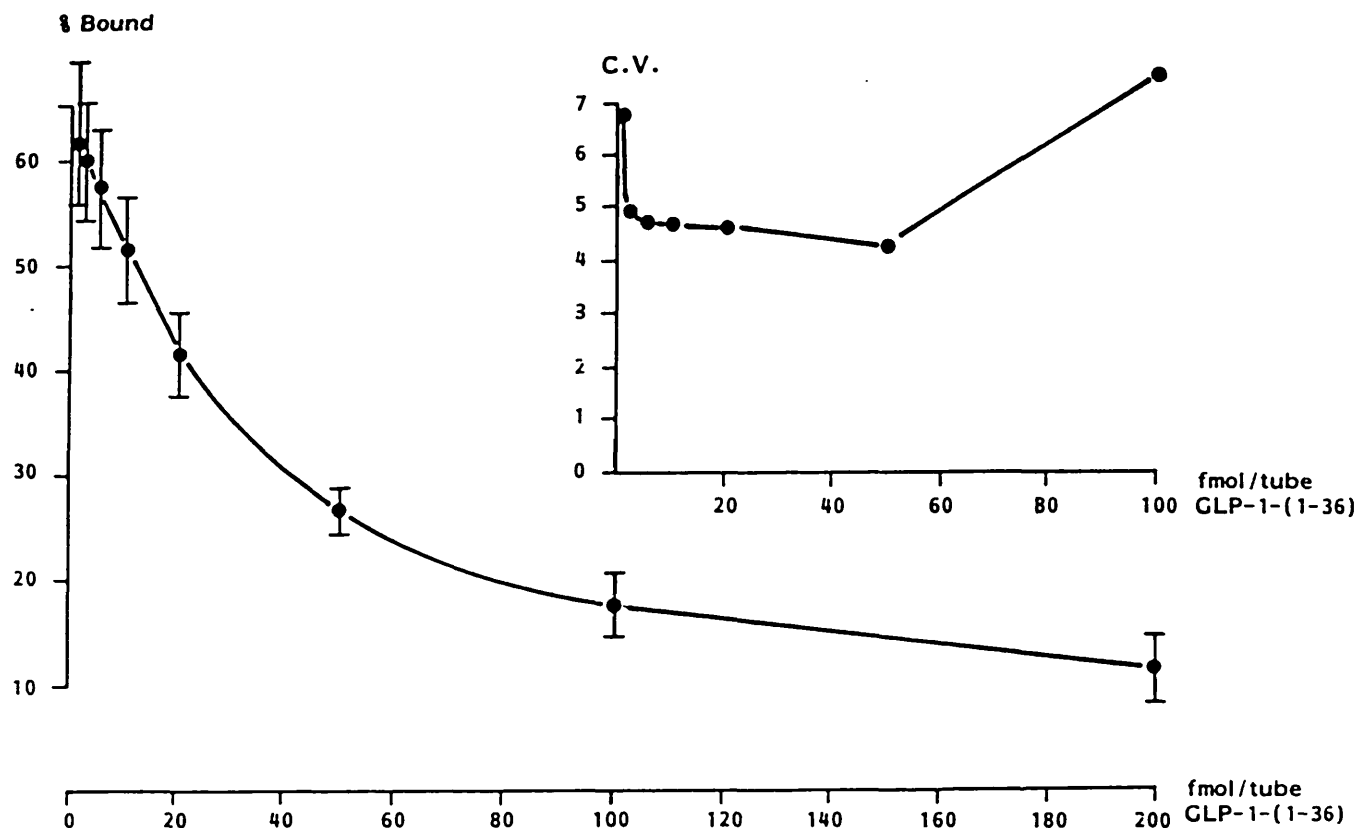


Figure 2:14 Mean GLP-1-(1-36) standard curve obtained from six replicate series of standard curves. The bars represent two standard deviations on either side of the mean % bound value. The inset figure is a precision profile for the GLP-1 assay. It illustrates the coefficient of variation of estimates of GLP-1 concentration at each of seven points on the standard curve. A level of maximum precision exists between concentrations of 2 and 50 fmol/tube.

combines with charcoal particles to occlude the pores, limiting access to small peptides. Along with the BSA of the assay buffer, it coats the outer surfaces of the charcoal particles, blocking potential adsorptive sites for antigen-antibody complexes.

The charcoal and dextran (the dextran comprising 10% of the charcoal weight) were mixed and stirred continuously in assay buffer at 4°C. The suspension was allowed to equilibrate for 20 min prior to the addition of 250 μ l to each assay tube. The optimum assay charcoaling conditions were determined by testing the effect of varying charcoal concentrations on assay parameters. Thus for the GLP-2-(1-9)+Y, GLP-1-(1-19) and GLP-1 whole molecule assays, 8 mg charcoal with 0.8 mg dextran per assay tube was used. However, in the case of the GLP-2-(1-34), 16 mg charcoal with 0.16 mg dextran per assay tube were required to achieve adequate separation of "bound" from "free" antigen (see fig 2:15).

Immediately following the addition of charcoal, the assay tubes were spun in a refrigerated (4°C) centrifuge at 1500 x g for 20 min. The supernatants were separated by means of a pasteur pipette and both fractions counted for 200 sec in a gamma counter. The quantity of radioactivity in the 'bound' fraction (ie the supernatant) was expressed as a percentage of the total radioactivity in both fractions. These percentage bound (%B) values were used as an expression of the assay "response" parameter.

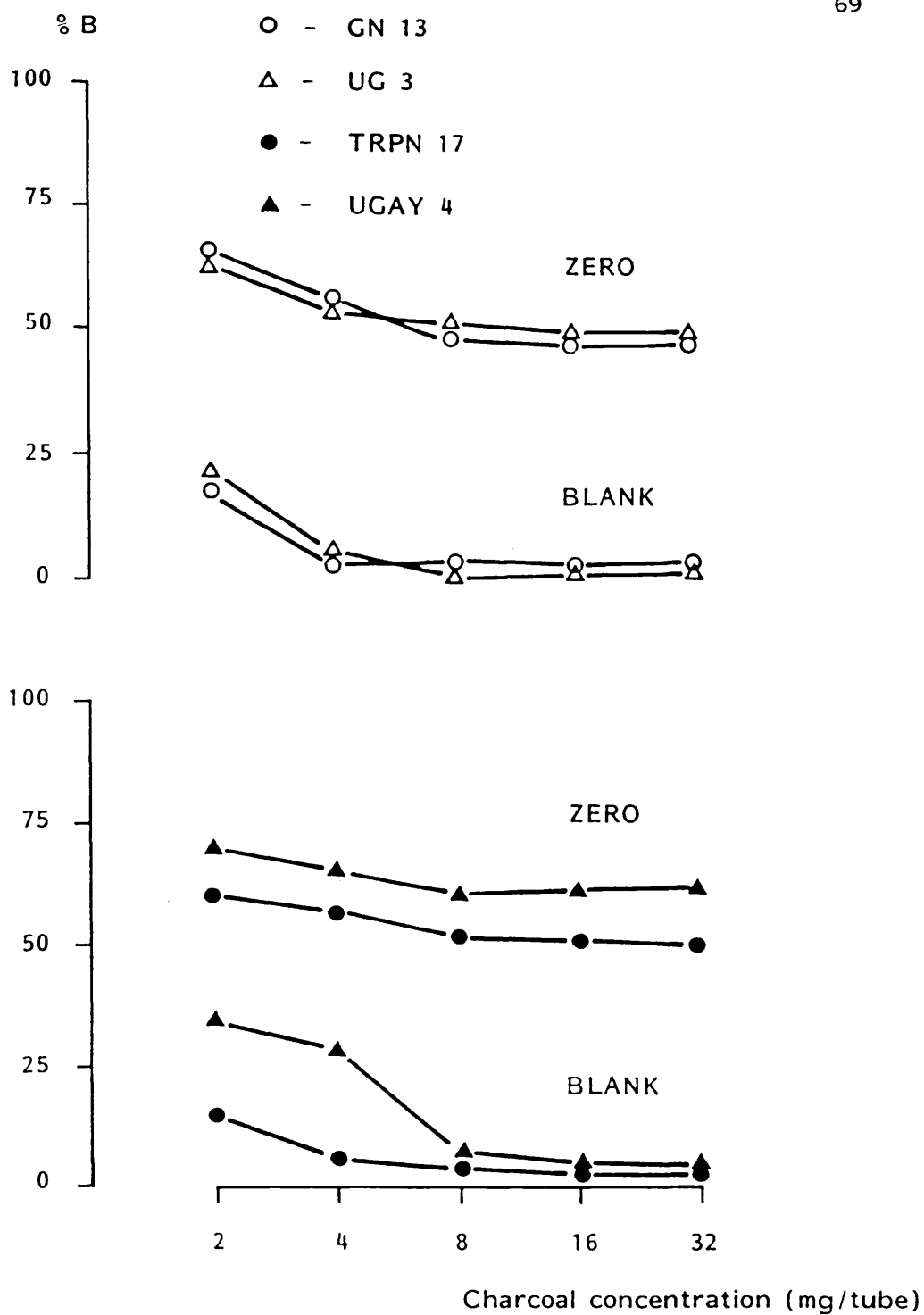


Figure 2:15 The effect of varying the amount of charcoal added to each assay tube on the % bound values obtained for the following assay parameters: radiolabel in the absence of antibody (blank) and radiolabel in the presence of the working titre of antibody (zero).

Assay Controls

All assays are performed according to the format illustrated in table 2:1. The respective assays are carried out in buffers containing 1% (v/v) BSA and 0.05% (w/v) sodium azide, that were previously shown to maximise antibody affinity (see fig 2:16). Each sample, standard and control was set up in duplicate and the various controls are included for the following reasons.

Blank: These tubes contain all reagents except antiserum in order to assess non-specific tracer binding.

1/2x and 2x Label: These two sets of tubes contain respectively half and twice the amount of radiolabel added to all the other assay tubes. They provide a means of assessing how the sensitivity of the assay may be affected by adding more or less radiolabel in subsequent assays. The percent bound value for the 2x label was also used to calculate the specific activity of the radiolabel.

Zero: These contain the radiolabel and antiserum but no standard or sample and were placed at regular intervals throughout the assay to assess assay drift.

Excess Ab: These tubes contain excess antibody with labelled peptide. They are used as a measure of integrity of the labelled peptide and in conjunction with the "blank" value enable the assessment of tracer quality.

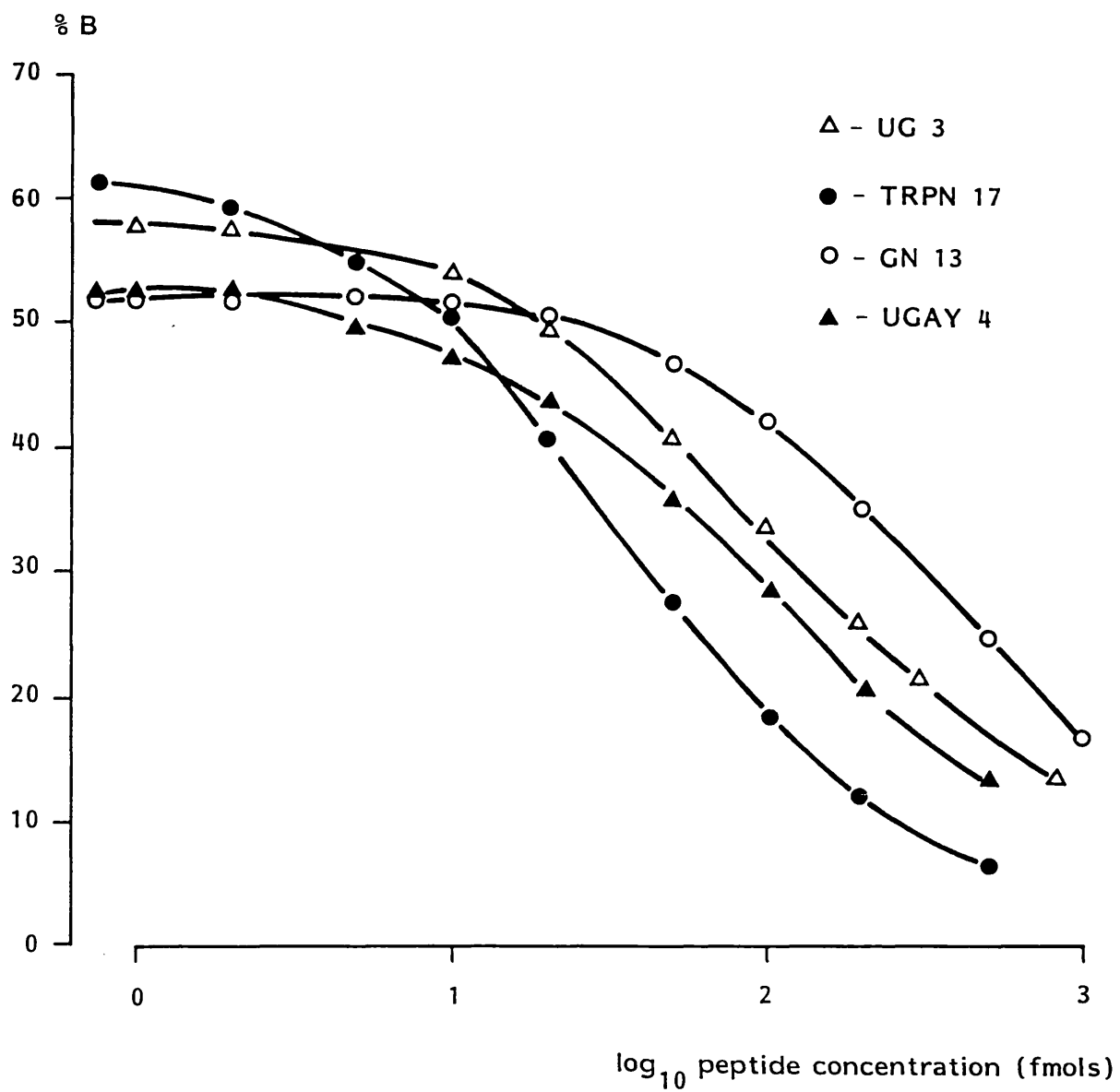


Figure 2:16 Representative standard curves for each of the respective antisera.

Table 2:1 - Assay format

	<u>Buffer Volume</u> (μ l)	<u>Label Volume</u> (μ l)	<u>Antibody Volume</u> (μ l)
Blank	700	100	NIL
¹ /2x label	650	50	100
2x label	500	200	100
Z	600	100	100
Excess Ab	600	100	100
Standards			
(from a solution			
of 1 pmol/ml)			
1,2,5,10,20 μ l	600	100	100
50 μ l	550	100	100
100 μ l	500	100	100
200 μ l	400	100	100
500 μ l	100	100	100
Samples	500	100	100
(100 μ l of column			
effluent)			

The above is the standard format used for all the assays. However, depending on the sensitivity of the assay the concentration of

standard added to the curve does vary. For the analysis of tissue extracts an equivalent amount of the extraction medium is added to all the other assay tubes to assess its effect on the antigen-antibody reaction. All the assays are performed in 2 ml polystyrene test tubes in a total volume of 800 μ l.

Preparation of Standards

All the synthetic peptides were received in freeze-dried form and stored at -20°C until used for the preparation of standards for use in radioimmunoassay. They were accurately weighed on an electronic balance and dissolved in appropriate volumes of freeze-drying solution to yield stock solutions of the desired strength.

Freeze-drying solution (FDS) consists of:

Lactose	50 g/l
Citric acid	2 g/l
BSA	25 g/l
L-cysteine hydrochloride	1 g/l
Aprotinin	8×10^5 KIU/l
Acetic acid.	0.5 M

The acetic acid helps to initially solubilise the peptide but is volatalised on freeze-drying. Citric acid is therefore added to maintain acidic conditions and peptide stability. The lactose helps to produce a large, freeze-dried, soluble pellet that dissolves readily. The albumin prevents adsorptive losses of peptide, aprotinin inhibits proteolytic enzymes and the reducing potential of cysteine prevents oxidation of methionine residues.

Prior to use, all apparatus and pipettes were pre-rinsed in FDS. Three batches of each peptide were weighed and reconstituted in FDS to yield the required concentration of stock solution. Aliquots were removed, transferred into freeze-drying vials and lyophilised. Two vials from each of the three batches of standard prepared (for the respective peptide) were compared in assay to check the accuracy of the process, and these calibrated vials were used as standards in future assays.

For the preparation of a standard curve, a calibrated vial is reconstituted in a precise volume of assay buffer. Duplicate aliquots of the required volume are then transferred into the appropriate tubes by means of a micropipette. In the case of the GLP-1 whole molecule assay, standard vials containing 4.0 pmol of GLP-1-(1-36) were prepared. Each of these vials was reconstituted in 4.0 ml of 0.2M glycine buffer yielding a standard solution of 1 pmol/mol. From this solution duplicate aliquots of 1, 2, 5, 10, 20, 50, 100, 200, and 500 μ l were transferred by micropipette into the respective assay tubes. It is essential that prior to transferring an aliquot the micropipette is thoroughly rinsed in the standard solution. This ensures that peptide losses are minimised by the saturation of potential binding sites. Similarly, inaccuracy is reduced, if after transference the micropipette is rinsed in the buffer of the assay tube.

Quality Control Samples

From stock solutions of the peptides, 50 or 200 fmol aliquots were transferred into a series of 36 polypropylene assay tubes, lyophilised and stored at -20°C . Groups of 6 tubes were

reconstituted in buffer and assayed on six separate occasions. The intra- and inter-assay variation could thus be determined. This is based on the mean peptide concentration estimated for these tubes within and between individual assays and may be calculated by dividing the standard deviation obtained for a series by the mean concentration.

Results and Discussion

By calculating the mean "Zero" % bound values within an assay the "minimum detection limit" at 95% confidence was 10 fmol/ml for the GLP-2-(1-9)+Y assay, 13 fmol/ml for the GLP-1-(1-19) assay, 40 fmol/ml for the crossreacting assay of antiserum GN-13 with that of GLP-1 whole molecule, 2.0 fmol/ml for the GLP-1 whole molecule assay and 5.0 fmol/ml for the h-GLP-2-(1-34) assay. With the exception of the h-GLP-2-(1-34) assay (16 mg/tube), charcoal concentrations of 8 mg/tube provided adequate separation of "bound" from "free" antigen. However, a considerable margin of tolerance is exhibited by all these assays with respect to the concentration of charcoal added (see fig 2:15).

A mean standard curve obtained from 6 replicate series of standards within a single assay is shown in figure 2:14. Also indicated are the approximate 95% confidence limits (ie $\pm$ two standard deviations). It exemplifies the fact that the variance of the response parameter (% bound) changes with dose (the concentration of peptide). The sensitivity of an assay thus varies along the standard curve. By dividing the standard deviation of each % bound value by the mean % bound value at each standard concentration, the

relative error or the intra-assay coefficient of variation in the estimates of peptide concentration may be determined across the detection range. Thus, the coefficient of variation (CV) is a measure of an assay's precision and varies with dose.

This is a common feature of radioimmunoassays (Rodbard 1974) and is referred to as the 'heteroscedacity' of an assay. Figure 2:14 demonstrates this principle as applied to the GLP-1 whole molecule assay. The precision is observed to vary from 6.8% at 1 fmol/tube to 4.2% at 50 fmol/tube. However, the next point on the curve produces a CV of 7.5% at 100 fmol/tube. A region of maximum precision (minimum CV in the estimates of concentration, 4.2-4.8%) therefore exists between concentrations of 2-50 fmol/tube of GLP-1.

Analysis of the data from the quality control samples gave respective intra- and inter-assay coefficients of variation of 9% and 15% at 200 fmol/tube for the GLP-1-(1-19) assay, 4.8% and 11.6% at 50 fmol/tube for the GLP-1 whole molecule assay and 5.5 and 13.5% at 50 fmol/tube for the h-GLP-2-(1-34) assay. It is of interest that the intra-assay CV calculated from quality control sample data for the GLP-1 whole molecule assay (4.8%) compares well with the relative error (4.2%) for the same concentration determined from the precision profile. Overall therefore, the sensitivity and precision of the respective radioimmunoassays developed was considered adequate for the studies intended.

b) EXTRACTION OF GLUCAGON-LIKE PEPTIDE IMMUNOREACTIVITY FROM TISSUE

INTRODUCTION

In order to accurately quantify the tissue content of these glucagon-like peptides, it was necessary to evaluate extraction methods which would allow for the maximal recovery of tissue immunoreactivity. Specific enzymatic degradative processes appear to exist for the biological breakdown of peptides (Mutt 1982). An efficient extraction protocol would thus have to render inactive such enzymes which, though not normally encountered by the peptide (under physiological conditions), may due to lysosomal disruption during the process of extraction result in its degradation.

The glucagon-like peptides (GLP-1 and GLP-2) are not proteins of a sufficient size to have a well defined tertiary structure. An extraction protocol utilising a boiling medium would thus afford a means of selectively destroying enzymatic activity (denaturing), whilst maintaining the structure of the peptide. However, boiling may cause chemical changes such as deamidation which may alter the immunoreactivity of the peptide. This possibility was investigated with the aid of reverse phase HPLC as well as by directly assessing the immunoreactivity of the extracted material. Therefore, alternative "cold" extraction protocols were also simultaneously assessed.

METHODS

Choice of Extraction Medium:

The following media were evaluated for their efficiency in recovering tissue GLP-like immunoreactivity.

- | | |
|--|----------|
| a) Water | - boiled |
| b) 0.5M Acetic Acid | - boiled |
| c) 0.2M Sodium Chloride | - boiled |
| d) 0.1M Sodium Hydroxide | - cold |
| e) 0.1M Hydrochloric Acid | - cold |
| f) Acid - Alcohol (80%(v/v) ethanol,
1%(w/v) ortho-phosphoric acid) | - cold |

The colons from 30 male Wistar rats were removed immediately on sacrifice, washed in saline and frozen on dry ice. For each extraction medium, 6 polypropylene tubes were allocated, each containing 10 ml of the respective medium. Those containing water, 0.5M acetic acid and 0.2M sodium chloride were held in a boiling water bath and allowed to reach a maximum temperature of 96°C. The rest of the media were maintained at room temperature.

The deep frozen colon tissue was broken up into pieces of approximately 100 mg in weight and thoroughly mixed to ensure uniformity. Batches of tissue of approximately 1g were accurately weighed and added to each tube. Great care was taken to ensure that no thawing of the tissue occurred prior to extraction. This is because other workers have shown that thawing of frozen tissue induces a significant loss in tissue peptide stores (McGregor &

Bloom 1983). The tubes held at 96°C are maintained at this temperature for a further 10 minutes, then removed from the bath and allowed to cool. The other extraction media held at room temperature were maintained at 4°C for 12 hrs following the addition of tissue. From the tube weights (when empty and with their contents) the volume contained in each tube was accurately determined. Evaporative losses were restored such that the final volume of each solution was precisely 10 ml.

A similar procedure was carried out with liver tissue obtained from the same rats. Approximately 1g (frozen and treated identically to the colon tissue) was accurately weighed and added to each of a series of three tubes/extraction medium. Those containing water, 0.5M acetic acid and 0.2M sodium chloride were maintained at 96°C for 10 min, whilst the remainder were held at 4°C for 12 hrs. A 100 µl aliquot was removed from each extract for the analysis of endogenous peptide content. An addition of 50 µl (equivalent to 50 pmol of each peptide) was made to each tube from a cocktail containing GLP-1-(1-36), h-GLP-2-(1-34) and pancreatic glucagon. The respective tubes were either re-boiled for a further 10 minutes or maintained at 4°C for another 12 hours. Following adjustment for any evaporative losses, both the colonic and liver extracts were stored frozen (-20°C) until required for assay.

Radioimmunoassay

The tissue content of GLP-1 and GLP-2-like immunoreactivity was measured using the radioimmunoassays discussed previously (antisera TRPN-17 and UGAY-4). Total glucagon-like immunoreactivity was

measured using the R59 antiserum as described by Ghatei (1982). This antiserum was raised against a carbodi-imide conjugate of porcine pancreatic glucagon. It shows a crossreactivity of 100% with pancreatic glucagon and 94% with apparently pure porcine glicentin. Studies with N- and C-terminal fragments point to the antigenic determinant being localised within the N-terminal region of pancreatic glucagon. Scatchard analysis yielded a maximal K value of 1.2×10^{11} l/mol at pH 8.8 (0.2M glycine buffer). The assay uses porcine pancreatic glucagon as both a reference standard and radiolabel. The minimum detection limit of this assay was 2.0 fmol/ml at the 95% confidence level, and the intra- and inter-assay coefficients of variation were 8% and 12% respectively.

Reverse Phase High Pressure Liquid Chromatography

A pooled aliquot (2 ml) of liver extract (0.5M acetic acid) containing the added peptides was deproteinised prior to injection onto the HPLC column. This was achieved by applying the extract to a pre-equilibrated Sep-Pak cartridge. These cartridges contain octadecyl silylsilica beads. They are equilibrated by sequentially washing through with 10 ml of 100% acetonitrile, followed by 10 ml of distilled water (both containing 0.1% (v/v) TFA). Following application of the liver extract, the cartridge was eluted with 20 mls of H₂O, allowing for the selective removal of large hydrophilic proteins. The peptides which remain bound to the beads were eluted off with 1 ml of 70% (v/v) aqueous acetonitrile and diluted with water to yield 2.5 ml of a 28% solution of aqueous acetonitrile. This is to enable sample loading onto the HPLC column at a concentration below that of 30% acetonitrile, at which the column is equilibrated for commencement of the run.

Peptide recovery off the sep-pak was assessed for GLP-1-(1-36), h-GLP-2-(1-34) and PG by determining the losses incurred on applying their respective radiolabelled peptides to sep-paks and processing them in an identical manner. In each case greater than 90% of the applied radioactivity was recovered.

Reverse phase HPLC was performed on a Techsil 5 μ m pore size octadecyl sililylsilica column (0.39 x 30 cm), to which was coupled a two solvent delivery system (model 6000 A), a universal chromatography septumless injector (model U6K) with a 2.0 ml injection loop and a gradient programmer (model 660). Following injection of the sample, the column was eluted with a linear gradient of aqueous acetonitrile (30-60 %), containing 0.1% TFA at a flow rate of 1 ml/min and over the course of 60 min. The concentration was then held at 60% for 10 minutes followed by a further gradient run of 60-100% acetonitrile in 10 minutes. Fractions of 2 mls were collected in polypropylene tubes, and these were taken to dryness in an evacuated centrifuge. They were reconstituted in assay buffer prior to radioimmunoassay. The losses incurred in the "drying down" process were considered to be negligible, as column recoveries approaching 100% (determined from the remnants of the sample loaded which had not been through the drying process) were regularly obtained.

Results and Discussion

Water, 0.5M acetic acid and 0.2M sodium chloride proved equally efficient in extracting the total glucagon (TG), GLP-1 and GLP-2 like immunoreactivity present in rat colonic tissues, whilst

the recovery with acid-alcohol, 0.1M hydrochloric acid and 0.1M sodium hydroxide was comparatively poor (see fig 2:17). Similarly, the recovery of exogenously added peptide from liver extracts was again most effectively achieved within the same group of media; recoveries of the three peptides being in excess of 90% (see fig 2:18). On the basis of the marginally better results obtained with 0.5M acetic acid and the long-established acid extraction protocols used in this laboratory (McGregor & Bloom 1983) this extraction medium was selected for further analysis.

Radioimmunoassay of the acetic acid liver extracts containing the exogenously added peptides produced dilution curves in parallel with each of the respective standards. A similar result was obtained with acetic acid colonic extracts. An equivalence in immunoreactivity with the reference standard is, therefore, indicated. Furthermore, no detectable immunoreactivity was present in aliquots of acetic acid liver extracts withdrawn prior to the addition of peptide. It appears, therefore, that boiling in acetic acid does not adversely affect the immunoreactivity of the recovered material, and this is irrespective of any chemical modifications that may have occurred.

However, the degradation of a peptide may yield a fragment with the antigenic determinant intact. In a radioimmunoassay, its immunoreactivity may be indistinguishable from that of the reference standard. Chromatographic studies to determine the precise nature of the extracted immunoreactivity are, therefore, also required. Reverse phase HPLC of the peptide containing acetic acid liver extracts produced three peaks of immunoreactivity, each

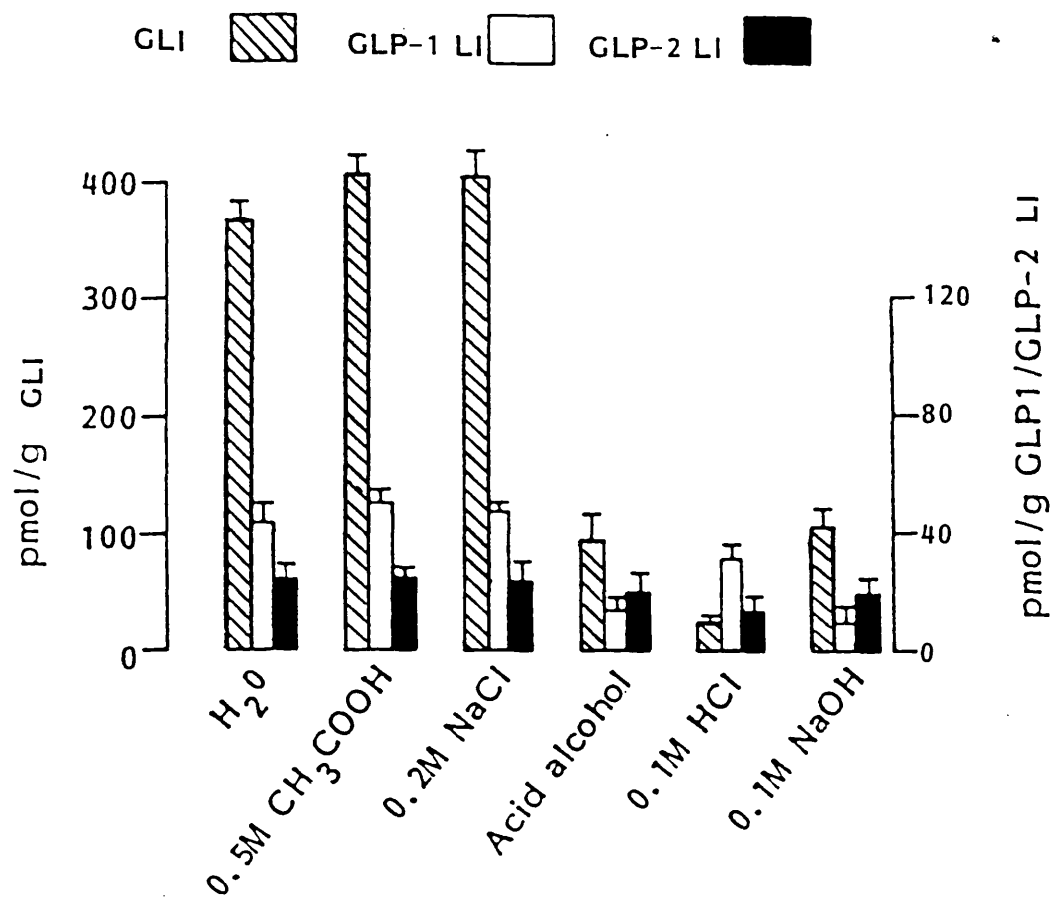


Figure 2:17 The efficiency of various media in solubilising rat colonic tissue peptide stores. Bars represent mean $\pm$ SEM.

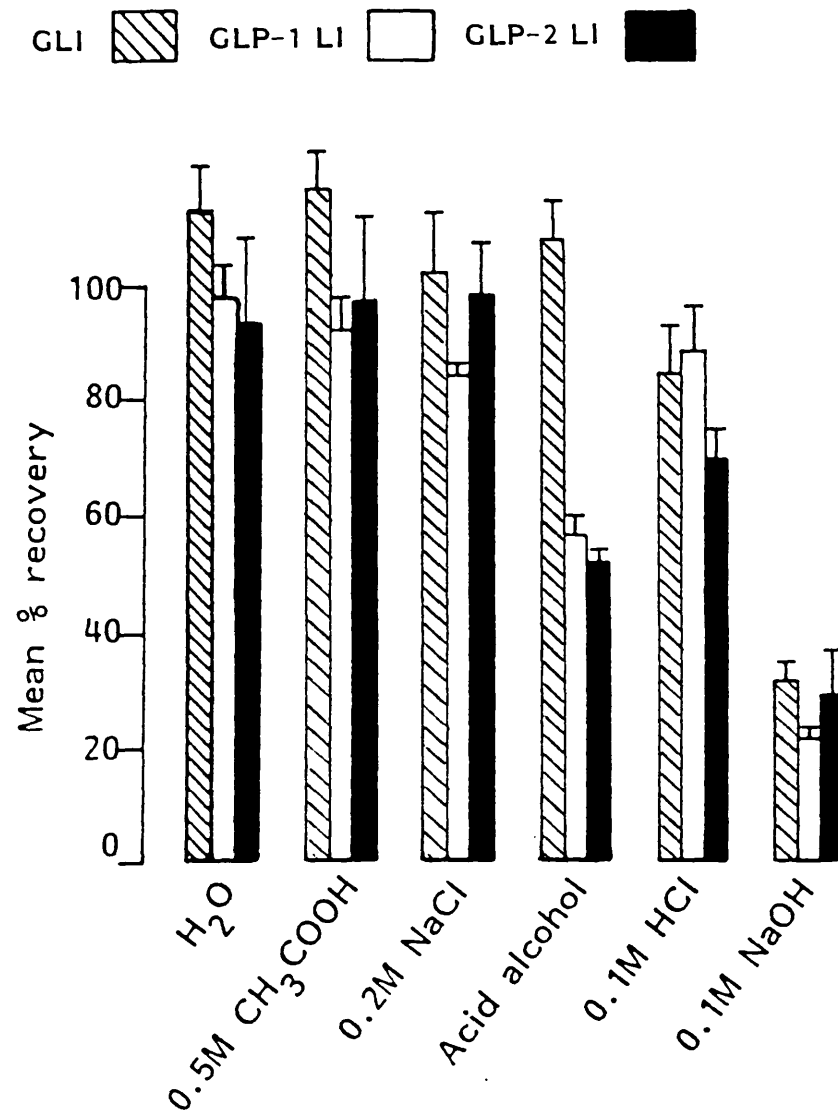


Figure 2:18 The efficiency of various media in recovering exogenously added peptides from their respective liver extracts. Bars represent mean $\pm$ SEM.

corresponding in position to that of its respective reference standard (see fig 2:19). On the basis of the above evidence, it may be concluded that acetic acid extraction is an efficient means of solubilising tissue GLI, GLP-1 and GLP-2-like immunoreactivities. It is capable of their efficient recovery, maintaining both their chromatographic and immunochemical properties and simultaneously allows for the adequate destruction of peptidase activity.

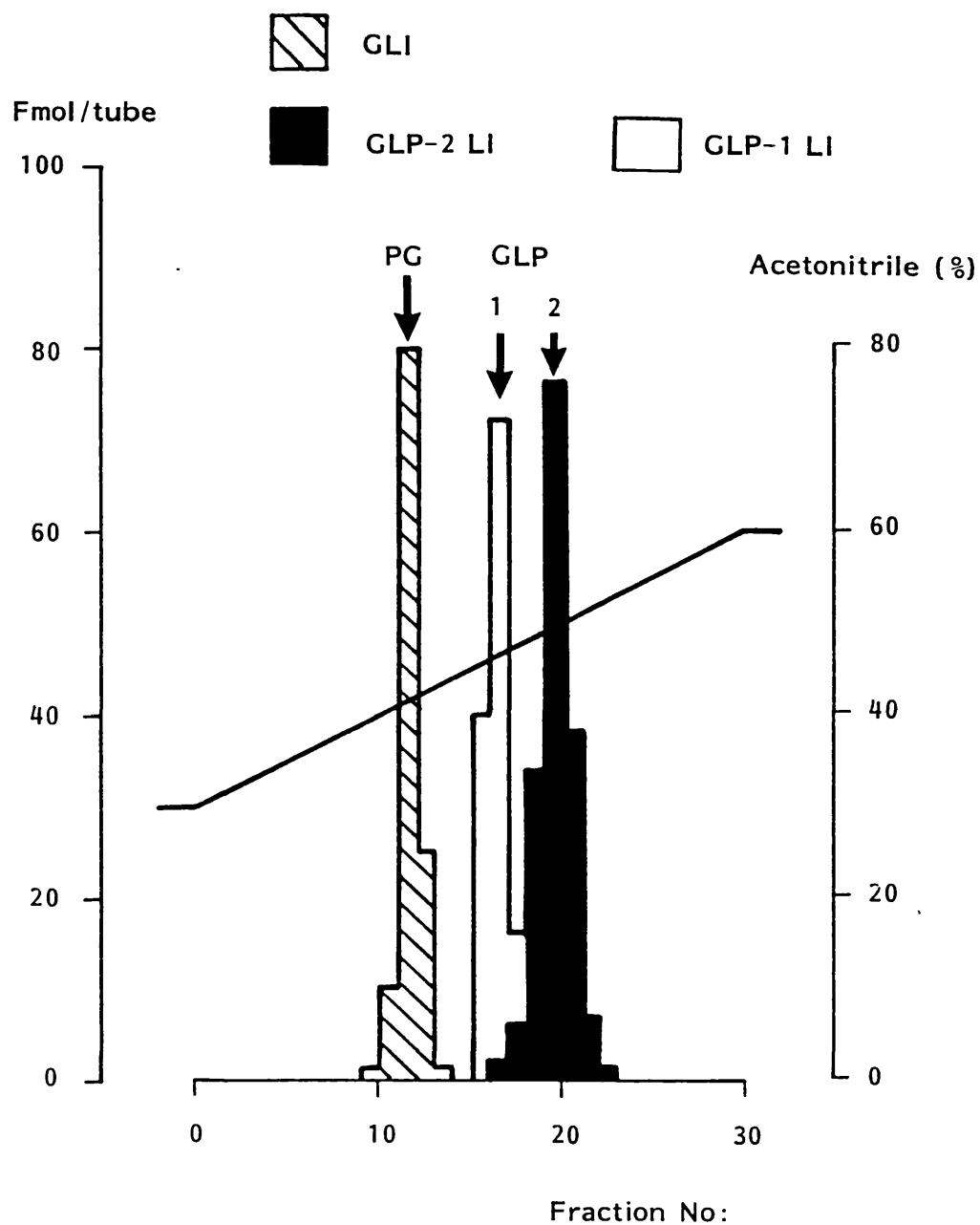


Figure 2:19 Reverse phase high pressure liquid chromatographic analysis of the immunoreactivity recovered by an acetic acid (0.5M) liver extract containing the exogenously added peptides.

CHAPTER 3

THE POST-TRANSLATIONAL PROCESSING OF MAMMALIAN PROGLUCAGON

INTRODUCTION

Although the post-translational processing of the N-terminal 69 amino acids of mammalian proglucagon corresponding to "glicentin" in the pig has been extensively studied (see chapter 1), the fate of the C-terminal portion of the precursor that contains the glucagon-like peptides GLP-1 and GLP-2 remains unknown. Are these glucagon-like peptides cleaved from the precursor and secreted as such by glucagon-secreting cells or does enzymatic processing result in larger or smaller fragments? As the -Arg-Arg- residues at position 17 and 18 of the glucagon sequence do not seem to form a cleavage site (known cleavage points in the N-terminal region of proglucagon being -Lys-Arg- residues), is it feasible that the -Arg-Arg- residues separating the GLP-1 and GLP-2 sequences are cleaved within the golgi apparatus of glucagon producing cells? Furthermore, might the C-terminal portion of the precursor be processed differently between the pancreas and the gut, as observed with the N-terminal portion of mammalian proglucagon?

To answer these questions, the radioimmunoassays developed to the synthetic peptide sequences of GLP-1-(1-19), GLP-1-(1-37) and human GLP-2-(1-34) (see chapter 2), were used to investigate the occurrence and chromatographic properties of their respective antigenic determinants in extracts of human pancreas,

gut, glucagonomas and plasma and, similarly, in porcine gut and pancreas.

METHODS

Tissue Samples and Extraction Procedure

Specimens of histologically normal pancreas ($n = 5$), ileum, colon and rectum ($n = 3$), and pancreatic glucagonomas ($n = 2$) removed at surgery were extracted according to the protocol described previously (see chapter 2b). The glucagonomas came from two patients (A, B) presenting with the "glucagonoma syndrome" (Mallinson et al 1974) and were shown by immunocytochemistry and radioimmunoassay to contain significant amounts of pancreatic glucagon (PG). Pre-treatment blood samples (10 ml) from the above two patients (A, B), and a third patient (C) with radiological and biochemical evidence of a glucagon-secreting tumour and blood samples from ten healthy volunteers were collected in lithium-heparin-coated glass tubes containing 4,000 kallikrein inhibitor units of aprotinin. The tubes were immediately centrifuged and the separated plasma stored at -20°C . Specimens of porcine pancreas, ileum, colon, caecum and rectum ($n = 4$) were also collected and extracted.

Chromatography

i) Gel Filtration

Gel filtration of tissue extracts (neutralised by the addition of half their volume of 1M NaHCO_3) and plasma samples was performed at

4°C on a column (90 x 1.4 cm) containing a 130 ml bed volume of Sephadex G-50 superfine, in either 0.05M sodium barbitone buffer [pH 8.0, for the analysis of GLP-1-(1-19)-like immunoreactivity] or 0.2M glycine buffer [pH 8.8, for the analysis of GLP-1 and GLP-2-like immunoreactivities], containing 1% BSA at a flow rate of 5.0 ml/hr collecting 2.5 ml fractions. The column was pre-calibrated with dextran blue (mol wt 67,000) as a void volume marker, horse heart cytochrome C (mol wt 12,384) and NaI¹²⁵ as a total volume marker. Elution volumes were plotted in terms of K_{av} according to Laurent and Killander (1964).

ii) High Pressure Liquid Chromatography - (Reverse Phase)

Chromatography of all samples on this system was carried out as described previously (see chapter 2b).

RESULTS

Human Pancreas

Figure 3:1 shows a representative gel filtration profile of GLP-1-(1-19)-like immunoreactivity (LI) in human pancreatic extracts eluted with 0.05M sodium barbitone buffer (pH = 8.0). The profiles from four separate specimens were similar; peaks eluting in the same positions but differing slightly in relative amounts. As only the crossreactivity with antiserum GN13 of the immunoreactive material eluting in the position of synthetic GLP-1 was known, it was chosen to present the results in terms of GLP-1 whole molecule

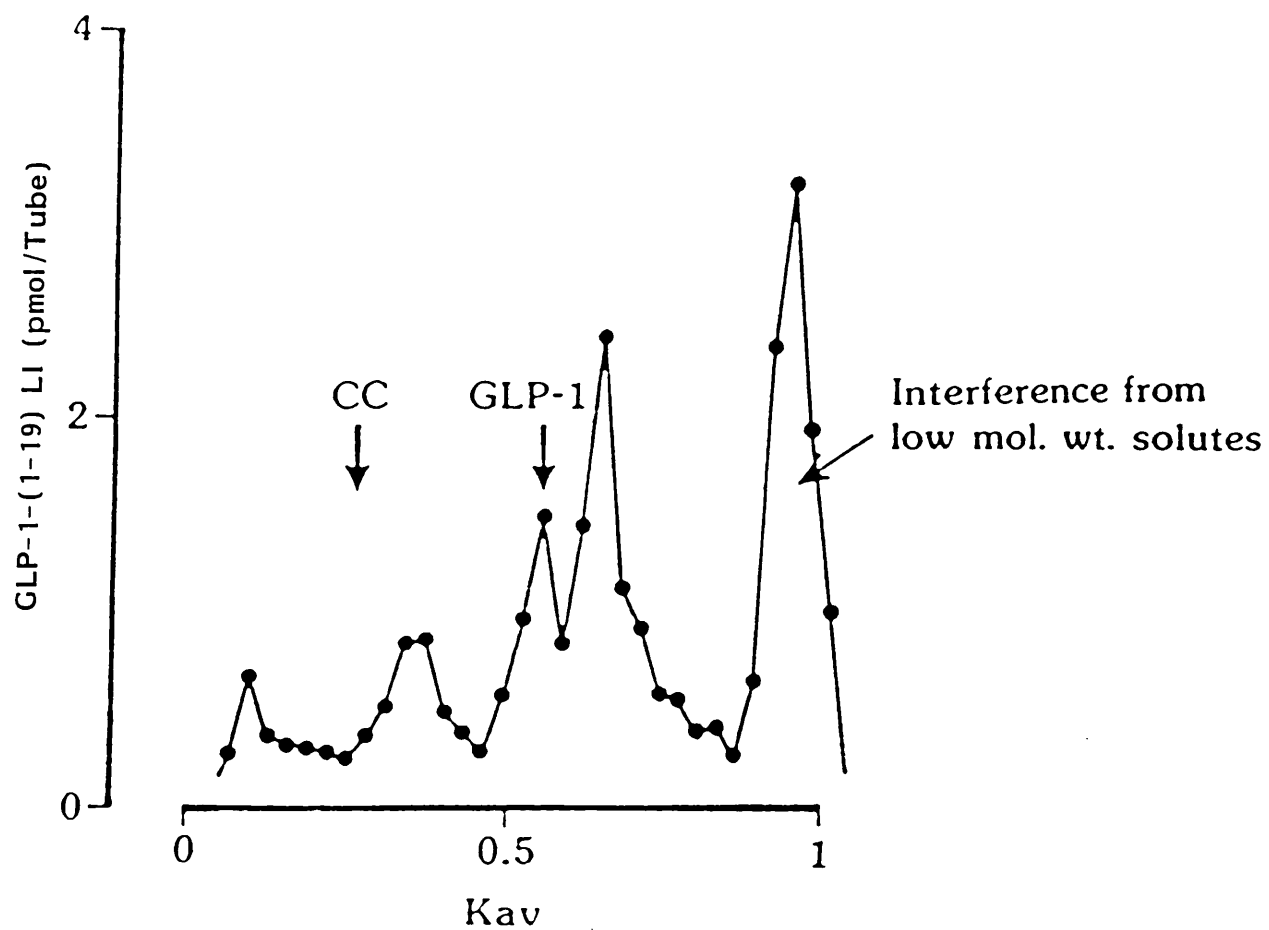


Figure 3:1 Representative sephadex G-50 gel filtration profile of GLP-1-(1-19) LI in human pancreatic extracts.

standard equivalents. Peak 1 (K_{av} 0.10) which was bifid in one case contained $20 \pm 8\%$ (mean $\pm$ SEM), peak 2 (K_{av} 0.36) $21 \pm 5\%$, peak 3 (K_{av} 0.57) corresponding to the elution position of synthetic GLP-1, $20 \pm 3\%$ and peak 4 (K_{av} 0.65) $35 \pm 8\%$ of the total recovered immunoreactivity. The concentration of peak 3 material from these pancreas specimens represents 94 ± 7 pmol equivalents of GLP-1 whole molecule, per gram wet weight of tissue.

Gel filtration of these pancreatic extracts (eluted with 0.2M glycine buffer, pH 8.8) analysed for GLP-1 (antiserum TRPN-17) and GLP-2 (antiserum UGAY-4) like immunoreactivities (LI) yielded more variable profiles (see fig 3:2). However, common to all these pancreases was:-

- a) A peak at K_{av} 0.15, the most prominent, containing equivalent amounts of both GLP-1 and GLP-2 LI and consisting of 60-80% (134.5 ± 12.0 pmol/g) of their respective total recovered immunoreactivities. In four cases the ratio of GLP-1 to GLP-2 LI within this peak was approximately 1:1, although in one case it was 1:0.3.
- b) A peak at K_{av} 0.43, the second most prominent, coinciding with the elution position of synthetic GLP-1 standard, and varying between 2-20% (30.5 ± 14.0 pmol/g) of the total recovered GLP-1 LI.

Other minor peaks of GLP-1 LI of K_{av} 0.29, 0.57, 0.61 and 0.88 were also obtained. Besides the principal form of GLP-2 LI observed eluting within the K_{av} 0.15 peak, variable amounts of GLP-2 LI of

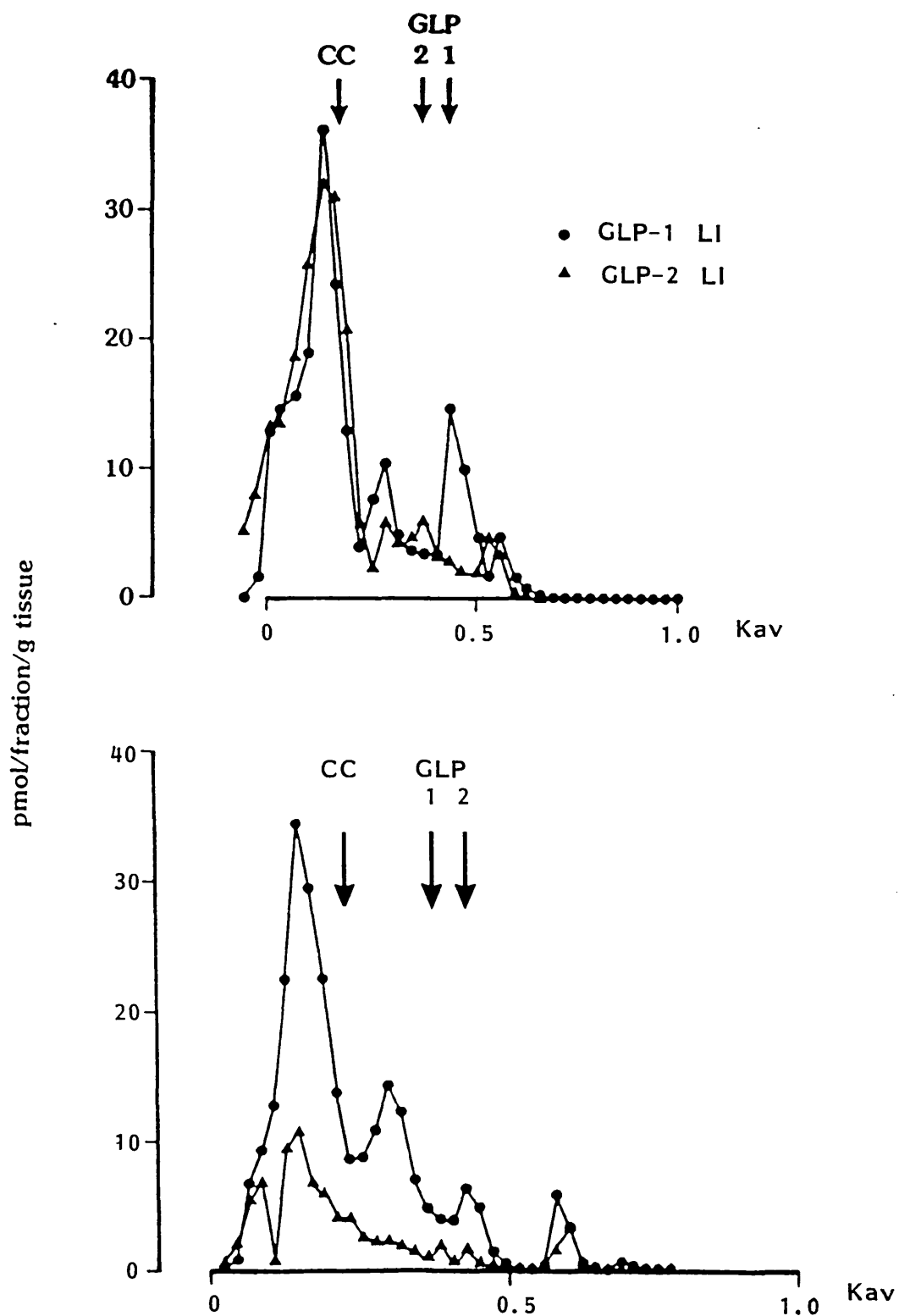


Figure 3:2 Representative sephadex G-50 gel filtration profile of GLP-1 and GLP-2 LI in human pancreatic extracts.

Kav 0.30, 0.37 [the elution position of synthetic human GLP-2-(1-34) standard, consisting of 2-5% (9.0 ± 4.5 pmol/g) of the total recovered GLP-2 LI] with an occasional peak eluting at Kav 0.53, were also obtained.

HPLC analysis of these pancreatic extracts demonstrated a peak (#19-22) eluting in the position of synthetic human GLP-2-(1-34) standard. It contained equivalent amounts of both GLP-1 and GLP-2 LI, consisting of 60-80% (95 ± 16 pmol/g) of the total recovered GLP-1 LI and 95-100% of the total recovered GLP-2 LI (see fig 3:3). An earlier peak of GLP-1 LI (#16-18) was also consistently obtained containing between 2-20% (27 ± 11.0 pmol/g) of the total recovered GLP-1-LI, eluting in the position of synthetic GLP-1. Under the elution conditions used it was not possible to differentiate between GLP-1-(1-36) amide and GLP-1-(1-37), and thus ascertain the precise native state of this molecular species. Glucagon-like immunoreactivity (GLI) emerged as a peak of pancreatic glucagon (#12-13) preceded by material that may correspond to N-terminally extended forms of pancreatic glucagon. The mean ratio of the total recovered GLI: GLP-1: GLP-2 LI in these chromatograms was 100:62:48.

Rechromatography on Sephadex G-50 of HPLC #20 (corresponding to the peak fraction of the immunoreactive material containing both the GLP-1 and GLP-2 antigenic determinants), yielded a major peak eluting at a Kav of 0.15 containing equivalent amounts of GLP-1 and GLP-2 LI accompanied by a small amount of GLP-2 LI eluting in the position of synthetic h-GLP-2-(1-34) standard, Kav 0.37 (see fig 3:4). Similar assessment of HPLC #17 yielded a single peak of GLP-1 LI, Kav 0.43, eluting in the position of synthetic GLP-1

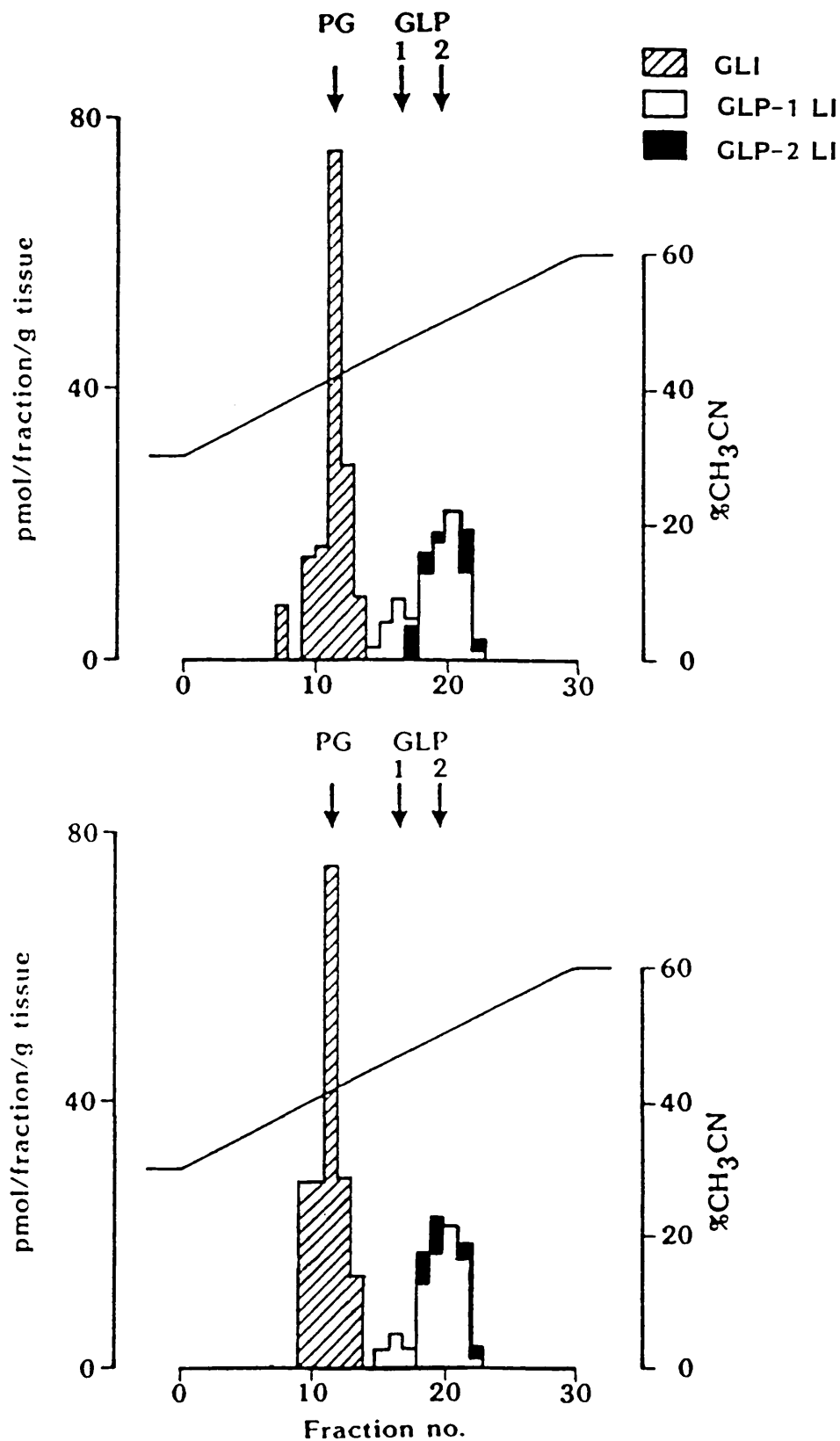


Figure 3:3 Representative reverse phase HPLC profiles of GLP-1 and GLP-2 LI in human pancreatic extracts.

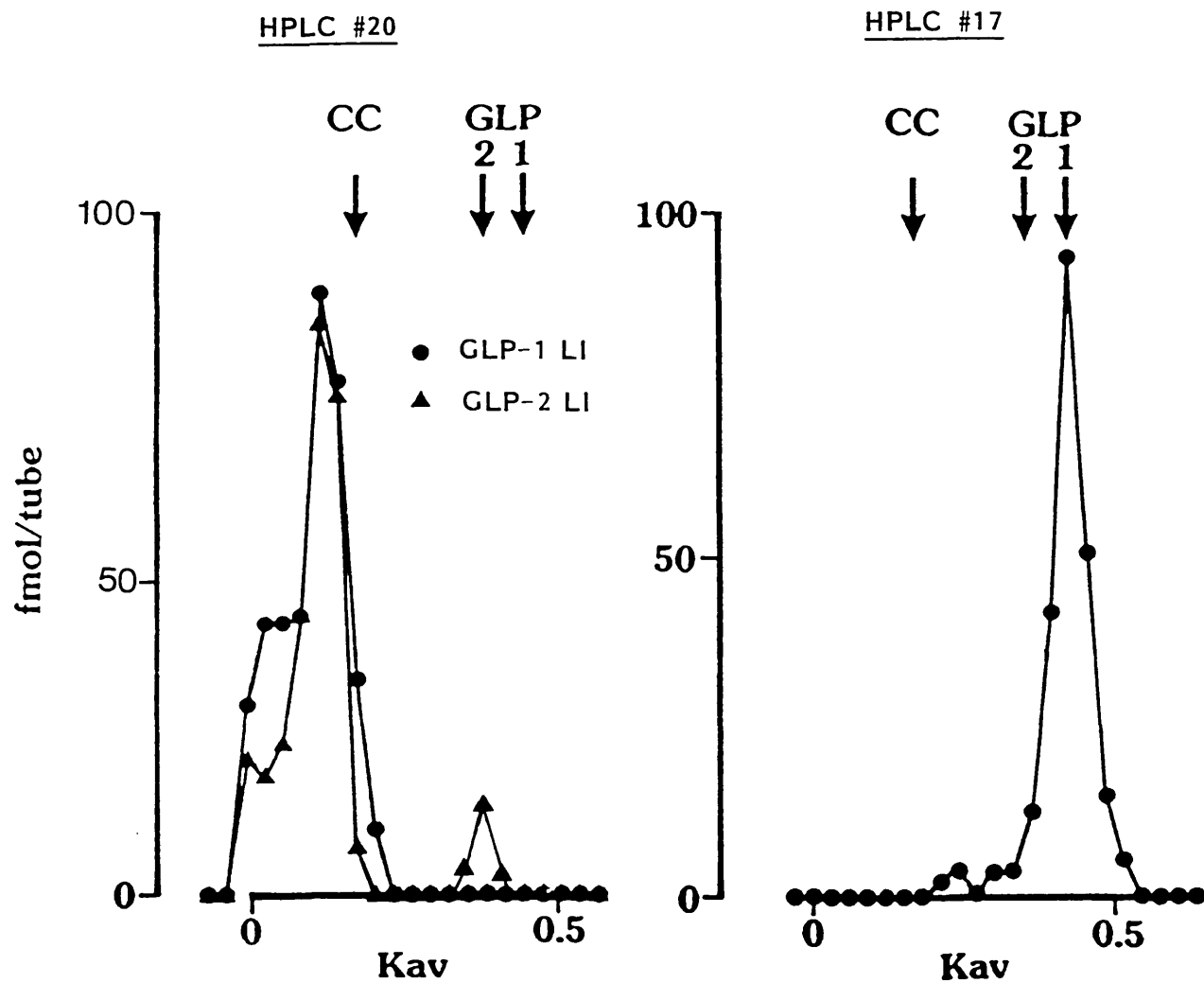


Figure 3:4 Rechromatography on sephadex G-50 of the peak fractions of HPLC recovered GLP-1 (# 17) and GLP-2 (# 20) LI from a human pancreatic extract.

(see fig 3:4). It is apparent that under the HPLC conditions used, both synthetic h-GLP-2-(1-34) standard and the large molecular weight component (K_{av} 0.15) have similar column retention times and therefore chromatograph identically.

Human Gut

i) Ileum

HPLC analysis revealed the presence of three prominent peaks of GLP-2 LI (see fig 3:5). A peak eluting in the position of synthetic h-GLP-2-(1-34) standard (#19-22) consisting of $75 \pm 10\%$ (30.0 ± 4.0 pmol/g) of the total recovered GLP-2 LI, with other earlier peaks accounting for $8 \pm 3\%$ (#11-13, 3.2 ± 1.2 pmol/g) and $6 \pm 2\%$ (#6-7, 2.4 ± 0.8 pmol/g) respectively. The majority of the recovered GLP-1 LI ($92 \pm 12\%$, 70.3 ± 9.2 pmol/g) eluted in the position of synthetic GLP-1 standard (#16-18) with a further $5 \pm 1\%$ (3.8 ± 0.8 pmol/g) eluting earlier (#5-6). GLI emerged as a single broad peak of immunoreactivity (#8-11, 181.8 ± 16.5 pmol/g) presumably corresponding to the human equivalent of porcine glicentin-like immunoreactivity. The mean ratio of GLI: GLP-1: GLP-2 LI in these specimens was 100:42:22.

ii) Colon

On HPLC analysis, the bulk of GLP-2 LI ($75 \pm 8\%$, 1.3 ± 0.14 pmol/g) emerged in the position of synthetic h-GLP-2-(1-34) standard (#19-22) along with earlier peaks constituting $18 \pm 4\%$ (#11-12, 0.32 ± 0.07 pmol/g) and $5 \pm 0.5\%$ (#6, 0.1 ± 0.008 pmol/g) respectively (see fig 3:5). GLP-1 LI emerged as a major peak eluting in the

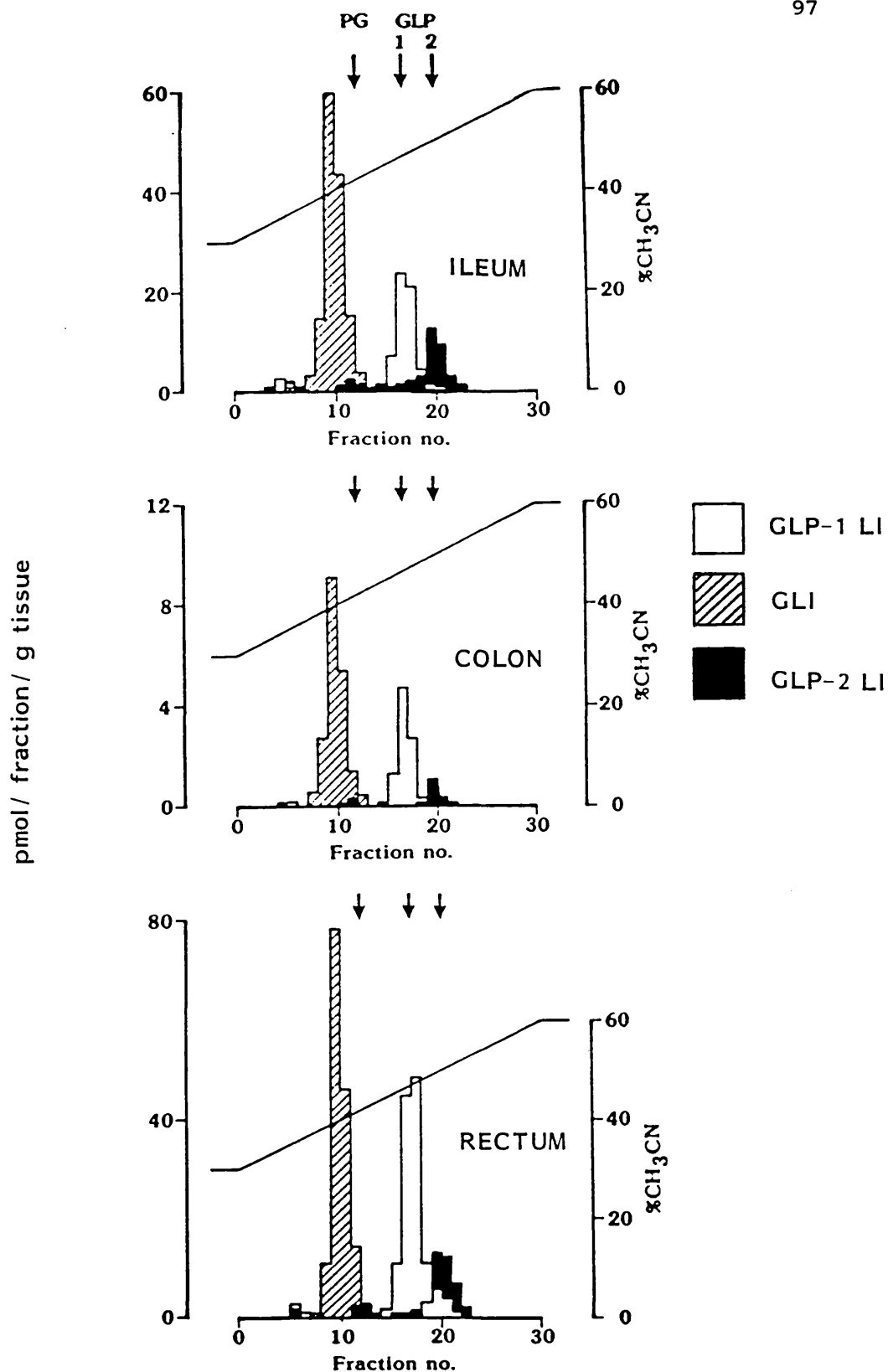


Figure 3:5 Representative reverse phase HPLC profiles of GLI, GLP-1 and GLP-2 LI in extracts of human intestine.

position of synthetic GLP-1 standard (#16-18) comprising $96 \pm 9\%$ (9.6 ± 0.9 pmol/g) of the total recovered GLP-1 LI, accompanied by an earlier eluting peak accounting for a further $4 \pm 1\%$ (#5-6, 0.4 ± 0.1 pmol/g). GLI emerged as a single peak (#9-11, 21.3 ± 4.2 pmol/g) as observed with ileal extracts. The mean ratio of GLI: GLP-1: GLP-2 LI in these specimens was 100:47:8.

Rectum

HPLC analysis revealed the major proportion of recovered GLP-2 LI ($75 \pm 11\%$, 31.1 ± 4.6 pmol/g) to elute in the position of synthetic h-GLP-2-(1-34) standard (#19-22), although other earlier eluting peaks accounting for $10 \pm 3\%$ (#11-12, 4.2 ± 1.2 pmol/g) and $6 \pm 2\%$ (#5-6, 2.5 ± 0.8 pmol/g) were also obtained (see fig 3:5). GLP-1 LI emerged as a peak eluting in the position of synthetic GLP-1 standard (# 16-18) representing $94 \pm 13\%$ (139.1 ± 19.2 pmol/g) of the total recovered GLP-1 LI, whilst an earlier eluting peak accounted for a further $4 \pm 1\%$ (#5-6, 5.9 ± 1.5 pmol/g). GLI (#9-11, 180.4 ± 12.6 pmol/g) was observed to elute similarly to ileal and colonic extracts; the mean ratio of GLI: GLP-1: GLP-2 LI being 100:82:23.

Rechromatography on Sephadex G-50 of HPLC #17 from representative ileal, colonic and rectal samples (corresponding to the peak fraction of GLP-1 LI emerging in the position of synthetic GLP-1 standard) resulted in its elution as a single peak (see fig 3:6) in the position of synthetic GLP-1 standard (K_{av} 0.43). However, similar analysis of HPLC #20 (corresponding to the peak fraction of GLP-2 LI emerging in the position of synthetic h-GLP-2-(1-34)

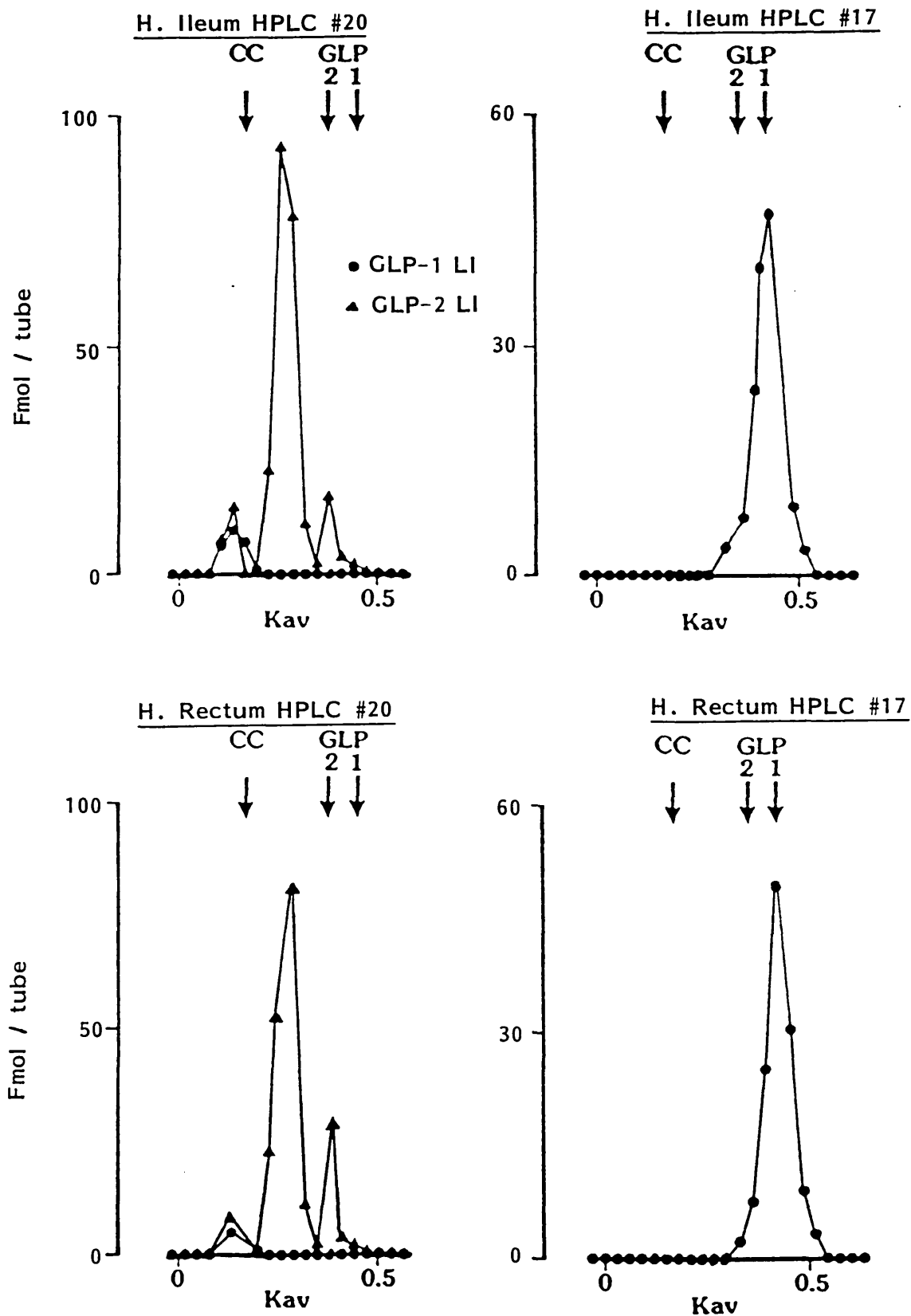


Figure 3:6 Rechromatography on sephadex G-50 of the peak fractions of HPLC recovered GLP-1 (# 17) and GLP-2 (#20) LI from extracts of human intestine.

standard) resulted in its resolution into two peaks of GLP-2 LI (Kav 0.26 and 0.37) containing no detectable GLP-1 LI. The Kav 0.37 peak (eluting in the position of synthetic h-GLP-2-(1-34) standard), represented approximately 12% of the total recovered GLP-2 LI, whilst that at Kav 0.26 accounted for the remaining 88% (see fig 3:6).

Normal Human Plasma

Early gel filtration studies on normal plasma revealed a single peak of GLP-1 LI eluting with a Kav of 0.15. Subsequent HPLC analysis (n = 4) produced a peak (#19-22) containing both GLP-1 and GLP-2 LI in a mean ratio of 1:1.7, accounting for $81 \pm 18\%$ (42 ± 9.3 pmol/l) of the total recovered GLP-1 LI and $50 \pm 13\%$ (70 ± 18.2 pmol/l) of the total recovered GLP-2 LI (see fig 3:7). An earlier eluting peak of GLP-1 LI was also obtained eluting in the position of synthetic GLP-1 standard (#16-18) consisting of $19 \pm 7\%$ (10 ± 3.6 pmol/l) of the total recovered GLP-1 LI. Other peaks of GLP-2 LI eluting at #6-8 (36 ± 12.6 pmol/l) and #11-14 (30 ± 8.4 pmol/l) were also present, accounting for 26 ± 9 and $21 \pm 6\%$ respectively of the total recovered GLP-2 LI. GLI emerged as a single peak (#9-10) containing material which may correspond to C-terminally extended forms of circulating pancreatic glucagon-like material (Ghatei et al 1983). The ratio of the total recovered GLI: GLP-1: GLP-2 LI was 100:220:726.

Rechromatography of HPLC #20 on Sephadex G-50 revealed two peaks of immunoreactivity eluting at a Kav of 0.15 and 0.26 (see Fig 3:8). The Kav 0.15 peak consisted of equivalent amounts of GLP-1 and

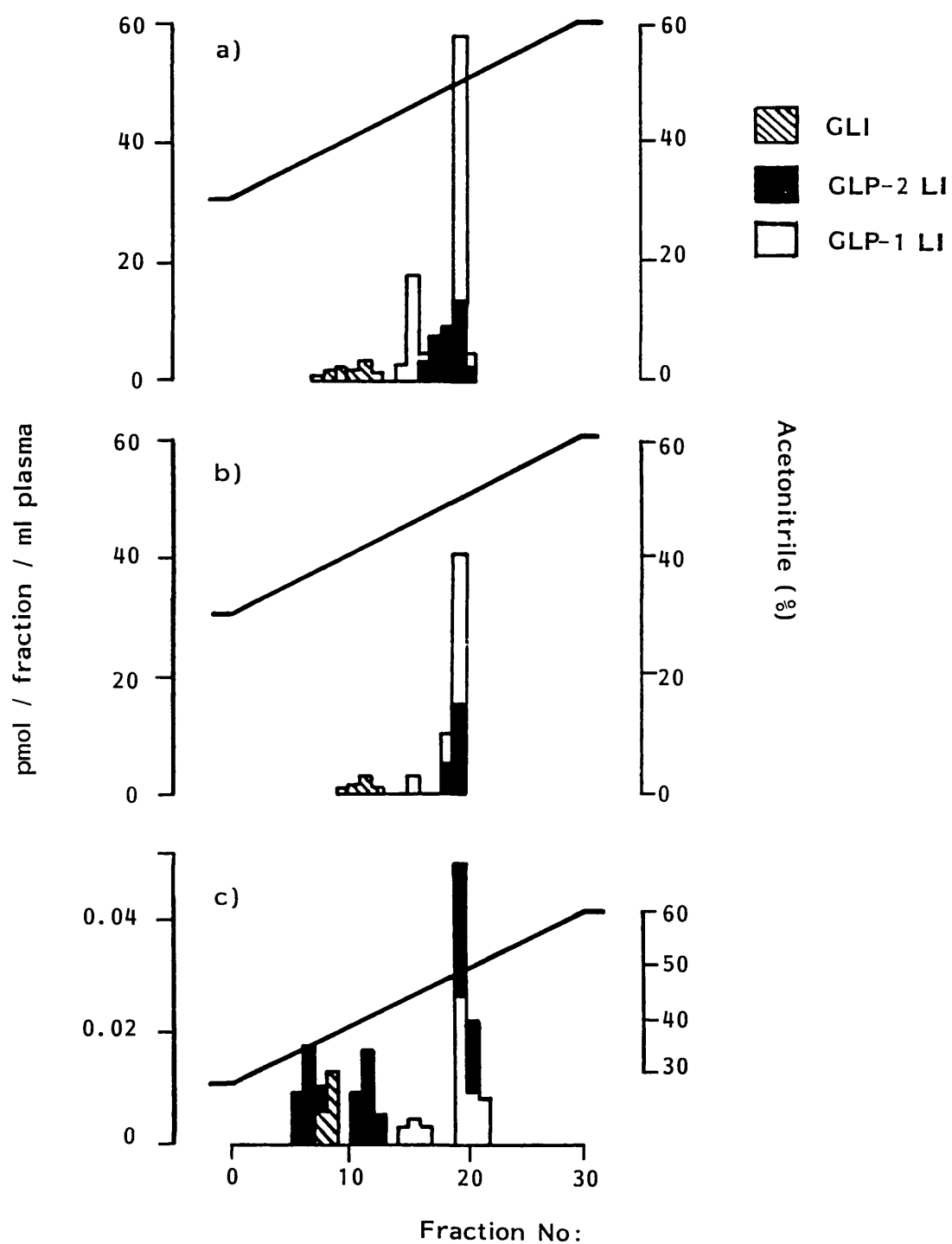


Figure 3:7 Reverse phase HPLC profiles of GLI, GLP-1 and GLP-2 LI
in a) Glucagonoma plasma A b) Glucagonoma plasma B and
c) Normal human plasma.

GLP-2 LI, accounting for 100% of the total recovered GLP-1 LI, but only 60% of the total recovered GLP-2 LI. The remaining 40% of GLP-2 LI was eluted within the Kav 0.26 peak.

Human Glucagonoma Plasma

Sephadex G-50 gel chromatography of pre-treatment plasma samples from glucagonoma patients (A, B and C) revealed peaks of GLP-1-(1-19) LI of Kav 0.02, 0.12, 0.21 and 0.56 (the elution position of synthetic GLP-1 (see fig 3:8); the peaks at Kav 0.12 and 0.21 being incompletely resolved. Integration of the peak eluting at Kav 0.56, allowed estimates of the molar concentrations of free GLP-1 LI in the plasma of glucagonoma patients to be made. These are compared with the corresponding plasma concentrations of immunoreactive glucagon (see table 3:1), as measured with the C-terminally reactive glucagon antiserum RCS5 (Christofides 1982). In each case, the plasma concentration of free GLP-1 LI was consistently higher than that of RCS5 immunoreactive glucagon.

HPLC analysis of plasmas A and B revealed a peak (#19-22) containing both GLP-1 and GLP-2 LI, consisting of 74% (74.0 nmol/l) of the total recovered GLP-1 LI and 100% (32 nmol/l) of the total recovered GLP-2 LI in plasma A, and 96% (50 nmol/l) and 100% (15 nmol/l) respectively in plasma B (see fig 3:7 & table 3:1). The ratios of GLP-1: GLP-2 LI within this peak in both plasmas were similar, being 1:0.4 (plasma A) and 1:0.3 (plasma B). Both samples also revealed an earlier peak (#16-18), eluting in the position of synthetic GLP-1 standard, consisting of 24% (24 nmol/l) of the total recovered GLP-1 LI in plasma A and 5% (2.6 nmol/l) in plasma B.

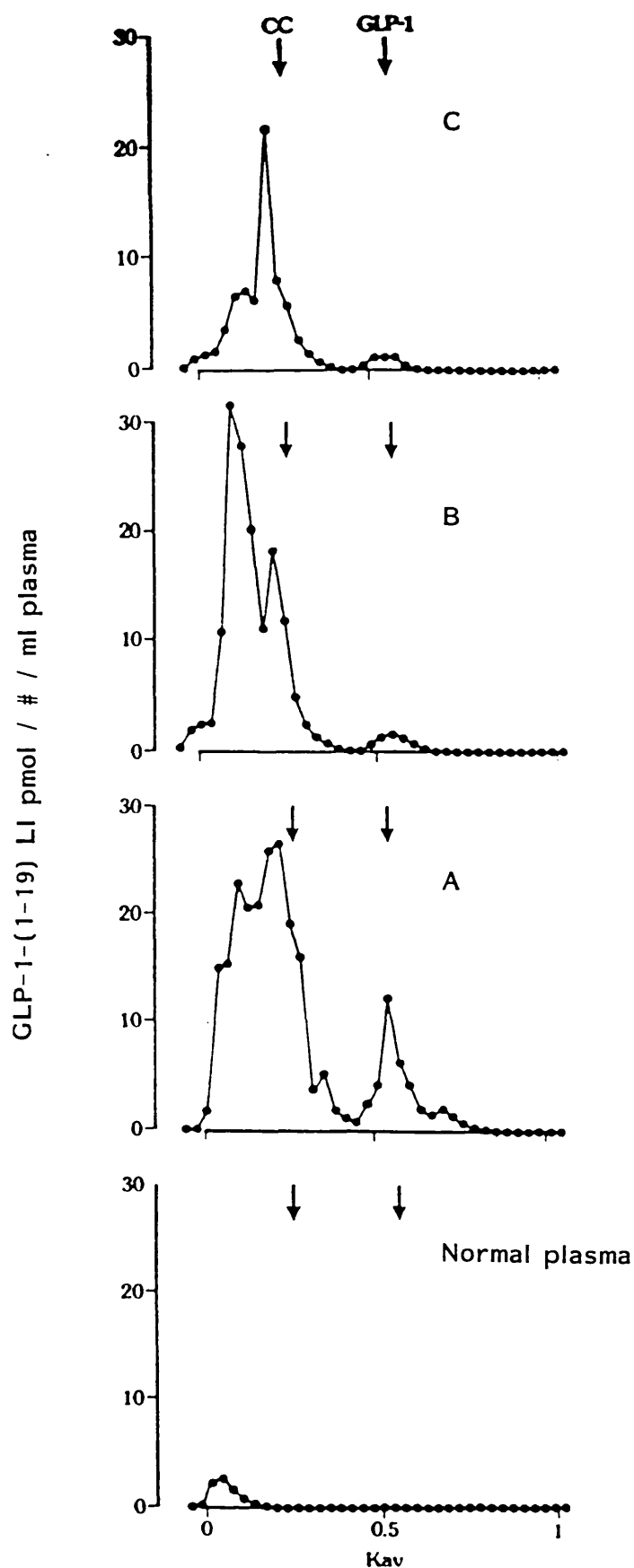


Figure 3:8 Sephadex G-50 gel filtration profiles of GLP-1-(1-19) LI in the plasma of three glucagonoma patients (A B and C) and in normal human plasma (D).

	<u>PG</u>	<u>Free GLP-1 (Kav 0.43)</u>		<u>Large molecular size</u>		
		<u>peak</u>		<u>(Kav 0.15) peak</u>		
	(nmol/l)	(nmol/l)		(nmol/l)		
<u>Patient</u>		<u>GLP-1-(1-19)</u>	<u>GLP-1</u>	<u>GLP-(1-19)</u>	<u>GLP-1</u>	<u>GLP-2</u>
A	7.6	26.8	24.0	183.0	74.0	32.0
B	3.2	4.9	3.0	139.0	50.0	15.0
C	2.2	3.1	?	60.0	?	?

Table 3:1 The relative concentrations of pancreatic glucagon, GLP-1-(1-19) GLP-1 and GLP-2 LI (nmol/l) within the plasma of glucagonoma patients A,B and C.

GLI emerged as a peak of PG-LI (#12-13) preceded by material that may correspond to C-terminally extended forms of pancreatic glucagon. Ratios of the total recovered GLI: GLP-1: GLP-2 LI were 100:1000:470 (plasma A) and 100:760:290 (plasma B).

Rechromatography on Sephadex G-50 of HPLC #20 yielded a single peak of K_{av} 0.15, containing both GLP-1 and GLP-2 LI, and confirmed the deficiency in GLP-2 LI. HPLC #17 emerged as a single peak eluting in the position of synthetic GLP-1 standard, K_{av} 0.43.

Human Glucagonomas

Gel filtration profiles of GLP-1-(1-19) LI in extracts of two glucagonomas (A,B) are shown in figure 3:9. In tumour A, the most prominent peak containing 55% (1.4 nmol/g) of the total recovered immunoreactivity emerged in the position of synthetic GLP-1, along with smaller amounts of material of larger molecular size. In tumour B, there was a preponderance of larger molecular forms that were poorly separated with only a relatively small amount (18%, 0.6 nmol/g) of the recovered immunoreactivity eluting in the position of synthetic GLP-1.

Similar chromatographic analysis for GLP-1 and GLP-2 LI within these tumour extracts, revealed two major peaks of GLP-1 LI eluting at K_{av} 0.15 and 0.43 (the elution position of synthetic GLP-1) (see fig 3:10). In both tumours, the ratio of GLP-1: GLP-2 LI within the K_{av} 0.15 peak was 1:0.25, although, in tumour A this peak consisted

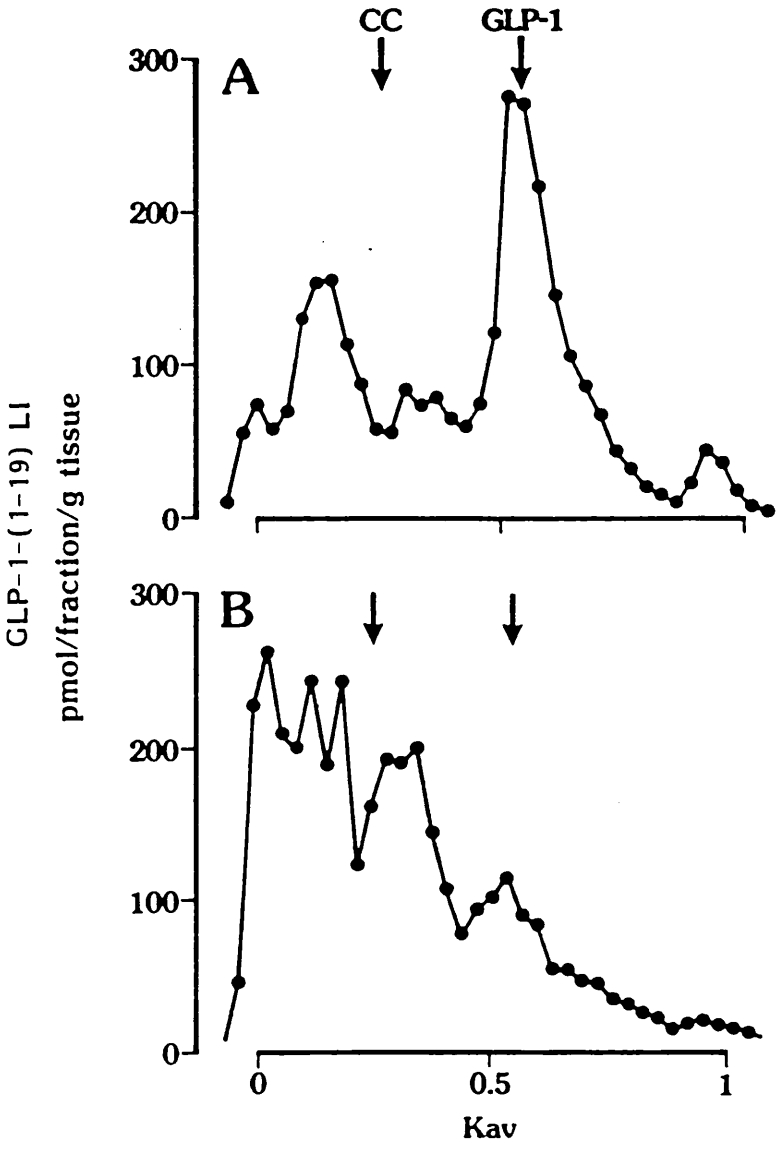


Figure 3:9 Sephadex G-50 gel filtration profiles of GLP-1-(1-19) LI
in extracts of two human pancreatic glucagonomas (A & B)

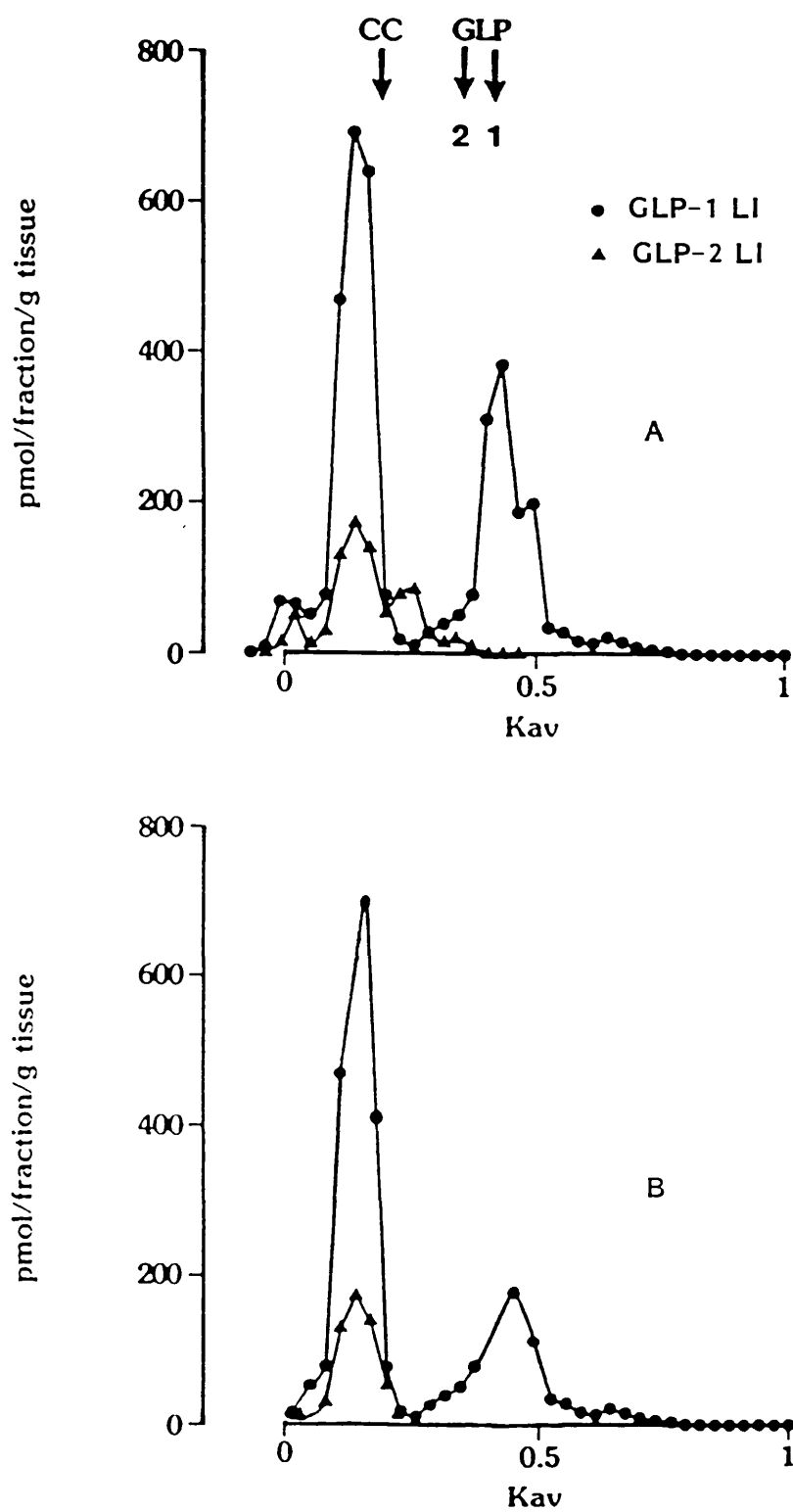


Figure 3:10 Sephadex G-50 gel filtration profiles of GLP-1 and GLP-2 LI in extracts of two human pancreatic glucagonomas (A & B)

of 57% (2.0 nmol/g) of the total recovered GLP-1 LI and 63% (0.5 nmol/g) of the total recovered GLP-2 LI, whilst in tumour B they were 65% (1.9 nmol/g) and 100% (0.5 nmol/g) respectively. The peak at K_{av} 0.43, consisted of 37% (1.3 nmol/g) of the total recovered GLP-1 LI (tumour A), whilst in tumour B this represented only 22% (0.6 nmol/g). Other minor peaks of GLP-2 LI eluting at K_{av} 0.02 and 0.26 were also obtained (tumour A), although neither tumour contained immunoreactive material eluting in the position of synthetic human GLP-2-(1-34), K_{av} 0.37.

HPLC analysis of tumour extracts A and B yielded a peak (#19-22) containing both GLP-1 and GLP-2 LI (see fig 3:11). In tumour A, this peak consisted of 40% (0.8 nmol/g) of the total recovered GLP-1 LI and 95% (0.3 nmol/g) of the total recovered GLP-2 LI, whilst in tumour B respective values of 62% (1.1 mol/g) and 95% (0.3 nmol/g) were obtained.

However, as observed with the gel filtration (K_{av} 0.15) peak, a similar disparity in the ratio of GLP-1: GLP-2 LI of approximately 1:0.3 was obtained in each case. An earlier peak of GLP-1 LI (#16-18) was also consistently obtained, comprising 48% (1.0 nmol/g) in tumour A and 28% (0.5 nmol/g) in tumour B, of the total recovered GLP-1 LI. Both tumours also contained small amounts (< 5%) of the total recovered GLP-1 LI eluting between #7-9. GLI emerged as a peak of pancreatic glucagon (#12-13), preceded by material that may correspond to N-terminally extended forms of PG (Ghatei et al 1983). The ratio of the total recovered immunoreactivities (GLI: GLP-1: GLP-2) in tumour A was 100:250:38 and in tumour B, 100:46:8.

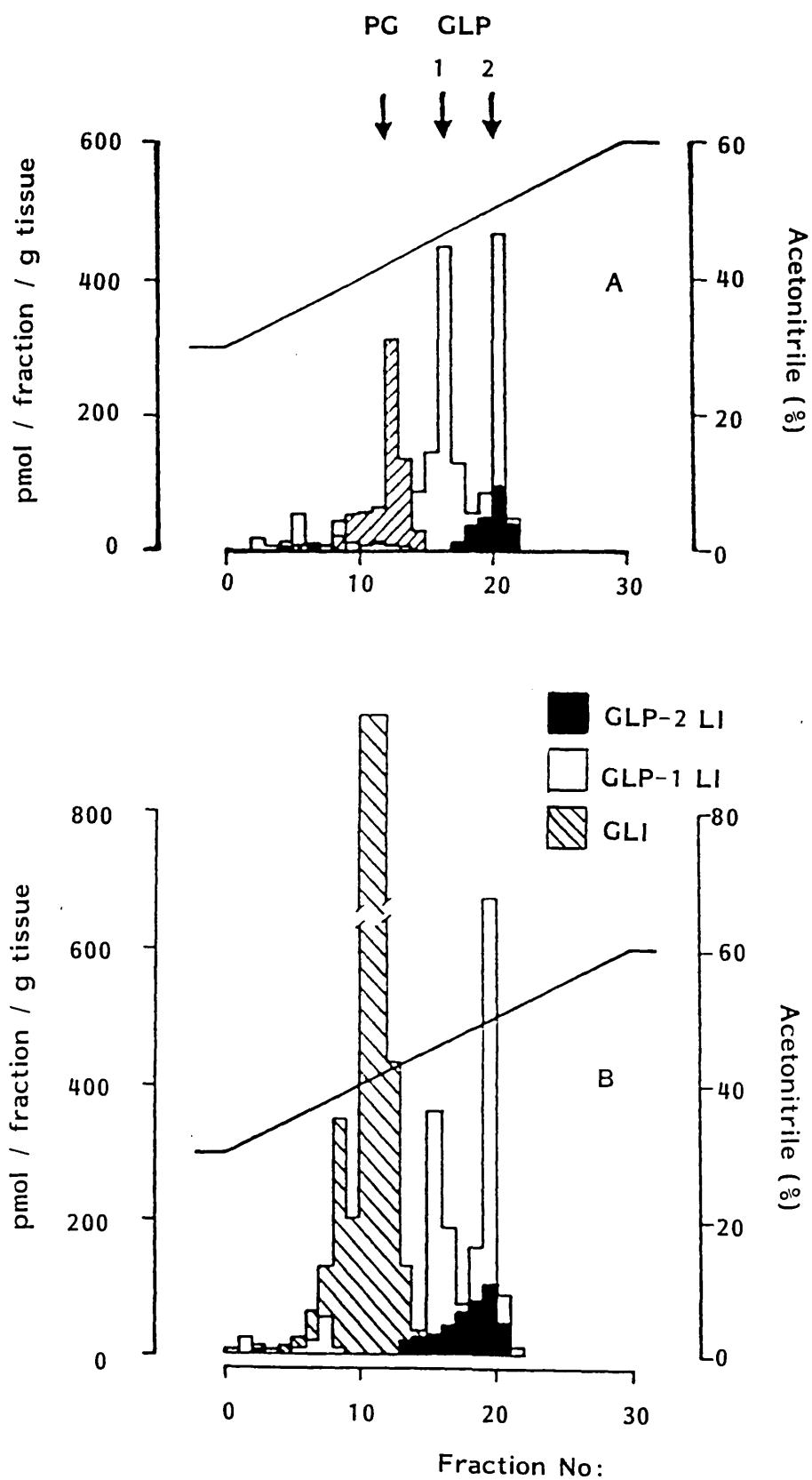


Figure 3:11 Reverse phase HPLC profiles of GLI, GLP-1 and GLP-2 LI in extracts of two human pancreatic glucagonomas (A & B).

Rechromatography on Sephadex G-50 of HPLC #20 (corresponding to the peak fraction of the immunoreactive material containing both GLP-1 and GLP-2 antigenic determinants) yielded a single peak eluting with a K_{av} of 0.15, containing GLP-1: GLP-2 LI in an approximate ratio of 1:0.3. Similar assessment of HPLC #16 yielded a peak of K_{av} 0.43 eluting in the position of synthetic GLP-1.

Porcine Pancreas

HPLC analysis of porcine pancreatic extracts revealed a peak (#19-22) containing both GLP-1 and GLP-2 LI, consisting of $37 \pm 6\%$ (24.5 ± 4.0 pmol/g) of the total recovered GLP-1 LI and $50 \pm 8\%$ (9.0 ± 1.4 pmol/g) of the total recovered GLP-2 LI (see fig 3:12). The ratios of GLP-1: GLP-2 LI within this peak were approximately 1:0.3. Other peaks of GLP-1 LI containing $10 \pm 3\%$ (#4-6, 6.6 ± 2.2 pmol/g) and $47 \pm 9\%$ (31.7 ± 6.0 pmol/g, eluting in the position of synthetic GLP-1 standard, (#16-18) were also observed. A further peak of GLP-2 LI (#23-25) consisting of $46 \pm 7\%$ (8.3 ± 1.3 pmol/g) of the total recovered GLP-2 LI, eluting later than synthetic h-GLP-2-(1-34) standard was also obtained. GLI emerged as a peak of pancreatic glucagon (#12-13) preceded by material that may correspond to N-terminally extended forms of PG. The mean ratio of GLI: GLP-1: GLP-2 LI in these specimens was 100:15:4.

Porcine Gut

HPLC analysis of extracts of porcine ileum, caecum, colon and rectum revealed the presence of two separate peaks of immunoreactivity

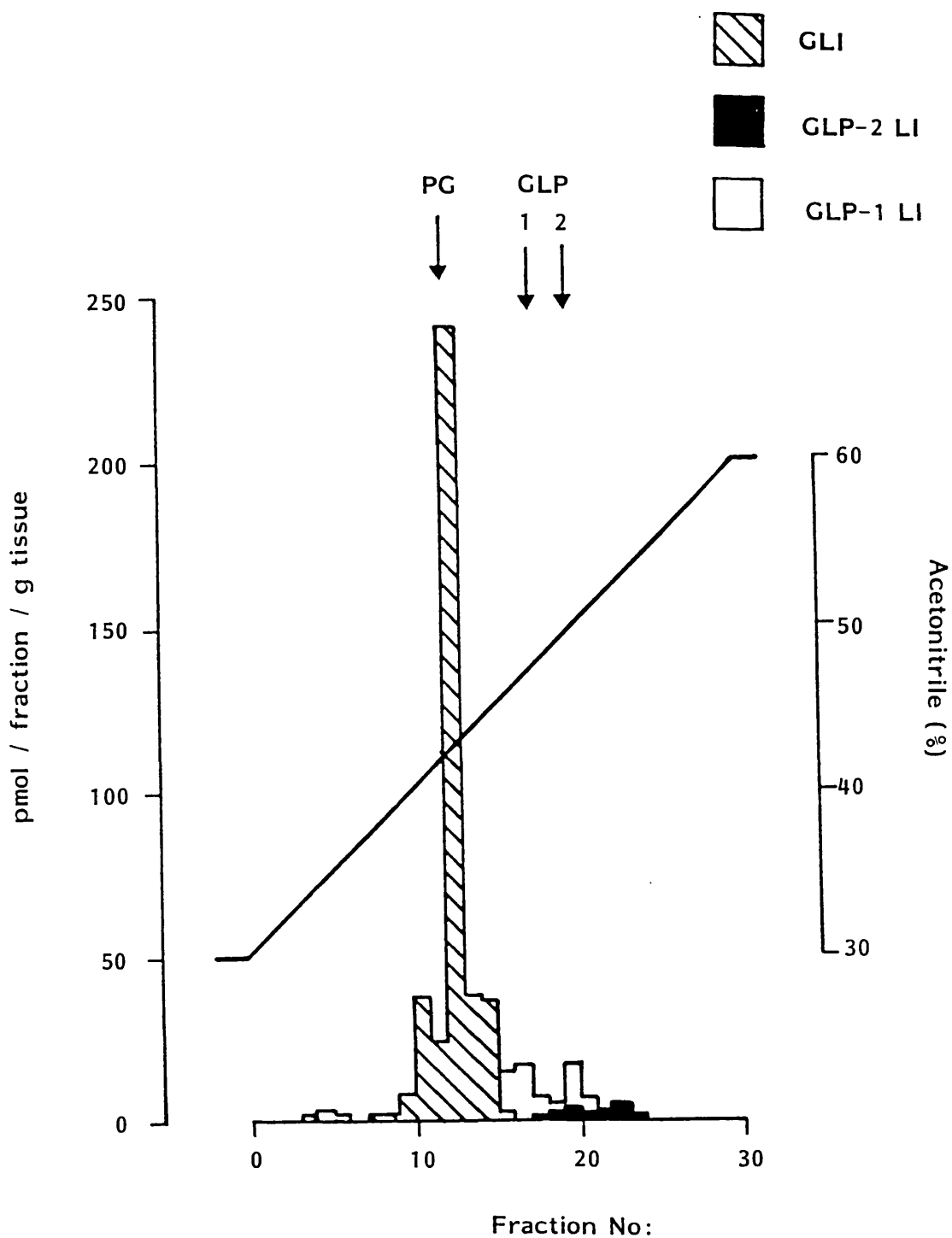


Figure 3:12 Representative reverse phase HPLC profile of GLI, GLP-1 and GLP-2 LI in porcine pancreatic extracts.

corresponding to GLP-1 LI (#16-18) and GLP-2 LI (#19-22), eluting in the positions of their respective synthetic standards (see fig 3:13). GLI emerged as a single peak (#9-10), eluting earlier than the PG standard, presumably corresponding to glicentin-like immunoreactivity. The mean ratios of GLI: GLP-1: GLP-2 immunoreactivities within these extracts were ileum (100:20:6), caecum (100:20:5), colon (100:20:7) and rectum (100:21:3).

DISCUSSION

When these results are interpreted in the light of the deduced structure of human pre-proglucagon (Bell et al 1983), it is apparent that the human pancreas consistently produces a proglucagon fragment (Kav 0.15) that emerges before horse heart cytochrome C on gel filtration, contains both GLP-1 and GLP-2 LI, but is devoid of GLI. It is the principal product resulting from the post-translational processing of the C-terminal portion of the pre-proglucagon molecule and is compatible with the production by the human pancreas of a cleavage product consisting of the GLP-1 sequence linked to that of GLP-2 via the intervening "spacer" peptide II.

Pulse chase studies on proglucagon biosynthesis in isolated rat islets have also demonstrated this fragment to be the sole processing product originating from the C-terminal portion of rat pre-proglucagon (Patzelt et al 1984). However, the situation in the human pancreas is more complex as there is also some production of free GLP-1 (ranging from 2-20% of the total recovered GLP-1 LI) and free GLP-2 (ranging from 2-5% of the total recovered GLP-2 LI).

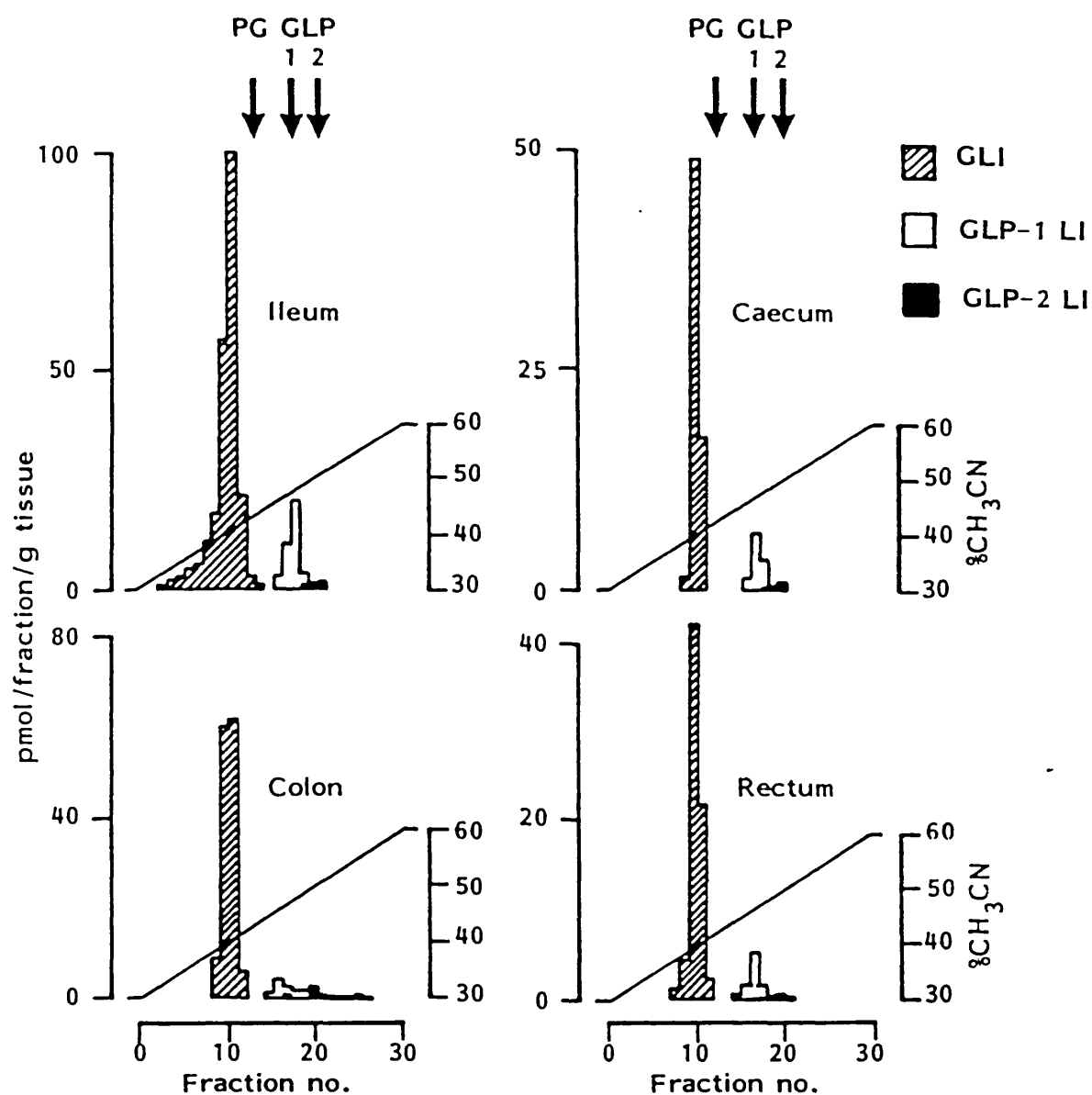


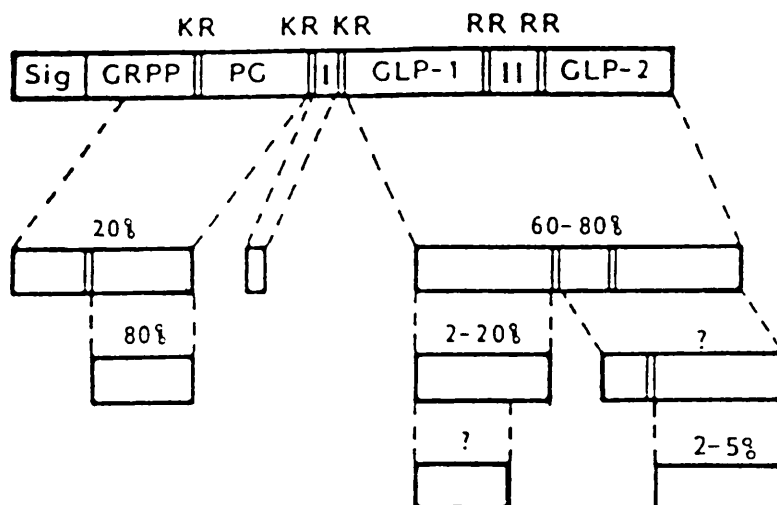
Figure 3:13 Representative reverse phase HPLC profile of GLI, GLP-1 and GLP-2 LI in porcine intestinal extracts.

This implies that within the human pancreas, the C-terminal portion of proglucagon is primarily cleaved at the -Lys-Arg- residues separating the sequence of the intervening "spacer" peptide I from that of GLP-1 (see fig 3:14). However, some cleavage does also occur at the -Arg-Arg- residues separating the sequence of the intervening "spacer" peptide II from those of GLP-1 and GLP-2. Further supportive evidence for this processing scheme is provided by the results obtained on rechromatography on Sephadex G-50 of the HPLC resolved immunoreactive components.

Although most human pancreatic extracts showed an equivalence in their GLP-1: GLP-2 LI within the K_{av} 0.15 peak, there was an occasional deficit in GLP-2 LI. Furthermore, all the pancreatic extracts showed a deficit in the total recovered GLP-1 LI with respect to GLI, and GLP-2 LI with respect to GLP-1 LI; and the expected equimolar production of GLP-1 and GLP-2 with that of pancreatic glucagon (PG) was not observed. This suggests that within the human pancreas, some degradation, derivatisation or post-translational modification of the GLP-1 and GLP-2 sequences occurs which significantly reduces their recovered immunoreactivities with respect to GLI. It is therefore of interest that GLP-1 extracted from the catfish pancreas is also observed to be in deficit to the pancreatic glucagon content (Andrews et al 1985) and may, therefore, be processed similarly.

HPLC analysis of porcine pancreatic extracts demonstrated the presence of molecular forms of GLP-1 and GLP-2 LI similar in size, hydrophobicity and immunochemical character to those present in the human pancreas. However, in contrast to the human pancreas there

PANCREAS



GUT

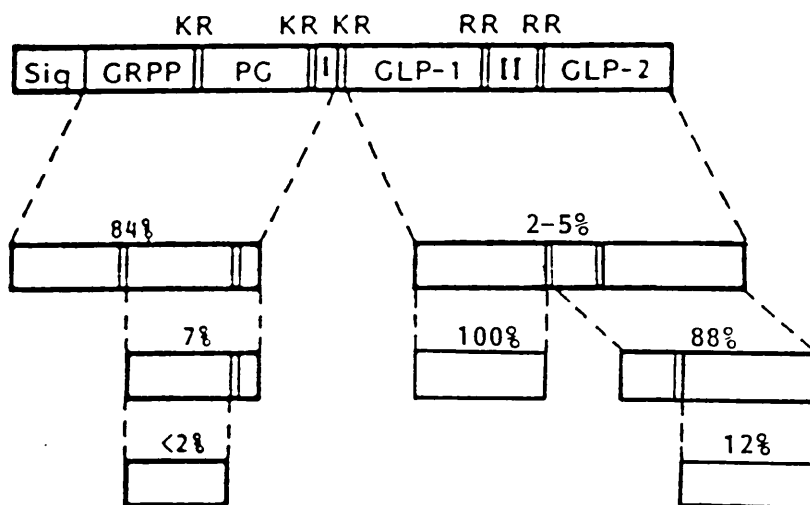


Figure 3:14 Schematic representation of the post-translational processing of pre-proglucagon within the mammalian gut and pancreas. Incorporated within is the deduced processing scheme for the GLP-1 and GLP-2 containing C-terminal portion. The quantities of GLP-1 and GLP-2 LI recovered are expressed as a percentage of their total respective tissue concentrations.

was a reduction in the proportion of the large molecular weight GLP-1 LI (Kav 0.15, HPLC #19-22), with a corresponding increase in the proportion eluting in the position of free GLP-1 (see fig 3:12). Furthermore, the ratio of GLP-1: GLP-2 LI within the large molecular weight component was reduced to 1:0.3, suggestive of further post-translational processing having occurred. This may account for the GLP-2 immunoreactive component eluting later than synthetic human GLP-2-(1-34) standard (see fig 3:12, #23-25) not observed in human pancreatic extracts.

The reduced immunoreactivity of these processing products may partly explain the lower levels of GLP-1 and GLP-2 LI detected in the porcine pancreas. They are considerably less than those observed in the human pancreas (see table 3:2), with the deficit of GLP-2 LI with respect to GLP-1 LI increased three-fold. Moreover, the sequence of the C-terminal portion of porcine proglucagon is yet to be established, and differences between the sequences of human and porcine GLP-1 or GLP-2 may significantly alter antibody crossreactivities, resulting in the estimation of reduced tissue levels of these peptides.

It is concluded that the post-translational processing of human and porcine proglucagon are in essence similar, though further modifications within the porcine pancreas occur, which may contribute to the reduced levels of GLP-1 and GLP-2 LI recovered. This may also partly be explained if non-identities between the sequences of the human and porcine glucagon-like peptides are shown to exist.

Concentration (pmol/g) - (Mean ± SEM)

Human

	<u>GLI</u>	<u>GLP-1 LI</u>	<u>GLP-2 LI</u>	<u>(GLI:GLP-1:GLP-2)</u>
pancreas (n=5)	198.0 ± 22.5	122.0 ± 12.6	95.0 ± 14.3	100 : 62 : 48
ileum (n=3)	181.8 ± 11.5	76.4 ± 8.6	40.0 ± 6.7	100 : 42 : 22
colon (n=3)	21.3 ± 4.2	10.0 ± 1.9	1.7 ± 0.2	100 : 47 : 8
rectum (n=3)	180.4 ± 12.6	148.0 ± 22.5	41.5 ± 6.1	100 : 82 : 23

Porcine

pancreas (n=4)	450.0 ± 36.2	66.2 ± 7.9	18.0 ± 2.6	100 : 15 : 4
ileum (n=4)	83.3 ± 11.3	16.7 ± 4.2	5.0 ± 1.2	100 : 20 : 6
caecum (n=4)	40.0 ± 4.9	8.0 ± 1.5	2.0 ± 0.1	100 : 20 : 5
colon (n=4)	100.0 ± 11.4	20.0 ± 2.5	7.0 ± 1.7	100 : 20 : 7
rectum (n=4)	100.0 ± 8.0	21.0 ± 4.7	3.0 ± 0.4	100 : 21 : 3

Table 3:2 The concentration in pmol/g of GLI, GLP-1 and GLP-2 LI in human
and porcine pancreas and intestine.

In contrast to the human pancreas, the human intestine shows a characteristic absence of the K_{av} 0.15 peak containing both GLP-1 and GLP-2 immunoreactivities; the two major components on HPLC analysis corresponding in elution position to those of the respective synthetic standards of GLP-1 and GLP-2. As observed with the pancreas, there is a deficit in total recovered GLP-1 LI when compared with GLI, whilst the same is true of the total recovered GLP-2 LI with respect to GLP-1 LI. Human ileum and colon contain GLP-1: GLP-2 LI in a mean ratio of 1:0.5 and 1:0.2 respectively, whilst this ratio approaches 1:0.8 in the rectum. The concentration of GLP-1 and GLP-2 immunoreactivity in the human gut is highest in the rectum, lower in the ileum and markedly reduced in the colon (see table 3:2). The significance of this concentration gradient is unknown, but may reflect a regulatory role within the gastrointestinal tract.

The situation in porcine gut is similar, but as observed between the human and porcine pancreas, the deficit in both GLP-1 and GLP-2 immunoreactivities with respect to GLI is greater (see table 3:2). As before, this may be the result of further modifications within the porcine gut and/or non-identities between the C-terminal portions of porcine and human proglucagon yielding processing products which show diminished crossreactivities with the respective antisera.

Rechromatography of the HPLC resolved immunoreactive peaks on Sephadex G-50 confirmed that the human intestine did indeed produce GLP-1 LI in the form of free GLP-1. This combined with the demonstration that synthetic GLP-1-(1-36) has a glucose-dependent

insulinotropic effect on isolated rat islets (Schmidt et al 1985), makes GLP-1 a candidate "incretin", ie a hormone increasing the insulin response to oral nutrient. In vivo studies demonstrating rises in glucose-induced plasma GLP-1 levels are, however, required to substantiate this claim. Rechromatography of the HPLC recovered GLP-2 LI indicates the majority of human intestinal GLP-2 LI to be of a larger molecular size than synthetic h-GLP-2-(1-34). It may therefore correspond to an N-terminally extended form of the GLP-2 sequence, inclusive of that of the intervening "spacer" peptide II.

It is, therefore, proposed, that the post-translational proteolytic processing of the C-terminal portion of proglucagon within the mammalian intestine, results primarily in the cleavage of the N-terminal -Lys-Arg- and C-terminal -Arg-Arg- residues delimiting the GLP-1 sequence. As a result, the mammalian intestine produces mainly immunoreactive material consistent in size and immunochemical character to free GLP-1, and the N-terminally extended form of GLP-2. However, the presence of small amounts of immunoreactivity eluting in the position of free GLP-2 suggests that further processing of this N-terminally extended GLP-2 sequence may occur (see fig 3:14).

In comparison, the post-translational processing of the C-terminal portion of mammalian proglucagon within the mammalian pancreas yields as its major product, immunoreactive material consistent in size and immunochemical character to that of the GLP-1 sequence linked to that of GLP-2 via the intervening "spacer" peptide II. This occurs as a result of the cleavage of the

-Lys-Arg- residues at the N-terminus of the GLP-1 sequence.

However, further processing of this entity whereby some cleavage of the -Arg-Arg- residues at the C-terminus of the GLP-1 sequence takes place, results in variable amounts of free GLP-1 being produced.

The fate of the remaining N-terminally extended GLP-2 sequence is not clear, and very little free GLP-2 is obtained. The degree of cleavage that occurs at the C-terminal -Arg-Arg- residues of the GLP-1 sequence is, therefore, the limiting factor which differentiates between proglucagon processing within the mammalian pancreas and gut. It is concluded that like the N-terminal portion, differential post-translational processing of the C-terminal portion of proglucagon occurs between the mammalian gut and pancreas (see fig 3:14).

Normal human plasma contains circulating GLP-1 and GLP-2 LI at a concentration of 52 ± 12.5 and 136 ± 32.2 pmol/l respectively. From the combined chromatographic and immunochemical studies it is apparent that contributions from both the human gut and pancreas account for these circulating levels. These include

- 1) A component of pancreatic origin eluting with a K_{av} of 0.15, thought to consist of the GLP-1 sequence linked to that of GLP-2 via that of the intervening "spacer" peptide II.
- 2) A component of either pancreatic or intestinal origin (K_{av} 0.43) corresponding to free GLP-1.
- 3) A component of intestinal origin (K_{av} 0.26) corresponding to the predominant form of GLP-2 LI produced by the human gut, thought to consist of the GLP-2 sequence extended at its N-terminus to include that of the intervening "spacer" peptide II.

although the nature and origin of the other circulating forms of GLP-2 LI present remain to be determined.

The concentrations of GLP-1 and GLP-2 immunoreactivities in normal human plasma are significantly higher than that of GLI and may reflect their longer half-lives in relation to circulating forms of GLI. However, the bioactivities of these molecular forms of GLP-1 and GLP-2 are yet to be determined, and their putative roles as classical hormones should not be discounted. The occurrence of similar forms of GLP-1 and GLP-2 LI in normal and glucagonoma plasma, differing only in their relative concentrations, further emphasises their possible clinical relevance.

Analysis of pre-treatment plasma from glucagonoma patients, revealed the presence of a major component similar in immunochemical and chromatographic properties to the predominant (K_{av} 0.15) material of pancreatic and tumour origin. As observed with both tumours, this material exhibited a characteristic deficiency in GLP-2 LI, and is, therefore, likely to be a secretory product of the tumour. Although both tumours produced significant amounts of free GLP-1 LI, only plasma A demonstrated the presence of appreciable amounts of this component. This might be a reflection of the difference in secretory potential between these tumours. It is likely that the GLP-1 and GLP-2 immunoreactive components present in normal plasma contribute to the circulating levels of immunoreactivity in these patients. However, their grossly elevated plasma levels of tumour produced GLP-1 and GLP-2 LI would suggest these contributions to be minimal.

The proportion of immunoreactivity eluting in the position of synthetic GLP-1 was alone higher than the concentration of immunoreactive glucagon in each of these plasma samples (see table 3:1). Furthermore, good agreement on its concentration was obtained between gel filtration and HPLC analysis, though losses (due to the pre-HPLC deproteination step) of the larger molecular form (K_{av} 0.15) were observed (see discussion on glucagonomas). The total recovered plasma GLP-1 and GLP-2 LI greatly exceeded plasma GLI levels (see table 3:1), and although correlation of the pattern of peptide production with clinical features was not possible in these cases, analysis of plasma GLP-1 and GLP-2 concentrations may serve as a useful biochemical tool in the diagnosis of patients with the "glucagonoma syndrome".

Both human pancreatic glucagonomas (A and B), in addition to free GLP-1, produce a cleavage product similar in immunochemical and chromatographic character to the K_{av} 0.15 material of pancreatic origin (see fig 3:10). However, in contrast to human pancreatic extracts, both tumours showed a 66-75% deficit of GLP-2 LI within this (K_{av} 0.15) peak. This may be the result of reduced antibody crossreactivity due to the occurrence of post-translational modifications within the GLP-2 sequence.

On comparing the amount of large molecular weight (K_{av} 0.15, HPLC #19-22) immunoreactive material recovered on subjecting these tumour extracts to gel filtration chromatography or HPLC, it is evident that HPLC induces a loss of almost 40% of the constituent GLP-1 and GLP-2 immunoreactivities, whilst maintaining the ratio of GLP-1: GLP-2 within this peak at approximately 1:0.3. HPLC column

recoveries were consistently high (85-90%) implying that the pre-HPLC deproteination step, although not affecting the recovery of free GLP-1 or GLP-2 immunoreactivities (see chapter 2b), does induce losses of this larger molecular form. It is of related interest that in the human pancreas, where there is an equivalence in the ratio of GLP-1: GLP-2 LI within this Kav 0.15 peak, no significant losses of this material on pre-HPLC deproteination were observed. These losses may, therefore, result from the different chemical characteristics of this GLP-2 LI deficient material of similar size.

Neither tumour produced immunoreactive material corresponding to free GLP-2 (see fig 3:10), although the peak at Kav 0.26 (Tumour A) may represent the sequence of GLP-2 linked at its N-terminus to that of the intervening "spacer" peptide II. The total recovered GLP-1 LI in tumour A was 2.5x that of GLI, whilst in tumour B the ratio was approximately 0.5:1. Both tumours showed a gross deficit in GLP-2 LI with respect to both GLI and GLP-1 LI, although the ratio of recovered GLP-2: GLP-1 LI in both tumours was approximately 1:6.

It is concluded that the major cleavage products resulting from the processing of proglucagon within the human pancreas and human pancreatic glucagonomas are similar. Furthermore, the variability between these tumours in the ratio and tissue concentrations of their GLP-1 and GLP-2 related processing products is consistent with previous reports on their diversity in processing the N-terminal portion of proglucagon (Holst 1983c).

In summary, these combined chromatographic and immunochemical studies clearly indicate that

- a) Differential post-translational proteolytic processing of proglucagon occurs between the mammalian pancreas and gut.
- b) GLP-1 and GLP-2 related components of both pancreatic and intestinal origin are present in normal human plasma.
- c) Patients with the "glucagonoma syndrome" have highly elevated circulating levels of GLP-1 and GLP-2 LI of tumour origin, which may be of diagnostic value.

CHAPTER 4

BIOLOGICAL STUDIES ON THE GLUCAGON-LIKE PEPTIDES

a) EFFECT OF GLP-1 ON CARBOHYDRATE METABOLISM

Introduction

The considerable sequence homology of GLP-1 with glucagon (see fig 4:1) and the possibility that it may be co-released with glucagon, suggested that it should be tested for any intrinsic glucagon-like bioactivity on carbohydrate metabolism. One of the most suitable bioassays for glucagon depends on its hyperglycaemic effect when given subcutaneously to rabbits. The sensitivity and reproducibility of this assay is increased when liver glycogen stores are increased by pre-treatment with cortisone (Tarding et al 1969). Synthetic GLP-1 was, therefore, tested for glucagon-like bioactivity in this bioassay system.

Methods

Synthetic GLP-1-(1-37) was tested for its effect on plasma glucose and insulin by a modification of the twin cross-over bioassay for glucagon (Tarding et al 1969), using crystalline glucagon as a standard. Twelve rabbits were injected (25 mg subcutaneously) on day 0 with cortisone acetate. The assay was performed on days 2 and 3, the rabbits being deprived of food for 18 hr before each part of the assay but with free access to water.

GLUCAGON	H S Q G T F T S D Y S K Y L D S R R A Q D F
GLP-1	H D E F E R <u>H</u> <u>A</u> <u>E</u> <u>G</u> <u>T</u> <u>F</u> <u>T</u> <u>S</u> <u>D</u> <u>V</u> <u>S</u> <u>S</u> <u>Y</u> <u>L</u> <u>E</u> <u>G</u> <u>Q</u> <u>A</u> <u>A</u> <u>K</u> <u>E</u> <u>F</u>
GLP-2	<u>H</u> <u>A</u> <u>D</u> <u>G</u> <u>S</u> <u>F</u> <u>S</u> <u>D</u> <u>E</u> <u>M</u> <u>N</u> <u>T</u> <u>I</u> <u>L</u> <u>D</u> <u>N</u> <u>L</u> <u>A</u> <u>A</u> <u>R</u> <u>D</u> <u>F</u>
GLUCAGON	V Q W L M N T
GLP-1	I A <u>W</u> <u>L</u> V K G R G
GLP-2	I N <u>W</u> <u>L</u> I Q <u>T</u> K I T D R

Figure 4:1 The amino acid sequences of human Glucagon, GLP-1 and GLP-2. The identities are underlined: the sequences of GLP-1 and GLP-2 showing homologies of 48% and 38% respectively with that of glucagon.

The peptides were dissolved immediately before each experiment in 1.6% (v/v) aqueous glycerine (containing 0.2% w/v phenol), adjusted to pH 3 with HCl.

The rabbits were randomised into four groups of three each. Each received a subcutaneous injection of 1 ml diluent, as a control, followed after 60 min by either GLP-1 (400 µg or 100 µg) or glucagon (24 µg or 6 µg) in the same volume. Blood samples (0.3 ml) were taken from a marginal ear vein at time points -40, 0, 20 and 60 min in relation to both diluent and peptide injections. The next day the experiment was repeated with a twin cross-over, so that rabbits that had received the higher dose of glucagon now received the lower dose of GLP-1. The blood samples were collected into fluoride-oxalate centrifuge tubes, containing dried aprotinin (200 kallikrein inhibitor units - Trasylol) and centrifuged immediately at 1600 x g for 10 min. The plasma was frozen on solid CO₂ and stored at -20°C. Plasma glucose concentrations were determined with a glucose analyser (Beckman Instruments), and immunoreactive insulin was determined by radioimmunoassay (Albano et al 1972).

Results were analysed by Student's paired t-test on log-transformed data.

Results

Plasma glucose and immunoreactive insulin concentrations obtained at 20 min and 60 min after the injection of diluent controls, GLP-1 at

doses of 400 μ g and 100 μ g, and glucagon at doses of 24 μ g and 6 μ g are shown in figure 4:2.

Glucagon produced the expected significant increases in plasma glucose and insulin concentrations, whereas GLP-1 had no effect on plasma glucose at either dose. The slight increase in the mean plasma insulin concentration seen 20 min after GLP-1 injections persisted at 60 min with the 400 μ g dose though not with the 100 μ g dose. However, these small apparent changes in insulin concentration were not statistically significant on a paired basis.

Discussion

Despite the sequence homology between GLP-1 and glucagon (14 out of 29 comparable residues), GLP-1 does not affect plasma glucose concentrations, even at the high doses used in this study. It must, therefore, possess considerably less than 1% of the hyperglycaemic activity of glucagon, 2 μ g doses of which produce detectable responses in the assay used (Tarding et al 1969).

The similarity between glucagon and GLP-1 is greatest in the first nine residues of glucagon, where there are seven amino acid identities; but GLP-1 has a further six amino acid residues extending its N-terminal region beyond that of glucagon (see fig 4:1). As N-terminally extended forms of glucagon do not interact with hepatic glucagon receptors (Holst 1983b), the N-terminal extension of GLP-1 is, therefore, only one of several structural features that may prevent its binding to glucagon receptors.

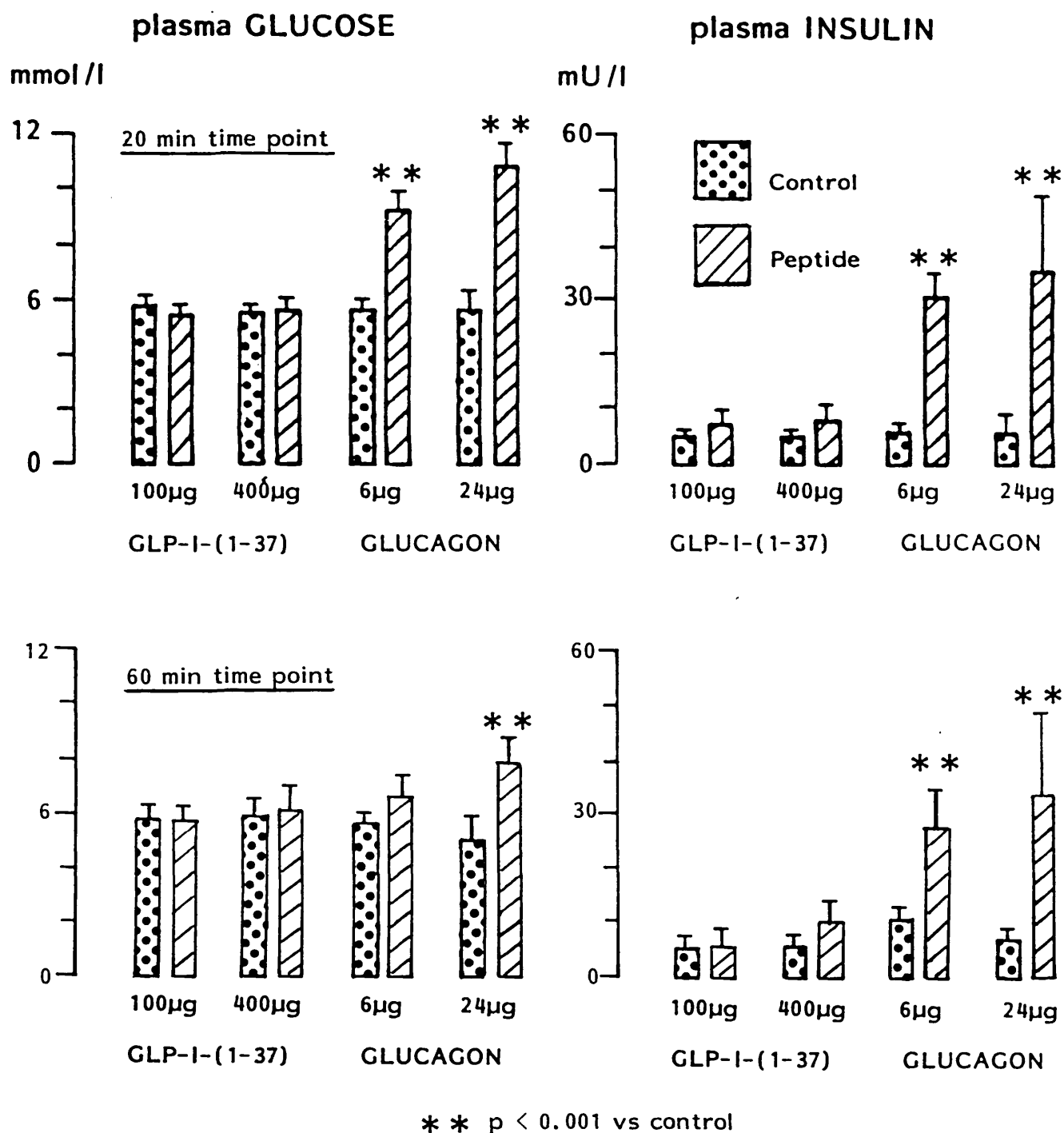


Figure 4:2 Plasma glucose and insulin concentrations in rabbits 20 and 60 min after injection of GLP-1 (100µg and 400µg) and glucagon(6µg and 24µg) compared with their respective control values after diluent injections. Bars represent mean $\pm$ SEM.

However, from the results of this study there are no indications that GLP-1 might produce a hyperglycaemic effect by an alternative mechanism, ie, through interaction with specific GLP-1 receptors.

GLP-2 shows a lesser degree of homology with glucagon (11 out of 29 comparative residues) but has no N-terminal extension that might interfere with its binding to glucagon receptors. Although it has a short C-terminal extension, it is known that C-terminally extended glucagon sequences are bioactive and do interact with hepatic glucagon receptors (Holst 1983b), though with considerably lesser potency than glucagon. Its effects may otherwise be mediated via specific hepatic GLP-2 receptors. In this context therefore, the testing of the effects of GLP-2 on carbohydrate metabolism might be indicated.

There remains the possibility that GLP-1 has a weak effect on insulin release, requiring higher doses or a larger number of animals than were available in this study for a statistically significant release to be demonstrated. That GLP-1 does in fact have a weak insulin-releasing effect has been recently shown by Schmidt and colleagues (1985). They were able to demonstrate a glucose-dependent stimulation of insulin release from isolated rat islets by high concentrations of GLP-1 in vitro. While this points to a possible role for GLP-1 within the islet, GLP-1 has thus far not been found to have a role in carbohydrate metabolism in the liver or other extrapancreatic tissues.

b) EFFECT OF GLP-1 ON GUT TROPHISM

Introduction

The report of delayed gastrointestinal transit and gross villous hypertrophy in a patient with an enteroglucagon-producing renal tumour first suggested that enteroglucagon might have a trophic effect on the small intestinal mucosa (Gleeson et al 1971; Bloom 1972). Resection of the tumour reduced the plasma enteroglucagon concentration and reversed the intestinal abnormalities. Plasma enteroglucagon is also markedly raised in a number of situations where there is increased enterocyte production in the small bowel mucosa, such as untreated coeliac disease (Besterman et al 1978); after jejunio-ileal bypass (Bloom 1980a) and after small bowel resection (Besterman et al 1982).

The enteroglucagon-producing cells have a distal distribution in the gut, being found in greatest concentration in the distal ileum and to a lesser extent in the colon (Bloom 1980b). These cells are stimulated by glucose and long-chain triglycerides (Holst 1978). It has been suggested that in situations where failure of absorption of nutrients occurs, such as after proximal small bowel resections, the enteroglucagon cells in the distal gut are stimulated by the greater amounts of these nutrients that come into contact with them.

If the enteroglucagons have a direct trophic action on the small intestinal enterocytes, this would provide a long-term

compensatory mechanism for enhancing small intestinal absorptive capacity. This hypothesis is supported by evidence from a number of studies of small intestinal adaptation, where significant correlations between circulating enteroglucagon concentrations and crypt cell production rates have been observed (Al-Mukhtar et al 1982). Moreover, an enteroglucagon-containing fraction of an extract of rat intestine produced a dose-dependent increase in DNA synthesis in cultured guinea pig jejunal mucosa (Uttenthal et al 1982), again, suggesting a possible association of a trophic effect with enteroglucagon.

The peptide sequences of GLP-1 and GLP-2 or sequences related to these peptides, may be co-secreted by the intestinal L cells, along with the enteroglucagons. These peptides are, therefore, also candidate trophic agents, in so far as they may be released whenever the enteroglucagons are released. Furthermore, the relatively crude fractions of intestinal or enteroglucagon tumour extracts that have been reported to have a trophic effect on small intestinal mucosa may also have contained components related to GLP-1 and GLP-2.

Owing to the inavailability of synthetic GLP-2 at this time, the following study employed only synthetic GLP-1. The protocol was designed to elevate plasma GLP-1 in rats by infusing the synthetic peptide via implanted osmotic mini-pumps, with the subsequent measurement of parameters related to effects on gut trophism and other glucagon-related peptides.

Methods

Male Wistar rats weighing (200–250g) were housed in wire-bottom cages under a 12 hr light/dark schedule of illumination. Water and a standard pelleted rat diet were freely available, except that food was withheld for 12 hrs before the animals were killed. Surgery was performed under intramuscular hypnorm (fentanyl and fluanisone - 0.5 ml/kg) and intraperitoneal diazepam (0.5 mg/kg) anaesthetic. The animals were weighed pre-operatively and immediately before being killed and weight gain expressed as the difference between these two weights.

Two groups of 8 rats were implanted subcutaneously with Alzet mini-osmotic pumps, model 2002. In the first group, pumps were filled with a solution of GLP-1-(1-37), 1.5 mg in 2.5 ml sterile normal saline containing 1% human serum albumin and 0.2% phenol (the vehicle), whilst the second control group were implanted with pumps filled with the vehicle alone. The pumps delivered 0.44 μ l/hr for 14 days, giving a calculated infusion rate of 5 pmol/kg/min of peptide.

Animals were killed on the 12th post-operative day by cardiac puncture exanguination under light ether anaesthesia, at timed intervals after vincristine treatment (see below). Blood samples (2 mls) were placed in heparinised tubes containing 4,000 KIU aprotinin (0.2 ml Trasylol), centrifuged, and the separated plasma stored at -20°C. Plasma obtained from normal rats was treated likewise, to estimate the recovery of exogenously added GLP-1-(1-37) from rat plasmas processed identically. Each recovery sample contained 0.5 pmol/ml of GLP-1-(1-37).

The duodenum, remaining small intestine, colon and pancreas were dissected free, washed in ice-cold saline and blotted dry prior to their weights being recorded. They were extracted in 0.5M acetic acid using the procedure described previously (see chapter 2b) and stored at -20°C . Samples for crypt cell production rate determinations were also taken from the duodenum (8 cm from the pylorus), mid small bowel and mid colon.

Crypt Cell Production Rate

A stathmokinetic method was used to assess the rate of production of cells in the crypts of Lieberkuhn; a widely used technique for determining proliferative status (Wright et al 1974). At 09.00 hrs on the morning of sacrifice each animal received an intra-peritoneal injection of vincristine, (1 mg/kg) which arrests cell division in metaphase, and the animals were killed from 30 min to 3 hr after injection. Samples of bowel were initially fixed in Carnoy's fluid for 4 hours and then transferred to 75% ethanol. The tissues were stained with Feulgen stain and the mucosa was stripped from the muscle coat. Individual crypts were dissected out under a dissecting microscope. The number of cells in metaphase within each crypt was counted and the mean of the metaphase counts of 10 crypts was taken as the reading for each individual rat. The number of cells arrested in metaphase per crypt was then plotted against time after vincristine administration. The crypt-cell production per hour (CCPR) for the group is given by the slope of the regression line, fitted by the method of least squares.

Assay Methodology

Plasma GLP-1 LI and tissue GLP-1, GLP-2 and total glucagon (TG)-like immunoreactivities were measured using the specific radioimmunoassays described previously (see chapter 2a & 2b). The plasma samples were concentrated on sep-pak C-18 cartridges and the eluates evaporated to dryness in an evacuated centrifuge. They were then reconstituted in buffer for radioimmunoassay. Tissue extracts were analysed directly without needing to concentrate the sample; the respective sample additions not producing any assay interference.

Chromatography

Gel filtration chromatography was performed on pooled plasma from the control and GLP-1 infused groups of rats. Plasma (4 mls) from each group was loaded onto a column (90 x 1.4 cm) containing 130 ml bed volume of Sephadex G-50 superfine. It was eluted with 0.2M glycine buffer (pH 8.8) containing 1% (v/v) BSA at a flow rate of 5 ml/hr and collecting 2.5 ml fractions. The column was precalibrated with dextran blue as a void volume marker, cytochrome C and NaI¹²⁵ as a total volume marker. Synthetic GLP-1-(1-37) was used to determine the elution position of GLP-1, and the elution volumes were plotted in terms of Kav (Laurent & Killander 1964).

Statistical Methods

Student's t test for unpaired data was used for intergroup comparison. Results are expressed as mean $\pm$ standard error of the mean (SEM).

Results

Plasma GLP-1 LI

Analysis of normal rat plasma containing 0.5 pmol/ml of exogenously added GLP-1-(1-37), treated identically to those plasmas obtained from the experimental groups of animals resulted in a mean recovery of $86.6 \pm 8.4\%$ ($n = 4$) of the added immunoreactivity. On correcting the plasma levels of GLP-1 LI in the peptide infused group for this level of recovery, a mean circulating level of 141.1 ± 28.8 pmol/l ($n = 8$) was achieved. There was no detectable GLP-1 like immunoreactivity in the plasmas obtained from the saline-infused group.

Chromatography

Chromatographic analysis on Sephadex G-50 of pooled plasma obtained from the normal saline infused group confirmed the absence of GLP-1 LI. In contrast, the pooled plasma from the GLP-1 infused group was eluted in a single peak of K_{av} 0.43, coinciding with the elution position of synthetic GLP-1-(1-37) (see fig 4:3).

Endogenous Peptide Content and Weight of Organs

Acid extracts of duodenum, small bowel, colon and pancreas from the control and peptide infused groups were analysed for GLP-1, GLP-2 and total glucagon (TG) -like immunoreactivities using specific radioimmunoassays described elsewhere (see chapter 2a). Statistical analysis of the results revealed no significant differences in the endogenous tissue content of the above peptides between the saline infused (control) and GLP-1 infused (treated) groups (see table 4:1)

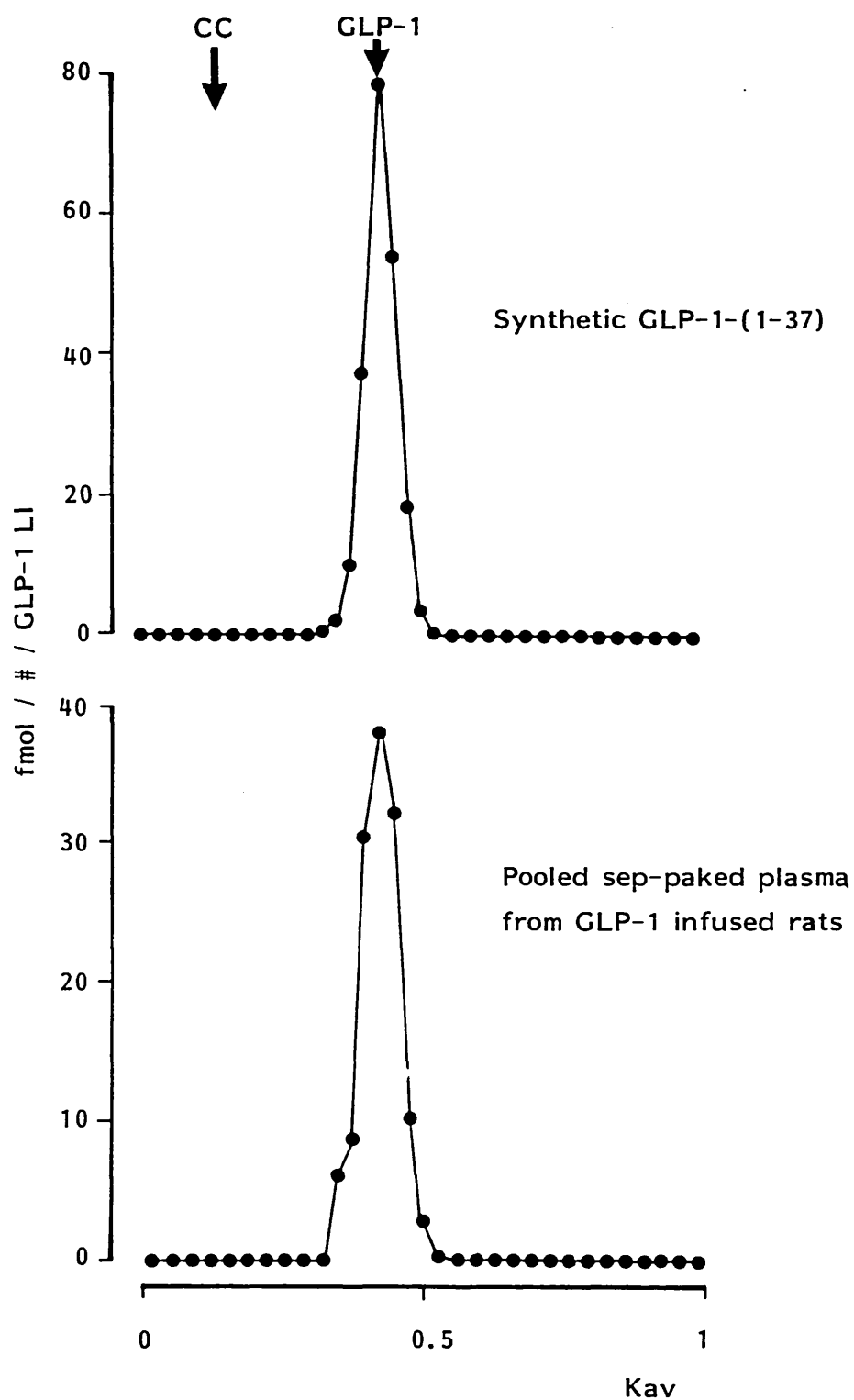


Figure 4:3 Co-elution on sephadex G-50 with synthetic GLP-1-(1-37) standard of the GLP-1 LI present in pooled plasma obtained from the GLP-1-(1-37) infused group.

<u>Organ peptide content</u>						
<u>Tissues</u>	<u>GLP-1 LI (pmol/g)</u>		<u>GLP-2 LI (pmol/g)</u>		<u>GLI (pmol/g)</u>	
(n=8)	<u>control</u>	<u>treated</u>	<u>control</u>	<u>treated</u>	<u>control</u>	<u>treated</u>
duodenum	1.4 ± 0.1	1.5 ± 0.2	3.6 ± 0.2	3.8 ± 0.4	19.8 ± 1.6	21.6 ± 1.7
ileum	22.3 ± 2.4	24.1 ± 1.1	28.4 ± 3.0	26.9 ± 1.4	328.2 ± 16.3	354.4 ± 8.0
colon	7.5 ± 0.5	6.3 ± 0.6	3.7 ± 0.4	3.7 ± 0.5	166.3 ± 10.2	144.1 ± 6.6
pancreas	163.4 ± 18.1	198.4 ± 15.9	50.1 ± 3.3	49.2 ± 4.0	561.5 ± 54.8	620.8 ± 52.4

<u>Organ weight (gms)</u>		
	<u>Control (n=8)</u>	<u>Treated (n=8)</u>
duodenum	0.74 ± 0.03	0.79 ± 0.06
ileum	0.59 ± 0.03	0.63 ± 0.06
colon	1.29 ± 0.05	1.36 ± 0.04
pancreas	1.19 ± 0.04	1.28 ± 0.08
initial animal wt:	207.30 ± 4.70	210.10 ± 3.70
terminal animal wt:	252.90 ± 4.50	255.80 ± 3.20

Table 4:1 Organ weight and peptide content for the saline infused (control) and GLP-1 infused (treated) groups. Results are expressed as mean ± SEM.

Similarly, no significant differences in organ weights were observed between the two groups, and the mean terminal weight of the animals in both groups was also indicative of no differential weight gain between the groups.

Crypt Cell Production Rates

The crypt cell production rates obtained for duodenum, mid small bowel and colon from both groups of animals are listed below:-

	<u>CCPR (cells/crypt/hr)</u> (Mean $\pm$ SEM)	
	<u>Saline Infused Group</u> (n = 8)	<u>GLP-1 Infused Group</u> (n = 8)
Duodenum	24.4 $\pm$ 7.4	21.6 $\pm$ 7.2
Small Bowel	15.4 $\pm$ 3.2	18.4 $\pm$ 4.3
Colon	6.9 $\pm$ 2.1	6.4 $\pm$ 1.5

There are no significant differences observed in the CCPR determined at various sites between the two groups.

Discussion

Radioimmunoassay of the plasma obtained from GLP-1 infused rats indicates that circulating levels of 141.1 ± 28.8 pmol/l of this peptide had been achieved. This figure contrasts with that for the plasma from the saline infused animals where no GLP-1 immunoreactivity could be detected. It is concluded that the infusion was effective in significantly elevating the plasma concentration of GLP-1 in the treated group.

As observed with humans, it is likely that GLP-1 LI circulates in normal rat plasma. However, on the basis of these results it would appear that its concentration in rat plasma is considerably lower than in man. The co-elution on gel filtration of plasma GLP-1 LI with synthetic GLP-1-(1-37) in a single peak of K_{av} 0.43, suggests that the immunoreactivity recovered from the plasma of GLP-1 infused rats consisted of intact GLP-1.

No significant differences between the saline and GLP-1 infused groups with respect to weight gain or organ wet weights was observed, indicating the absence of a trophic response. Similarly, no change between the groups in crypt cell production rates, a robust indicator of proliferative status, was apparent for the duodenal, ileal and colonic sites examined. If exogenous GLP-1 were having a significant biological effect, it is possible that feed-back mechanisms might alter tissue synthesis and secretion of the precursor and its products. However, the elevated circulating levels of GLP-1 LI in the peptide infused group had no effect on the endogenous tissue content of GLP-2 and total glucagon-like immunoreactivities. It should be noted that the deduced amino acid sequence of rat GLP-1 (Heinrich et al 1984) is identical to that of hamster, human and ox GLP-1, so that lack of effect could not be attributed to the infusion of a non-homologous peptide.

The description of an enteroglucagon-producing tumour in a patient with marked villous hypertrophy that resolved after tumour excision, led to the hypothesis that enteroglucagon might be a trophic hormone (Gleeson et al 1971). Since then other workers have shown that an

enteroglucagon-containing fraction of intestinal extract was capable of producing an increase in DNA synthesis by guinea-pig jejunal mucosa cultures (Uttenthal et al 1982). However, the infusion of GLP-1 over 12 days had no significant effect on weight gain, organ weight or the proliferative status of the intestinal tract, and the maintenance of TG and GLP-2 tissue levels showed no evidence that the procedure disturbed enteroglucagon/GLP precursor synthesis or secretion. There is, therefore, no evidence from this experiment that GLP-1 exerts a trophic influence within the gastrointestinal tract.

The trophic response is however, only one feature of intestinal adaptation and other aspects include functional adaptation of the mucosa to increase absorption (Dowling & Booth 1967) and a slowing of intestinal transit time to allow prolonged contact between food and mucosa (Nygaard 1967). Further studies may resolve whether GLP-1 has a role in mediating these other aspects of intestinal adaptation. Similar studies are also required with GLP-2, when synthetic rat GLP-2 becomes available, which differs by 6% in its amino acid composition from the human GLP-2 sequence.

c) EFFECT OF GLP-1 ON ISOLATED RAT HEPATOCYTES AND ITS EFFICACY IN
BINDING LIVER MEMBRANES

Introduction

The complete conservation of GLP-1 observed in the deduced amino acid sequences of hamster, man, ox and rat pre-proglucagon (see introduction) and its 48% homology with glucagon was suggestive of its possible involvement in a glucagon-like role for carbohydrate metabolism. This hypothesis was further strengthened when both the mammalian pancreas and gut were found to produce a free GLP-1-like component which is similar to that which occurs in normal human plasma (see chapter 3). It may therefore function as a classical hormone, capable of interacting with specific hepatic GLP-1 or glucagon receptors.

The inability of GLP-1 to exhibit a glucagon-like elevation of plasma insulin or glucose (see Chapter 4a) does, however, not negate the possibility of a regulatory role in carbohydrate metabolism. This might be mediated via a regulation of the actions of glucagon by competing for its receptors, or by binding to specific high affinity GLP-1 receptors which influence some other aspect of the glycogenolytic process. This study, therefore, sets out to investigate the binding characteristics of GLP-1 to rat liver membranes and to determine whether it is capable of stimulating cyclic AMP production in isolated rat hepatocytes.

The fact that several peptides cleaved from their respective prohormones are C-terminally amidated, and that this amidation occurs adjacent to a glycine residue within the prohormone sequence is well established (Steiner et al 1980). Moreover, supportive evidence demonstrating the existence of an enzyme capable of converting peptides terminating in a glycine residue to the corresponding des-glycine peptide amide has been presented (Bradbury et al 1982). As the GLP-1 sequence is a candidate for this type of C-terminal amidation, both synthetic GLP-1-(1-37) and synthetic GLP-1-(1-36)-peptide amide were evaluated in these studies.

Methods

Iodination

Radiolabelled GLP-1-(1-37) was prepared by iodination with carrier-free Na ^{125}I using the chloramine T method (see chapter 2). The iodinated peptide was separated from both unreacted iodide and unlabelled peptide by reverse-phase high pressure liquid chromatography. This batch of ^{125}I -GLP-1-(1-37) label at the time of use showed a specific activity of 26 Bq/fmol.

Monoiodinated ^{125}I -glucagon with a specific activity of 40 Bq/fmol was obtained according to the procedure of Notley and Rosselin (1971). Iodination was carried out in a total volume of 100 μl of 0.3M phosphate buffer (pH 7.4) containing 5 μg porcine glucagon, 2.5 mCi of ^{125}I Na and chloramine T (25 μg), for 30 secs.

This was immediately followed by the addition of sodium metabisulphite (50 μ g) and 100 μ l of 0.3M phosphate buffer (pH 7.4) containing 10 g/l bovine serum albumin (BSA). The reaction mixture was applied to a 0.9 x 30 cm DEAE cellulose column (Whatman 52) equilibrated with 0.05M tris-HCl and 7M urea buffer (pH 9.3) at 4°C. After the passage of 80 mls of buffer, a linear gradient of NaCl (from 0 to 0.2M) was run. Mono-iodinated glucagon obtained thus, was diluted in 0.05M phosphate buffer (pH 7.4) and stored at -20°C.

Preparation of "Partially Purified" Liver Plasma Membranes

This method is based on that described by Pohl et al (1971). As far as possible, all experimental procedures were carried out at 4°C. Male Wistar rats (120-150g), housed under constant conditions of lighting and temperature, receiving a standard pelleted diet were used throughout these studies. Rats were fasted overnight and sacrificed by cervical dislocation. The livers (10g) were immediately removed, mixed and homogenised in 25 ml of 1mM NaHCO₃ held at 4°C.

The homogenate was diluted to 250 ml, filtered through four layers of cheesecloth and spun at 1500 x g for 10 min. The resulting pellets were re-suspended in 25 mls of bicarbonate buffer and the process repeated. They were then re-suspended in a 69% sucrose solution (34 mls) and adjusted to $44 \pm 0.1\%$ sucrose (total volume = 60 ml). From this solution, 20 mls was poured into each of three centrifuge tubes and overlayed with 10 mls of a $42.3 \pm 0.1\%$

sucrose solution (checked with a refractometer) and spun at 90,000 x g for 2 hours. The float at the density interface was removed, and to it was added 8 ml of 1mM NaHCO₃ (4°C) and the tubes spun at 25,000 x g for 10 min to pack the sediment. The resulting pellet was re-suspended in 4 mls of 1mM bicarbonate buffer. To each of two tubes containing a cushion of 4.1 mls of a 50 ± 0.1% sucrose solution, over which a linear gradient of 37-33% sucrose was formed, was overlayed 2 mls of the re-suspended pellet. The tubes were spun at 550 x g for 1 hr with the brake off.

This results in "partially purified" liver membranes collecting at the cushion interface. They were collected and stored in liquid nitrogen. Membrane protein was determined by the method of Lowry et al (1951) and the functional integrity of the membrane preparation was assessed on its ability to bind ¹²⁵I-glucagon. All experimental work was carried out in polypropylene laboratory ware.

Isolation of Hepatocytes

Rats fasted for 48 hrs were anaesthetised with ether and the portal vein exposed and cannulated. The liver was initially perfused with calcium and magnesium-free Krebs-Ringer bicarbonate buffer (pH 7.4) for 4 min at the rate of 30 ml/min. Another 5 min perfusion with buffer containing 3.75 mg/ml of collagenase (type I 160 units/mg), was carried out at an identical rate. The liver was then removed, cut into fine pieces and incubated at 37°C in Krebs-ringer bicarbonate buffer (pH 7.4) containing 10 g/l BSA with slow stirring for 5 min. The digested liver was filtered and the cells spun down at 50 x g for 2 min. All procedures with isolated cells

were carried out in plastic laboratory ware and the cell number was determined by counting in a Neubauer chamber. Cell size was estimated after determining cell diameters visualised under a phase contrast microscope. The viability of the isolated hepatocytes was assessed by their ability to exclude 0.5% (w/v) of Trypan blue stain for 2 min, in the absence of albumin.

Measurement of Hormone Binding to Liver Membranes

The binding assay is carried out as described by Rubaclava and Rodbell (1973). Liver membranes (4150-4850 μg protein/ml) were incubated in Krebs-Ringer phosphate buffer (pH 7.5), containing varying concentrations of unlabelled glucagon, GLP-1-(1-37) or GLP-1-(1-36)-peptide amide (10^{-6}M to 10^{-11}M) in the presence of a constant concentration of mono ^{125}I -glucagon (0.188 pmol/ml) or ^{125}I -GLP-1-(1-37) (0.85 pmol/ml) for 4 hr at 10°C . At the end of the incubation period the samples were rapidly diluted out with 1 ml of ice-chilled Krebs-Ringer phosphate buffer (pH 7.5) containing 10g/l BSA and immediately filtered on oxoid filters (0.45 μm) soaked in 100 g/l BSA for 30 min prior to use.

To correct for non-specific adsorption of the peptides to liver membranes, the binding of radioactive hormone was determined in the presence of $1 \times 10^{-6}\text{M}$ unlabelled glucagon, a concentration which gave maximal inhibition of glucagon radiolabel binding. The difference between membrane bound radioactivity in the absence or the presence of an excess of unlabelled hormone was considered to represent binding of the labelled hormone to specific binding sites.

Incubation of Isolated Hepatocytes

Isolated hepatocytes ($7.5 \times 10^6/\text{ml}$) were incubated at 37°C for 2.5 min with Krebs-Ringer bicarbonate buffer (pH 7.4), supplemented with BSA (40g/l), alanine (10 mM) and theophylline (2mM) whilst being gently shaken and gassed with 95% O_2 and 5% CO_2 . GLP-1-(1-37), GLP-1-(1-36)-peptide amide or glucagon were added to yield final concentrations within the range of 10^{-6} to 10^{-11}M , respectively. The effect of glucagon (10^{-6} to 10^{-11}M) in the presence of a constant concentration of 10^{-6}M GLP-1-(1-37) or GLP-1-(1-36)-peptide amide was also assessed.

At the end of the incubation period, the cell suspensions were treated twice with 0.5 ml of a 100 g/l solution of trichloroacetic acid and the combined supernatants washed with ether saturated with water. Aliquots of the aqueous phase were analysed by radioimmunoassay to determine cyclic AMP content (Rianen cAMP [^{125}I] RIA kit, New England Nuclear Chemicals, Dreieich, W Germany).

Results

Under the conditions of incubation and separation, non-specific binding of ^{125}I -glucagon was less than 2% of the total added. Whilst unlabelled glucagon caused a 50% displacement (ED_{50}) of ^{125}I -glucagon from its specific receptors at a concentration of $3 \times 10^{-9}\text{M}$, neither GLP-1-(1-37) nor GLP-1-(1-36)-peptide amide at concentrations of up to 10^{-6}M displaced ^{125}I -glucagon (see fig 4:4).

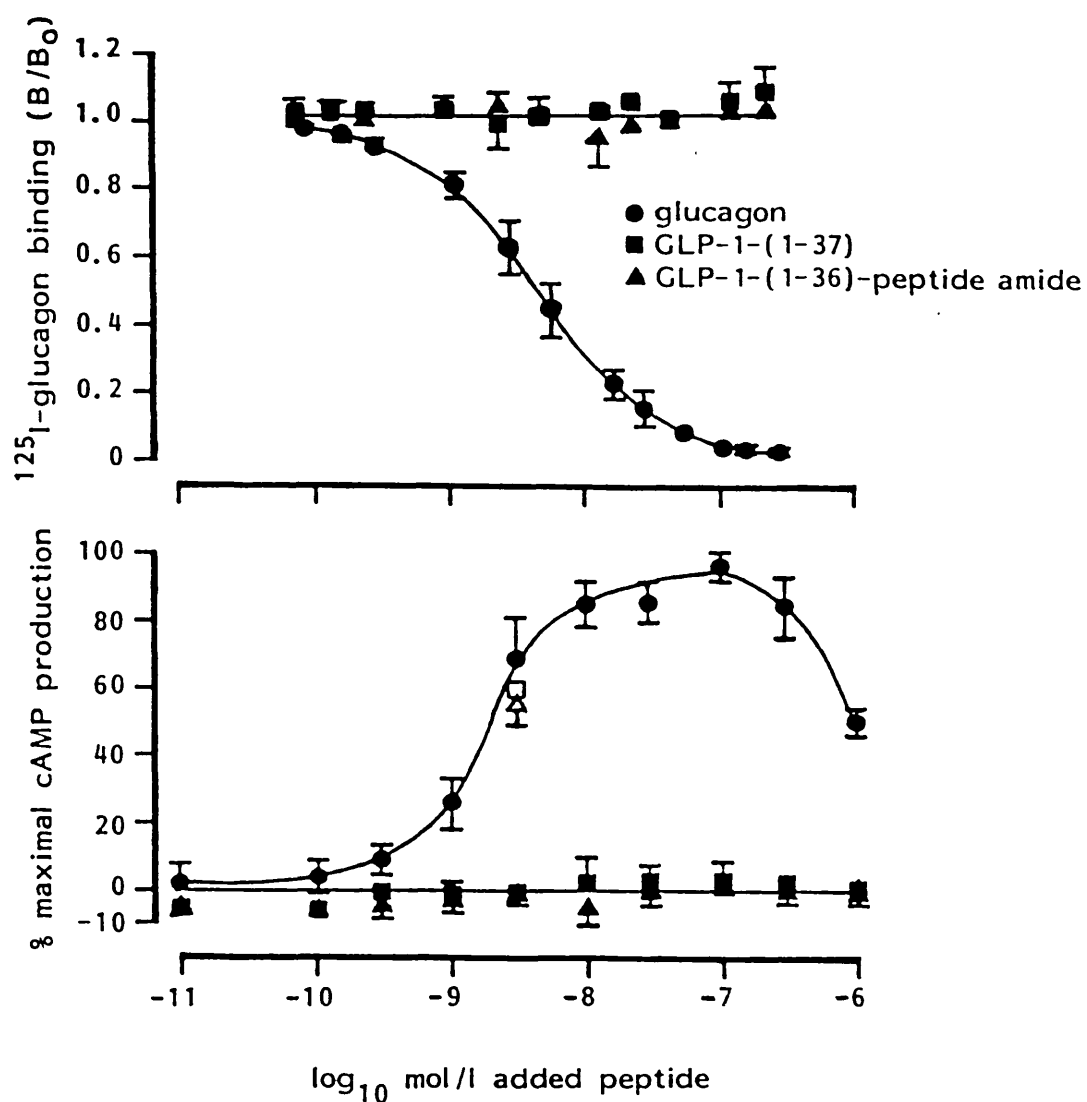


Figure 4:4 Upper panel: displacement of I^{125} -glucagon from liver membranes by unlabelled glucagon (●), GLP-1-(1-37) (■) or GLP-1-(1-36)-peptide amide (▲). B/B_0 is [tracer bound]/ [tracer bound in absence of added peptide]. Lower panel: production of cyclic AMP by isolated rat hepatocytes in the presence of glucagon (●), GLP-1-(1-37) (■) or GLP-1-(1-36)-peptide amide (▲), expressed as a percentage of maximum increment produced by glucagon. Effect of adding GLP-1-(1-37) (□) or GLP-1-(1-36)-peptide amide (Δ) at $10^{-6}M$ in the presence of glucagon ($3 \times 10^{-9}M$).

When liver membranes were incubated in the presence of ^{125}I -GLP-1-(1-37) at a concentration of $0.85 \times 10^{-9}\text{M}$, there was no displaceable binding of peptide to the membranes. Non-specific binding in the presence of $2.4 \times 10^{-7}\text{M}$ of unlabelled peptide was 3.2% of the total tracer added.

While glucagon stimulated the production of cyclic AMP in isolated hepatocytes over the same concentration range and with the same ED_{50} ($3 \times 10^{-9}\text{M}$) as for its interaction with specific glucagon receptors, neither GLP-1-(1-37) nor GLP-1-(1-36)-peptide amide stimulated cyclic AMP production (see fig 4:4). Moreover, when the hepatocytes were incubated with $3 \times 10^{-9}\text{M}$ glucagon, together with either GLP-1-(1-37) or GLP-1-(1-36)-peptide amide at 10^{-7}M and 10^{-6}M respectively, no alteration in glucagon stimulated cyclic AMP production was observed.

Discussion

The identification of tissue specific high affinity receptors for a peptide requires the demonstration of its time and temperature dependent, saturable, reversible binding to its target organ receptors. The fact that GLP-1 does not interact with liver membranes in this manner, is in accordance with the hypothesis of another group (Hoosein & Gurd 1984) and the absence of specific high affinity GLP-1 receptors in these membranes suggests that the liver is not a target organ for GLP-1. In support of this evidence, GLP-1 does not stimulate cyclic AMP production by isolated hepatocytes and

does not affect glucagon-stimulated cyclic AMP production. These results are also consistent with its lack of effect on plasma glucose concentrations at high doses in vivo (see chapter 4a).

All these data, therefore, suggest that despite its structural similarity to glucagon, the highly conserved GLP-1 sequence does not fulfil its biological role by exerting a direct action on hepatic carbohydrate metabolism. However, GLP-2 shows a 38% homology with glucagon and furthermore lacks the constraint of an extended N-terminus which might inhibit the binding of GLP-1 to hepatic glucagon receptors. The effect of GLP-2 on hepatic carbohydrate metabolism therefore prompts investigation.

d) EFFECTS OF GLP-1 AND GLP-2 ON PITUITARY HORMONE SECRETION

Introduction

It has recently been reported by Hoosein and Gurd (1984) that both GLP-1 and GLP-2, although not interacting with glucagon receptors on rat pituitary and hypothalamic membranes, show a remarkable potency in activating pituitary and hypothalamic adenylate cyclase. This data is further supported by their demonstration that the ED_{50} for this activation process effected by GLP-1 and GLP-2 ($30-50 \times 10^{-12}M$) is markedly different from that for glucagon [$5 \times 10^{-9}M$], and has led them to postulate the existence of specific GLP-1 and GLP-2 receptors within the central nervous system.

Large molecular forms of glucagon-like immunoreactivity have been previously isolated from rat brain. In particular, two molecular forms of 8 and 12 kD (Tager et al 1980), the latter of which may represent the proglucagon fragment consisting of GRPP and glucagon extended by the GLP-1 sequence. The existence of pancreatic glucagon-like immunoreactivity in the brain (see chapter 1) is suggestive of the presence of proglucagon processing enzymes capable of cleaving the double basic residues at the C-terminus of glucagon sequence. The 12 kD component could thus conceivably be that which remains after the excision of GLP-2, which gives rise to the 8 kD fragment owing to the subsequent cleavage of the GLP-1 sequence. Therefore, the existence of free GLP-1 and GLP-2 in the CNS can be visualised accounting for these potent actions.

The concentrations at which GLP-1 and GLP-2 activate pituitary and hypothalamic adenylate cyclase are of an order of magnitude to be of physiological relevance (5 pM). The accompanying increase in intracellular cyclic AMP levels would be expected to initiate an important intracellular event. That this, as in the case of GRF (Law et al 1984), might result in modulating the release of a pituitary hormone is conceivable and, therefore, the effects of GLP-1 and GLP-2 on pituitary secretion were investigated.

Methods

Peptides

Synthetic GLP-1-(1-36)-peptide amide and synthetic h-GLP-2-(1-34) were purchased from Peninsular Laboratories Inc, Belmont, CA, USA. GLP-1 in its amidated form was used throughout these studies so as to maintain comparability with the studies of Hoosein and Gurd (1984). Crystalline glucagon was obtained from Novo (Novo Industries, Copenhagen, Denmark) and synthetic hpGRF-(1-44) and CRF from the Salk Institute (USA). All peptides were freshly dissolved in the incubation media such that a 1 or 2 ml aliquot contained a peptide solution of the required concentration.

Incubation of Rat Pituitaries

The method used is a modification of that of Arimura et al (1983). Male wistar rats (22 day old, 50-60g) were sacrificed by cervical dislocation and immediate decapitation. The top of the skull was

removed with scissors and the brain carefully manoeuvred out to expose the hypophysis. The gland was dissected free with fine forceps and the posterior lobe lifted off. The anterior lobe was then halved along the mid line and each pituitary half placed in oxygenated Krebs-Ringer bicarbonate medium (pH 7.4), containing 100 mg% glucose and 250 mg% bovine serum albumin (KRBG-BSA). All incubations were conducted at 37°C under 95% O₂ and 5% CO₂. The pituitary halves were randomised in such a manner that no two related halves received the same dose of peptide.

After two preincubations of 1.5 hr in KRBG-BSA (to allow for equilibration), the pituitary halves were incubated for 1 hr in 1 ml of KRBG-BSA containing 2.5×10^{-4} M L-ascorbic acid (KRBG-BSA-C) to obtain basal values (period A). They were then incubated in KRBG-BSA-C alone or in solutions of the peptides dissolved in KRBG-BSA-C for a further 1 hour (period B). The incubation media from periods A and B were collected and stored at -20°C prior to assay.

Preparation of Rat Anterior Pituitary Cell Cultures

Female Wistar rats (180-220g) were decapitated, the anterior pituitaries removed and placed immediately in culture medium (Eagle's minimum essential medium containing 10% foetal calf serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (Glaxo) and 2.5 µg/ml fungizone (Squibb), buffered with 20 mM HEPES (Hopkins and Williams) and 20 mM sodium bicarbonate). The pooled anterior pituitaries were cut into pieces of approximately 1mm³, washed with phosphate buffered saline (pH 7.3) and dispersed by 6 consecutive 20

min incubations at 37°C in 20 ml phosphate buffered saline containing 0.125% trypsin (Wellcome reagents). Dispersed cells were aspirated and recovered by centrifugation (200 x g, 5 min). The resulting cell pellets were washed with 1 ml culture medium, pooled, recentrifuged and dispersed in 20 ml culture medium. Cells were counted using a haemocytometer and diluted to give 2×10^5 cells/ml medium. Cell viability determined by the trypan blue exclusion method was greater than 95%. Cells (2.5×10^5) were added to glass tubes (Labco S100 WC) and incubated at 37°C for 4 days to allow cell attachment prior to use in experiments.

Immediately before use, the cells were washed with 2 ml of culture medium. Experiments were performed at 37°C in closed tubes, using 2 ml culture medium containing 2.5×10^{-4} M ascorbic acid. All the peptide doses tested were freshly made up in ascorbic acid containing culture medium, such that a 2 ml aliquot contained the required peptide concentration. A minimum of 3 cultures were used per dose tested, and the incubation protocol involved a 4 hr pre-incubation (period A), followed by a 4 hr incubation (period B); when the medium contained the peptide doses under test. At the end of periods A and B, the media were decanted and stored at -20°C prior to assay.

Preparation-integrity

The functional integrity of incubated pituitary halves and cultures was determined under incubation conditions, by assessing their responses to pre-determined effective concentrations of pituitary hormone-releasing factors GRF and CRF (Arimura et al 1983; Turkelson et al 1981).

Radioimmunoassay

Rat growth hormone, prolactin and ACTH were measured against NIAMDD rat GH-RP-1, prolactin-RP-2 and synthetic α_{1-39} ACTH standards as per Powell et al (1981). The sensitivities of these assays were GH (4 ng/ml^{-1}), PRL (2 ng/ml^{-1}) and ACTH (0.2 ng/ml^{-1}); their intra-assay coefficients of variation were less than 7% and samples from entire experiments were analysed in a single assay. Reagents for the assay were a kind donation from the NIH Pituitary Agency (USA). The amount of pituitary hormone released during the second period (B), expressed as a percentage of the amount released during the first period (A), ie $(B/A\%)$ was used to index releasing activities.

To determine whether the desired concentrations of GLP-1 and GLP-2 had been achieved in the pituitary incubates, the relevant media were analysed using specific radioimmunoassays developed for these peptides (see chapter 2a).

Statistical analysis

Unpaired t-tests using pooled estimates of error of log transformed data were used to determine statistical significance.

Results

Assay of the incubation media for GLP-1 and GLP-2 immunoreactivity confirmed that the pituitary halves were incubated in approximately the desired concentrations of these peptides (see table 4:2).

However, GLP-1 and GLP-2 over the dose range 10^{-11}M to 10^{-7}M are observed to have no measurable effects on the secretion of GH, PRL and ACTH, both in the incubated pituitary halves and cultures. (see table 4:1 to 4:7). Glucagon (10^{-6}M) also shows a similar ineptitude, although the functional integrity of both preparations is well maintained as observed by their responses to the hypothalamic-pituitary hormone releasing factors; GRF and CRF.

In both the incubated pituitary halves and cultures, hpGRF at concentrations of 10^{-8}M and 10^{-9}M respectively, produced significant rises in growth hormone secretion ($p < 0.001$), and the dose response of GRF to GH release from incubated pituitary halves is illustrated in figure 4:5. In addition, a significant increase in ACTH secretion ($p < 0.001$) was also observed with pituitary cultures incubated with CRF (10^{-9}M). However, the GRF (10^{-9}M) and CRF (10^{-9}M) stimulated release of GH and ACTH respectively by pituitary cell cultures, was unaffected by simultaneous incubations with GLP-1 (10^{-9}M) or GLP-2 (10^{-9}M).

Discussion

Owing to their potency in activating pituitary adenylate cyclase, the testing of the effects of GLP-1 and GLP-2 on pituitary hormone secretion were initiated on rat pituitary cell cultures. However, as the process of trypsinisation used to liberate these cells may affect the cellular response by causing non-specific damage to ligand binding sites (receptors); the effects of GLP-1 and GLP-2 were also assessed on incubation with isolated rat pituitary halves.

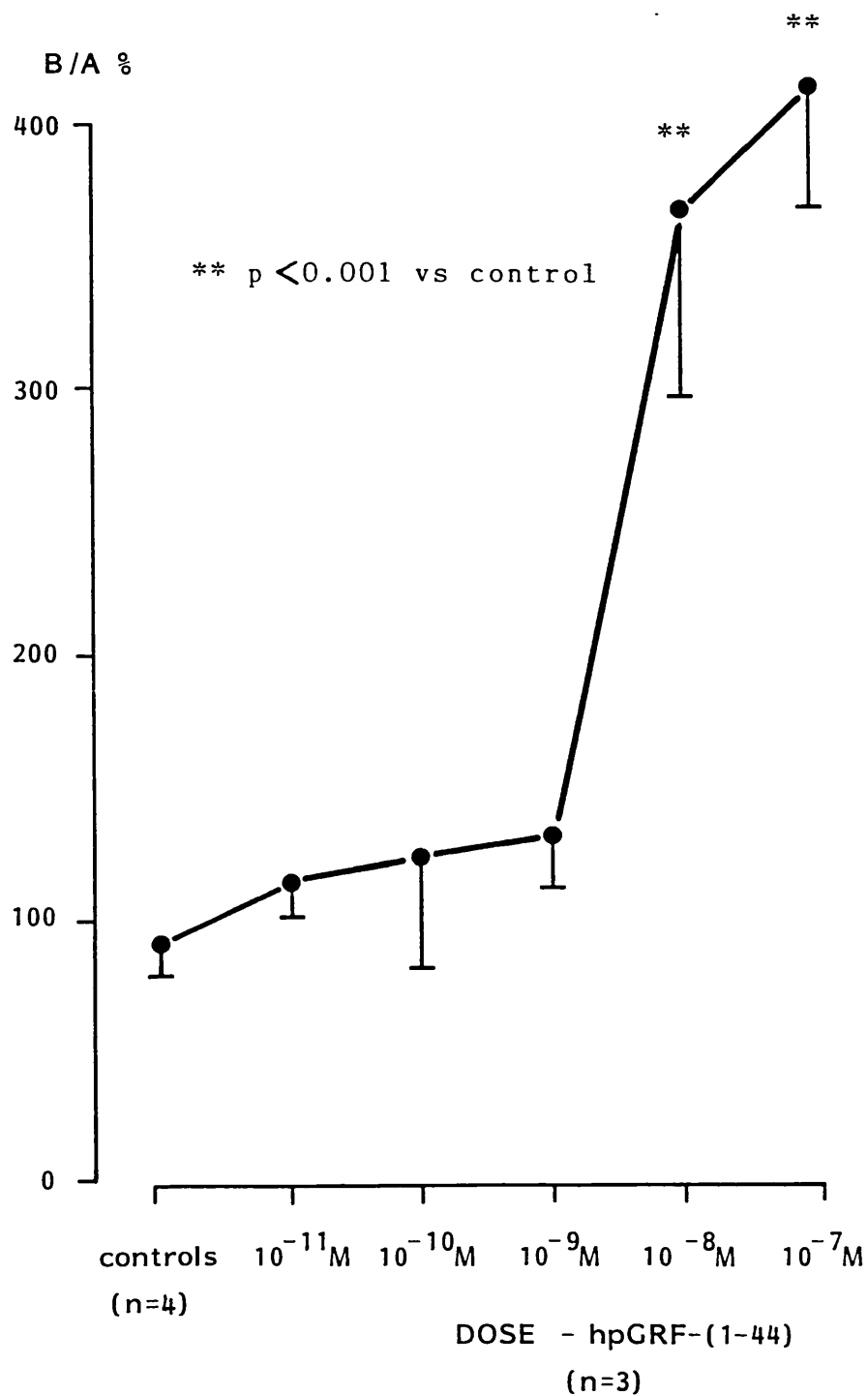


Figure 4:5 The dose-response of GRF to growth hormone release in isolated rat pituitary half incubates. Bars represent mean $\pm$ SEM.

Incubation of rat pituitary halves

Table 4:1 - Hormone (rGH)

GLP-1 treated

<u>Sample</u>		<u>Mean B/A% $\pm$ SEM</u>
Controls	(n = 12)	103.9 $\pm$ 18.6
10 ⁻⁸ M hpGRF	(n = 6)	354.5 $\pm$ 13.4
10 ⁻⁶ M Glucagon	(n = 2)	121.1 $\pm$ 14.1
10 ⁻¹¹ M GLP-1	(n = 6)	88.0 $\pm$ 5.0
10 ⁻¹⁰ M GLP-1	(n = 8)	107.9 $\pm$ 11.6
10 ⁻⁹ M GLP-1	(n = 8)	88.2 $\pm$ 20.9
10 ⁻⁸ M GLP-1	(n = 8)	160.4 $\pm$ 51.5
10 ⁻⁷ M GLP-1	(n = 8)	102.8 $\pm$ 15.6

GLP-2 treated

Controls	(n = 8)	80.1 $\pm$ 10.3
10 ⁻⁸ M hpGRF	(n = 4)	354.4 $\pm$ 68.5
10 ⁻¹¹ M GLP-2	(n = 4)	103.5 $\pm$ 31.8
10 ⁻¹⁰ M GLP-2	(n = 6)	105.6 $\pm$ 21.1
10 ⁻⁹ M GLP-2	(n = 6)	108.7 $\pm$ 11.9
10 ⁻⁸ M GLP-2	(n = 6)	86.8 $\pm$ 13.3
10 ⁻⁷ M GLP-2	(n = 6)	103.9 $\pm$ 11.7

Table 4:1 contd: - Hormone (rGH)hpGRF-(1-44) treated

<u>Sample</u>		<u>Mean B/A% ± SEM</u>
Controls	(n = 4)	90.6 ± 11.3
10 ⁻¹¹ M hpGRF	(n = 3)	114.7 ± 13.5
10 ⁻¹⁰ M hpGRF	(n = 3)	123.3 ± 42.1
10 ⁻⁹ M hpGRF	(n = 3)	130.1 ± 20.9
10 ⁻⁸ M hpGRF	(n = 3)	367.7 ± 68.4
10 ⁻⁷ M hpGRF	(n = 3)	44.8 ± 46.4

Table 4:2 - Concentrations of GLP-1 And GLP-2 achieved in the
pituitary halves incubation media

<u>projected concentration</u>	<u>actual concentration</u> (Mean ± SEM) (n = 4)
10 ⁻⁷ M GLP-1	0.69 ± 0.12 x 10 ⁻⁷ M
10 ⁻⁸ M GLP-1	0.61 ± 0.13 x 10 ⁻⁸ M
10 ⁻⁹ M GLP-1	0.63 ± 0.14 x 10 ⁻⁹ M
10 ⁻¹⁰ M GLP-1	1.17 ± 0.04 x 10 ⁻¹⁰ M
10 ⁻⁷ M GLP-2	1.41 ± 0.06 x 10 ⁻⁷ M
10 ⁻⁸ M GLP-2	1.43 ± 0.02 x 10 ⁻⁸ M
10 ⁻⁹ M GLP-2	1.05 ± 0.26 x 10 ⁻⁹ M
10 ⁻¹⁰ M GLP-2	1.44 ± 0.37 x 10 ⁻¹⁰ M

Table 4:3 - Hormone (rACTH)GLP-1 treated

<u>Sample</u>		<u>Mean B/A% ± SEM</u>
Controls	(n = 8)	113.9 ± 10.7
10 ⁻⁶ M Glucagon	(n = 2)	86.7 ± 22.4
10 ⁻¹¹ M GLP-1	(n = 4)	103.3 ± 6.4
10 ⁻¹⁰ M GLP ⁻¹	(n = 6)	136.5 ± 20.2
10 ⁻⁹ M GLP-1	(n = 6)	95.3 ± 18.0
10 ⁻⁸ M GLP-1	(n = 6)	126.9 ± 6.9
10 ⁻⁷ M GLP-1	(n = 6)	117.6 ± 29.1

GLP-2 treated

Controls	(n = 8)	95.5 ± 12.3
10 ⁻¹¹ M GLP-2	(n = 4)	121.2 ± 14.3
10 ⁻¹⁰ M GLP-2	(n = 6)	98.2 ± 11.2
10 ⁻⁹ M GLP-2	(n = 6)	104.6 ± 8.0
10 ⁻⁸ M GLP-2	(n = 6)	88.9 ± 13.7
10 ⁻⁷ M GLP-2	(n = 6)	114.2 ± 6.4

Table 4:4 - Hormone (rPRL)GLP-1 treated

<u>Sample</u>		<u>Mean B/A% $\pm$ SEM</u>
Controls	(n = 8)	92.8 $\pm$ 8.3
10 ⁻⁶ M Glucagon	(n = 2)	89.4 $\pm$ 5.1
10 ⁻¹¹ M GLP-1	(n = 4)	113.4 $\pm$ 9.0
10 ⁻¹⁰ M GLP-1	(n = 6)	97.3 $\pm$ 5.1
10 ⁻⁹ M GLP-1	(n = 6)	99.6 $\pm$ 13.6
10 ⁻⁸ M GLP-1	(n = 6)	81.6 $\pm$ 11.1
10 ⁻⁷ M GLP-1	(n = 6)	87.4 $\pm$ 4.0

GLP-2 treated

Controls	(n = 8)	104.6 $\pm$ 12.2
10 ⁻¹¹ M GLP-2	(n = 4)	113.3 $\pm$ 6.4
10 ⁻¹⁰ M GLP-2	(n = 6)	96.4 $\pm$ 4.0
10 ⁻⁹ M GLP-2	(n = 6)	87.0 $\pm$ 8.6
10 ⁻⁸ M GLP-2	(n = 6)	116.3 $\pm$ 6.1
10 ⁻⁷ M GLP-2	(n = 6)	106.4 $\pm$ 14.6

Incubation of rat pituitary cultures

Table 4:5 - Hormone (rGH)

<u>Sample</u>		<u>Mean B/A% ± SEM</u>
Controls	(n = 6)	82.7 ± 9.3
10 ⁻⁹ M GRF	(n = 4)	165.5 ± 12.3
10 ⁻⁷ M Glucagon	(n = 4)	84.4 ± 2.1
<u>GLP-1 treated</u>		
10 ⁻¹¹ M GLP-1	(n = 4)	96.8 ± 13.7
10 ⁻¹⁰ M GLP-1	(n = 4)	85.2 ± 4.4
10 ⁻⁹ M GLP-1	(n = 4)	90.4 ± 9.9
10 ⁻⁸ M GLP-1	(n = 4)	86.8 ± 4.9
<u>GLP-2 treated</u>		
10 ⁻¹¹ M GLP-2	(n = 4)	88.6 ± 4.8
10 ⁻¹⁰ M GLP-2	(n = 4)	91.3 ± 6.6
10 ⁻⁹ M GLP-2	(n = 4)	78.5 ± 1.5
10 ⁻⁸ M GLP-2	(n = 4)	91.4 ± 5.2
10 ⁻⁹ M GLP-1 + 10 ⁻⁹ M GRF	(n = 3)	170.5 ± 8.3
10 ⁻⁹ M GLP-2 + 10 ⁻⁹ M GRF	(n = 3)	209.6 ± 36.4

Table 4:6 - Hormone (rACTH)

<u>Sample</u>		<u>Mean B/A% ± SEM</u>
Controls	(n = 6)	89.3 ± 16.0
10 ⁻⁹ M CRF	(n = 4)	535.5 ± 120.6
10 ⁻⁷ M Glucagon	(n = 4)	99.4 ± 8.6
 <u>GLP-1 treated</u>		
10 ⁻¹¹ M GLP-1	(n = 4)	103.3 ± 14.6
10 ⁻¹⁰ M GLP-1	(n = 4)	113.3 ± 26.8
10 ⁻⁹ M GLP-1	(n = 4)	80.6 ± 10.8
10 ⁻⁸ M GLP-1	(n = 4)	94.4 ± 14.3
 <u>GLP-2 treated</u>		
10 ⁻¹¹ M GLP-2	(n = 4)	84.5 ± 6.3
10 ⁻¹⁰ M GLP-2	(n = 4)	89.3 ± 14.6
10 ⁻⁹ M GLP-2	(n = 4)	104.4 ± 8.7
10 ⁻⁸ M GLP-2	(n = 4)	97.5 ± 16.2
10 ⁻⁹ M GLP-1 + 10 ⁻⁹ M CRF	(n = 3)	411.5 ± 68.6
10 ⁻⁹ M GLP-2 + 10 ⁻⁹ M CRF	(n = 3)	614.2 ± 111.1

Table 4:7 - Hormone (rPRL)

<u>Sample</u>		<u>Mean B/A% ± SEM</u>
Controls	(n = 6)	87.0 ± 7.0
10 ⁻⁷ M Glucagon	(n = 4)	90.8 ± 10.2

GLP-1 treated

10 ⁻¹¹ M GLP-1	(n = 4)	89.8 ± 1.6
10 ⁻¹⁰ M GLP-1	(n = 4)	74.5 ± 6.3
10 ⁻⁹ M GLP-1	(n = 4)	94.0 ± 3.7
10 ⁻⁸ M GLP-1	(n = 4)	81.2 ± 4.8

GLP-2 treated

10 ⁻¹¹ M GLP-2	(n = 4)	91.2 ± 3.6
10 ⁻¹⁰ M GLP-2	(n = 4)	90.2 ± 4.3
10 ⁻⁹ M GLP-2	(n = 4)	86.4 ± 8.2
10 ⁻⁸ M GLP-2	(n = 4)	78.4 ± 2.5

From the observations of Hoosein and Gurd (1984), the concentration range of both GLP-1 and GLP-2 effective at activating brain adenylate cyclase activity ranged from 10^{-12} M to 10^{-7} M. However, over the same dose range, neither of these peptides had any demonstrable effects on growth hormone (GH), prolactin (PRL) and adrenocorticotrophic hormone (ACTH) secretion by either the incubated pituitary halves or cultures. These negative results are obtained despite the proven responses of the preparations to the hypothalamic-pituitary hormone-releasing factors, growth hormone-releasing factor (GRF) and corticotrophin-releasing factor (CRF) and neither GLP-1 nor GLP-2 were capable of modifying GRF and CRF-induced secretion in pituitary cell cultures.

These results suggest that although GLP-1 and GLP-2 may be potent activators of brain adenylate cyclase, the accompanying increase in intra-cellular cyclic AMP levels is not used to drive processes mediating the secretion of GH, PRL and ACTH. Furthermore, neither is any involvement in modulating the secretory effects of the hypothalamic-releasing factors (GRF and CRF) evident. GLP-1 and GLP-2 may not therefore be construed to have any regulatory effects on lactation, growth or the adrenal cortex brought about by modulating anterior pituitary hormone release.

The anterior pituitary however, contains other hormones such as luteinising hormone (LH), follicle stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), which were not analysed for in these studies. Possibly, the effects of GLP-1 and GLP-2 may result

in modifying their release or otherwise control a cellular process quite unconnected with that of hormone release. The end result of the potentiation of brain adenylate cyclase activity by these glucagon-like peptides is therefore yet to be determined.

e) EFFECT OF GLP-1 AND GLP-2 ON EXOCRINE PANCREATIC
SECRETION IN THE RAT

Introduction

The regulation of pancreatic exocrine secretion involves a complex interplay between nervous and hormonal mechanisms. Many circulating gut hormones, such as secretin, cholecystokinin (CCK) and gastrin have stimulatory effects on the exocrine pancreas. This is in addition to the neuropeptides such as vasoactive intestinal polypeptide (VIP), which may exert a similar stimulatory effect when released from peptidergic nerves localised within the pancreas. Also, glucagon and somatostatin have inhibitory effects. Together, therefore, these peptides may modulate the more direct regulation of exocrine pancreatic secretion mediated by the vagal cholinergic nerves (review - Greenberg 1983).

Guinea-pig pancreatic acinar cells contain two distinct categories of secretagogue receptors. The interaction of secretagogues with one type results in the mobilisation of intra-cellular calcium (eg CCK-like secretagogues), whilst interaction with the other causes activation of adenylate cyclase, resulting in an increase in intra-cellular cyclic AMP levels (VIP and secretin-like secretagogues); both resulting in an increased output of pancreatic juice (Gardner 1979). However, the influence on the composition of stimulated pancreatic juice differs markedly between these two classes of secretagogues. CCK-like agents cause an increase in the

protein content of the juice, whilst VIP and secretin selectively increase the percentage of bicarbonate (Greenberg 1983), although VIP exhibits a potency of only 1% relative to secretin (Holst 1982b).

The receptors interacting with VIP and secretin are subdivided into two further classes. They are characterised by their differential affinities for VIP and secretin and are termed the "VIP-preferring" and "secretin preferring" receptors; their distribution per acinar cell favouring the "secretin preferring" class by a ratio of almost 7:1 (Gardner 1977). Although occupation of these receptors by either VIP or secretin results in an increase in intra-cellular cAMP, only in the "VIP-preferring" case is an accompanying increase in pancreatic secretion observed. The cellular function mediated by interaction with the "secretin-preferring" class is unknown, but the substantial increase in intra-cellular cAMP, along with the abundance of this receptor class suggests involvement in an important physiological role (Jensen et al 1981).

Glucagon has been shown to inhibit pancreatic exocrine secretion in the rat (Shaw & Heath 1973) and man (Di Magno 1983), and shows no selectivity in its inhibition between the protein and bicarbonate content of pancreatic juice. As VIP, glucagon and secretin are all members of a family of structurally related peptides (see fig 4:6), it is therefore likely, that the ability to modulate pancreatic exocrine secretion is inherent within their structure. The amino acid sequences of GLP-1 and GLP-2, apart from their respective homologies of 48% and 38% with glucagon show distinct homologies with VIP and secretin (see fig 4:6 and Table 4:8). It is therefore

Secretin (1-27)	H S D G T F T S E L S R L R D S A R L Q R L L Q G
VIP (1-28)	H S D A V F T D N Y T R L R K Q M A V K K Y L N S
Glucagon (1-29)	H S Q G T F T S D Y S K Y L D S R R A Q D F V Q W
GLP-1 (7-37)	H A E G T F T S D V S S Y L E G Q A A K D F I A W
GLP-2 (1-29)	H A D G S F S D E M N T I L D S L A T R D F I N W

Secretin (1-27)	L V
Vip (1-28)	I L N
Glucagon (1-29)	L M N T
GLP-1 (7-37)	L V K G R G
GLP-2 (1-29)	L I Q T

Figure 4:6 comparison of amino acid sequences (partial) to establish identities between members of the "glucagon-secretin" family

	<u>% Homology</u>		
	<u>VIP</u>	<u>Secretin</u>	<u>Glucagon</u>
GLP-1	14	30	48
GLP-2	21	30	38

Table 4:8 percentage sequence homology of other members of the "glucagon-secretin" family with GLP-1 and GLP-2.

feasible, that both GLP-1 and GLP-2 might be capable of modulating exocrine pancreatic secretion. This hypothesis was thus examined.

Methods

The method was adapted from Dockray (1972). Male Wistar rats (200-300g) were fasted overnight and anaesthetised with intramuscular fentanyl (315 µg/kg)/fluanisone (10 mg/kg) - hypnorm and intraperitoneal diazepam (5 mg/kg). The jugular vein was catheterised and infused with 0.9% saline at a flow rate of 0.03 ml/min throughout the experiment, using a syringe ram pump. The common pancreatic bile duct was cannulated near its entrance to the duodenum and bile flow was blocked by ligating the upper end of the duct. The pylorus was also ligated in order to avoid endogenous stimulation of pancreatic exocrine secretion by flow of gastric contents into the duodenum. Pancreatic juice was collected in calibrated capillary tubes (20 µl) changed at 5 min intervals and the contents added to 1 ml aliquots of distilled water for estimation of protein output.

Intravenous bolus doses of synthetic GLP-1-(1-37) at doses of 0.024 (0.1 µg), 0.24 (1.0 µg), 1.2 (5.0 µg), 2.4 (10.0 µg), 4.8 (20.0 µg) and 9.6 (40.0 µg) nmoles, synthetic h-GLP-2-(1-34) at doses of 2.5 (10.0 µg) and 5.1 (20.0 µg) nmoles, and crystalline glucagon at a dose of 28.7 (100.0 µg) nmoles were administered via the jugular cannula. The functional integrity of each animal preparation was checked by injecting a dose of secretin [16.4 pmoles (50ng)]

after the response to the test dose had returned to basal levels. Peptides were administered in a vehicle of 0.9% saline containing 1% bovine serum albumin (BSA). Protein content of the diluted pancreatic juice was estimated by extinction at 280 nm against a BSA standard.

Expression of results

The effect of peptides on the rates of pancreatic juice volume output ($\mu\text{l}/5\text{min}$) and protein output (mg BSA equivalents/5 min) was recorded. In addition, total responses to an injection were calculated from the total volume and total protein collected during the period of the response minus that due to the basal output (Love 1957). The basal output was determined from the mean of the outputs before the injection and after the response had finished. All values are expressed as mean $\pm$ SEM. Unpaired t-tests using pooled estimates of error of log transformed data were used to calculate the significance of the difference between stimulated and basal rates of either volume output or protein secretion. A value of $p < 0.05$ was considered significant.

Results

Basal pancreatic juice output was $5.0 \pm 0.6 \mu\text{l}/5 \text{ min}$ ($n = 36$) and protein output was $0.7 \pm 0.04 \text{ mg}/5 \text{ min}$. Intravenous bolus doses of GLP-1-(1-37) showed marked effects on pancreatic juice volume output for the 5 min period coinciding with the peak of the response (see fig 4:7 to fig 4:9). GLP-1-(1-37) at a dose of $0.1 \mu\text{g}$ ($n = 3$) causes a decrease in the volume output (% Basal) to $84 \pm 7\%$, which

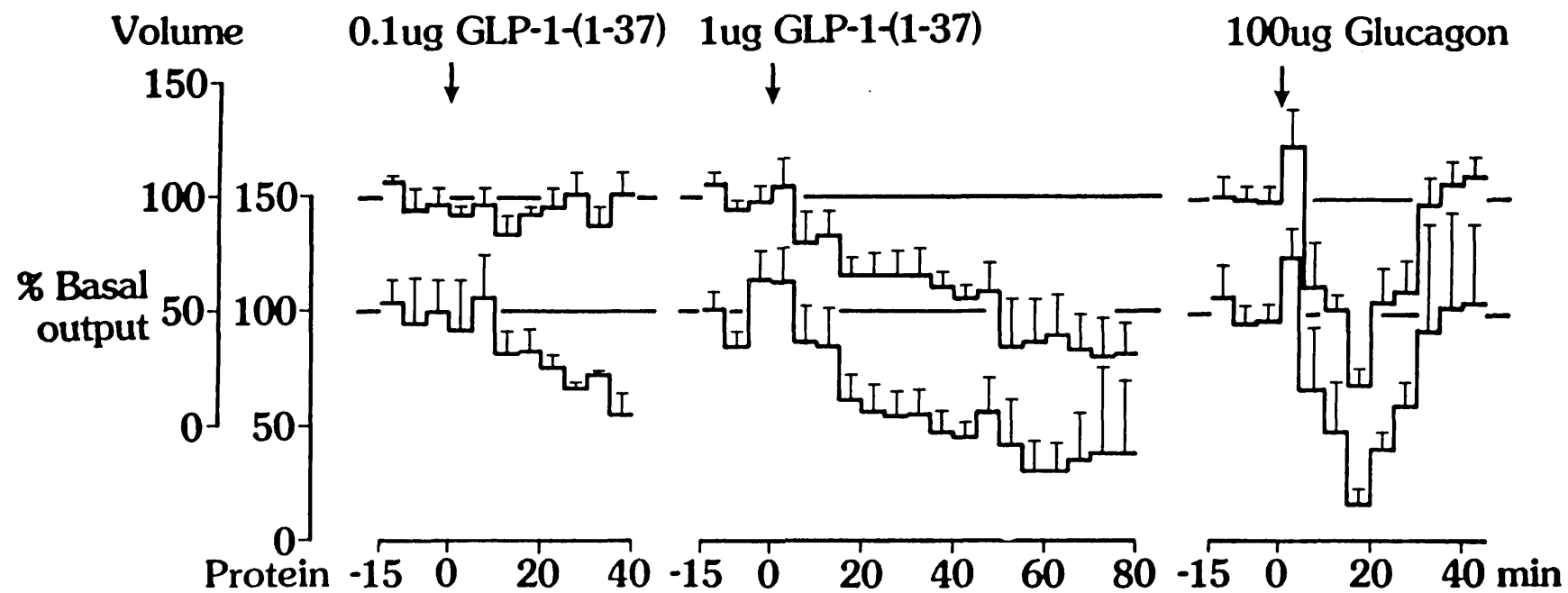


Figure 4:7 The effect of GLP-1 (0.1µg and 1µg) and glucagon (100µg) on pancreatic juice volume and protein output. Bars represent mean $\pm$ SEM and the results are expressed as a percentage of the basal output.

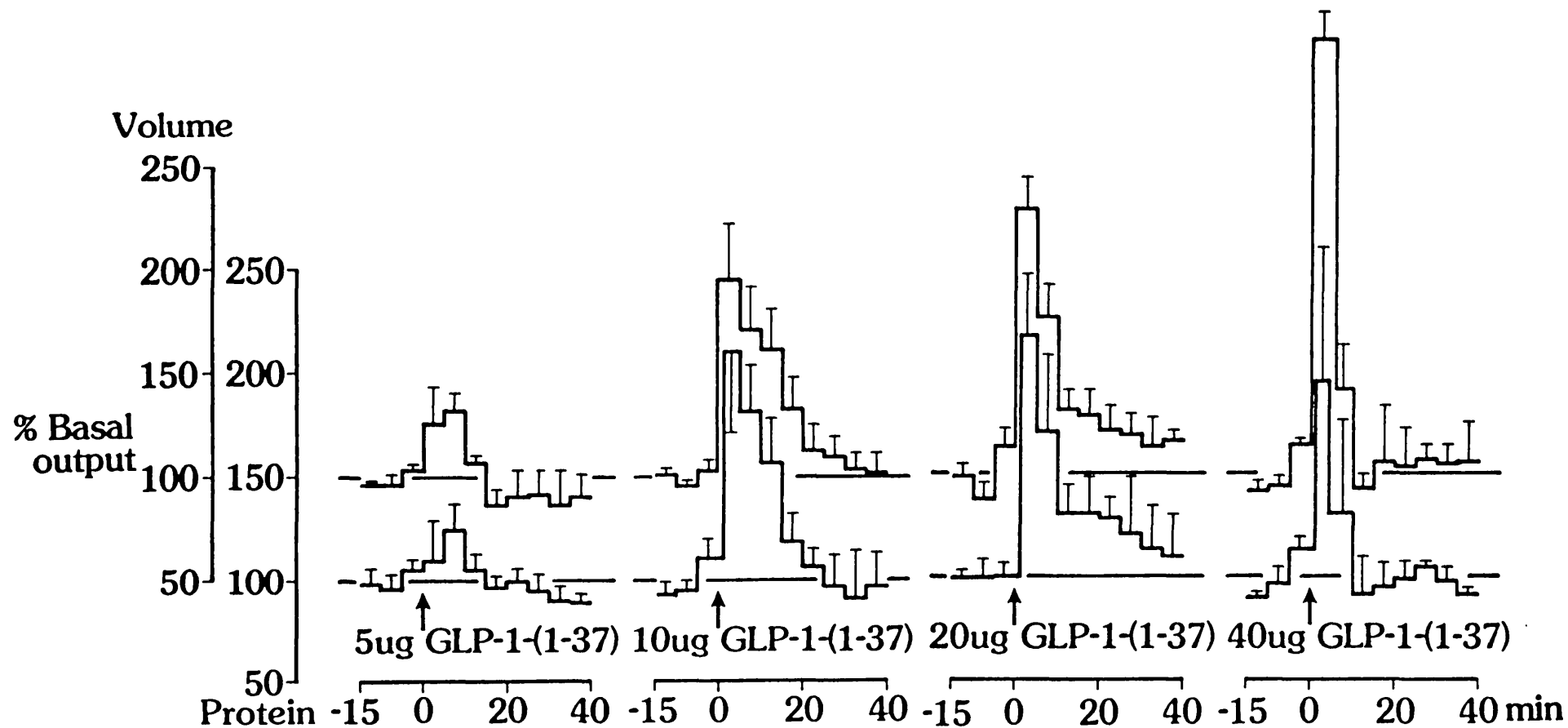


Figure 4:8 The effect of GLP-1 (5, 10, 20 and 40µg) on pancreatic juice volume and protein output. Bars represent mean $\pm$ SEM and the results are expressed as a percentage of the basal output.

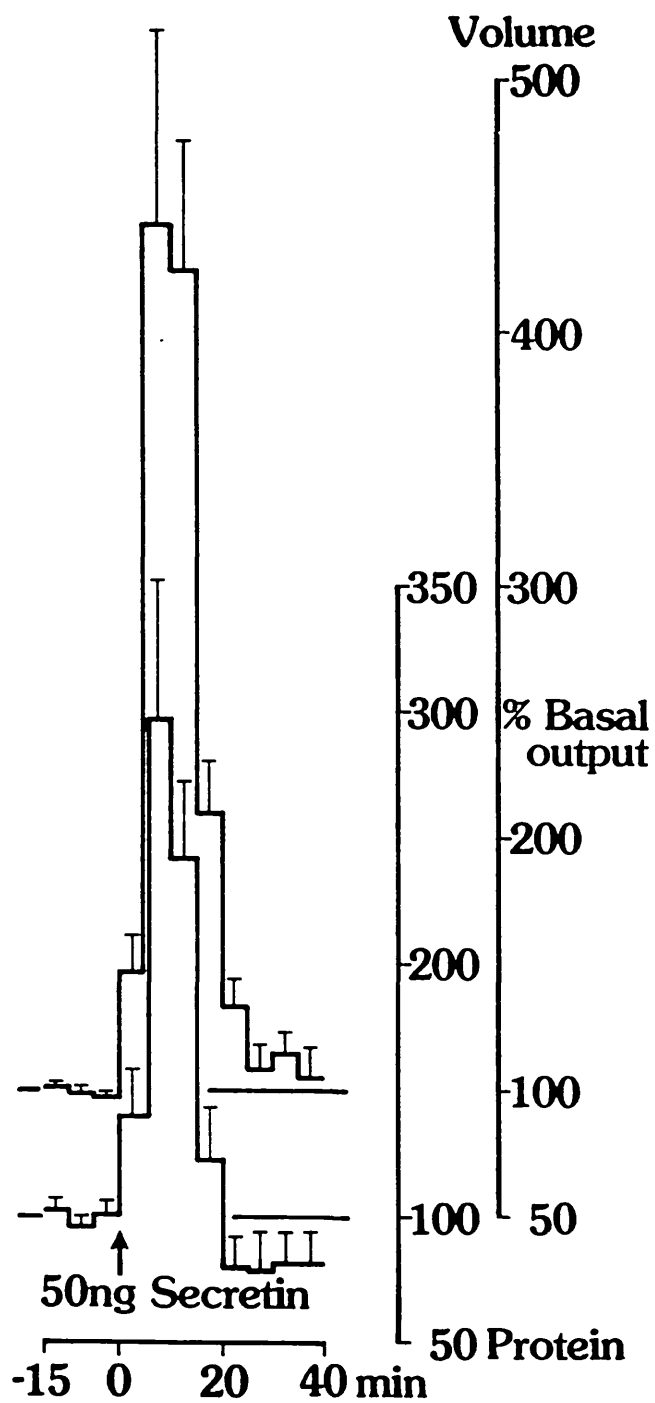


Figure 4:9 The effect of secretin (50ng) on pancreatic juice volume and protein output. Bars represent mean $\pm$ SEM and the results are expressed as a percentage of the basal output.

is amplified at the 1.0 μg dose ($n = 5$) to $31 \pm 16\%$ basal. With this dose, the onset of the response occurs within 5 min of injection and the inhibition of pancreatic juice continues for periods exceeding 80 min. A further increase in dose to 5 μg ($n = 4$) results in a reversal of the response and elicits the secretion of pancreatic juice, the volume output at the peak of the response rising to $132 \pm 8\%$ basal. This trend is continued with doses of 10 μg ($n = 9$), 20 μg ($n = 5$) and 40 μg ($n = 2$), the peak volume output increasing to $196 \pm 26\%$, $228 \pm 16\%$ and $313 \pm 13\%$ of basal respectively (see fig 4:10).

On examining the peak protein outputs, coinciding with the 5 min period for the peak of the response for volume output, the same general trend is observed. It rises from $30 \pm 12\%$ for the 1.0 μg dose (the point of maximal inhibition) to $217 \pm 29\%$ for the 20 μg dose; increases in protein output corresponding to similar changes in volume output. However, with the 40 μg dose, the peak protein output was no different from that for the 20 μg dose despite the significant difference in their respective volume outputs (see fig 4:10).

Support for the above data is obtained on examining the total stimulated protein and volume outputs for each dose (area under the curve). As observed between the volume and protein outputs at the peak of each response for doses of GLP-1-(1-37) ranging from 5-20 μg , an increase in total stimulated volume output is accompanied by a corresponding rise in the total stimulated protein output (see fig 4:11). Although, as seen between their respective peak volumes and their protein outputs (10 and 20 μg doses), no significant

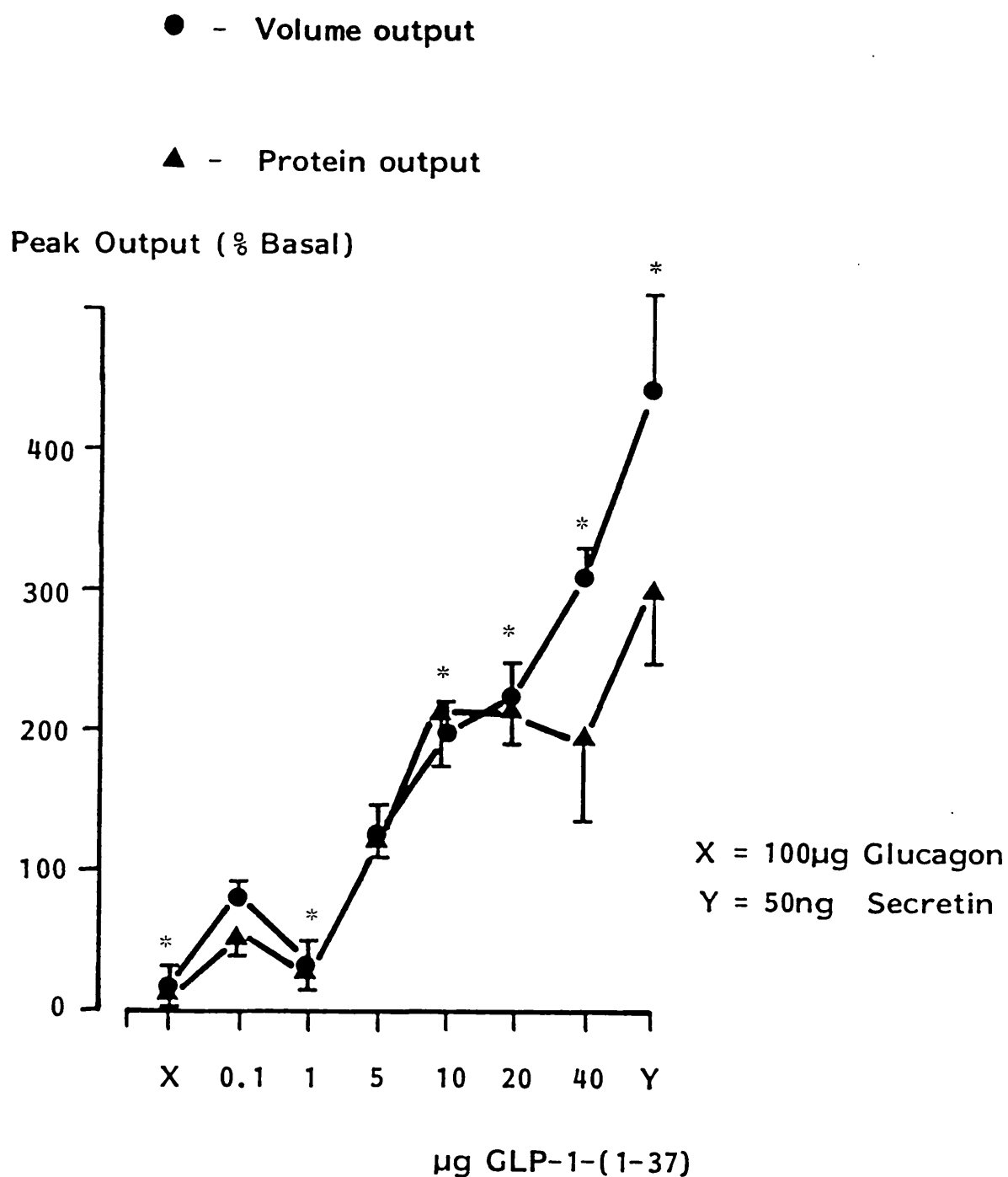


Figure 4:10 The effect of intravenous bolus doses of GLP-1 (0.1 - 40µg), glucagon (100µg) and secretin (50ng) on the peak of the response pancreatic juice volume and protein output (ie peak output). Bars represent mean $\pm$ SEM and the results are expressed as a percentage of the basal output. * represents a value of $p < 0.05$.

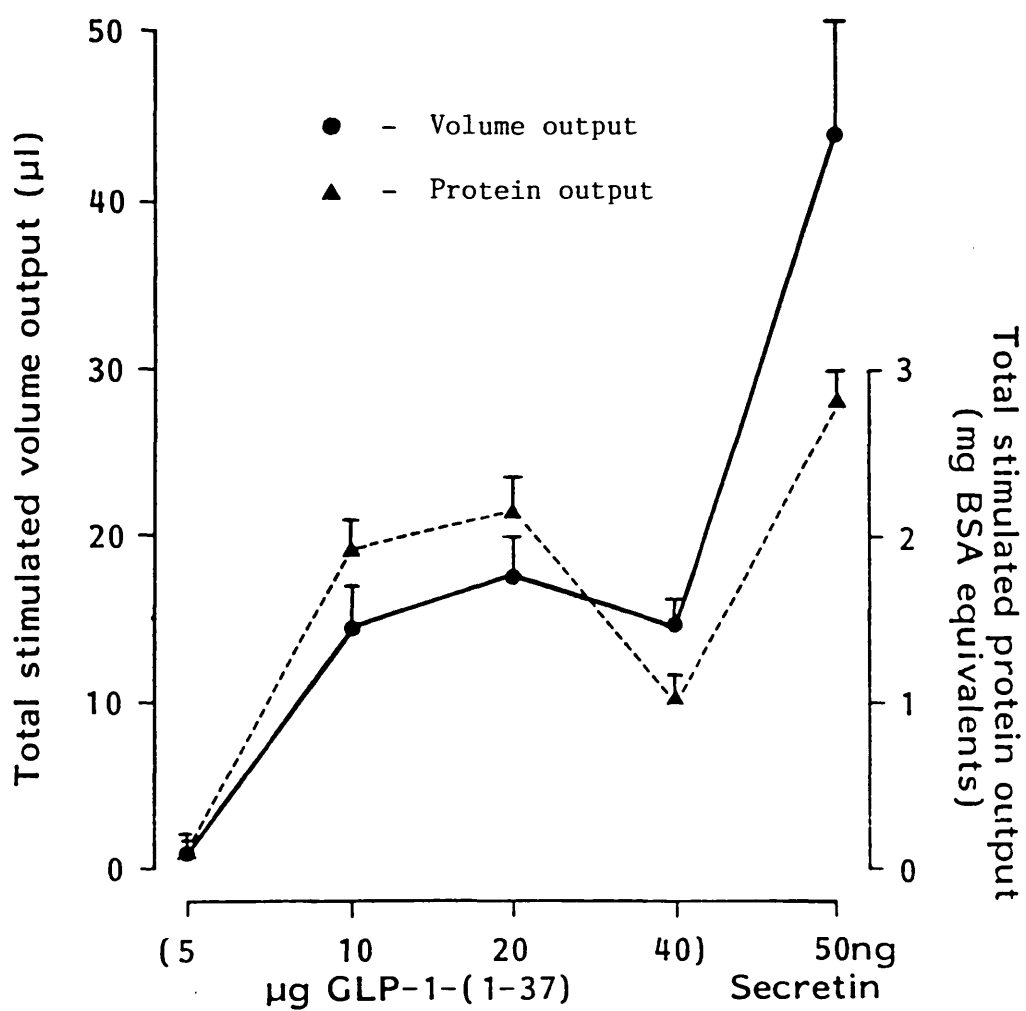


Figure 4:11 The effect of intravenous bolus doses of GLP-1 (5.0 - 40μg) and secretin (50ng) on the total stimulated pancreatic juice volume and protein output. Bars represent mean $\pm$ SEM.

differences were observed between their respective total stimulated volumes or protein outputs. The marked deficit in peak protein output (40 µg dose) when compared with the peak volume output was also reproduced between the total stimulated volume and protein outputs.

Human GLP-2-(1-34), at doses of 10 µg (n = 2) and 20 µg (n = 2) showed no significant change from basal secretory values for either protein or volume output, although fluid and protein outputs were integrated over a 60 min period after injection to check for minimal responses. Glucagon at a dose of 100 µg (n = 4) produced a maximal inhibition of pancreatic juice volume output to $19 \pm 6\%$ basal with a corresponding inhibition of protein output to $16 \pm 6\%$ basal (see fig 4:10).

Secretin at a dose of 50 ng (n = 8) produced a significant increase in volume output at the peak of the response to $441 \pm 14\%$ basal; the corresponding protein output reaching a level of only $297 \pm 54\%$ basal (see fig 4:10). These trends were mirrored on analysing the total stimulated protein and volume outputs for this dose (see fig 4:11). The efficacy of secretin in selectively stimulating pancreatic bicarbonate output is well documented (Greenberg 1983; Dockray 1972). This results in increased production of pancreatic juice which is deficient in protein when compared to basal levels (Dockray 1972). The demonstration of a similar response in the experimental animal model used in these studies testifies to its functional integrity.

Discussion

The amino acid sequence homology observed between the glucagon-like peptides (GLP-1 and GLP-2) and peptides such as secretin, VIP and glucagon (agents capable of modulating exocrine pancreatic secretion), prompted investigating their effects on exocrine pancreatic secretion. Although GLP-2 shows no demonstrable effects on either the volume or protein output of the exocrine rat pancreas, GLP-1 is shown to have effects similar to those exhibited by other members of the "glucagon-secretin" family.

Intravenous bolus doses of GLP-1-(1-37) ranging from 5-20 μg (1.2-4.8 nmol) produce a dose related rise in pancreatic juice peak volume output paralleled by an equivalent rise in peak protein output. This data therefore suggests, that for the 5 min periods corresponding to the peak of the response and over the dose range of 5-20 μg GLP-1-(1-37), the protein concentration of the pancreatic juice secreted remains unchanged (see fig 4:10). GLP-1 administered over this dose range may, therefore, be considered to act similarly to CCK (Dockray 1972). However, the onset of its response is quicker than with CCK, although sustained for a similar duration.

Although bolus doses of 40 μg GLP-1-(1-37) produce a further increase in the peak volume output of pancreatic juice, a corresponding increase in protein output is not observed. This same deficit is apparent on comparing the respective total stimulated protein and volume outputs. At this dose, therefore, the concentration of protein secreted is considerably lower than for the 5-20 μg dose range, and the response may be likened to that of

secretin. Secretin specifically increases exocrine pancreatic fluid and bicarbonate output with only small accompanying increases in protein output (Dockray 1972). Comparison of the peak output volume to protein ratios for the 40 μg GLP-1 dose with that of secretin (50 ng) yields good agreement (see fig 4:10). This may suggest that both these peptides at their respective doses, stimulate the production of pancreatic juice of a similar protein concentration.

Lower doses of GLP-1 produced a marked and significant inhibition of pancreatic juice output. The inhibition produced was detectable within 5 min of injection, and with the 1.0 μg dose continued for periods in excess of 80 min. It was accompanied by an equivalent decrease in protein output suggestive of the maintenance of the pancreatic juice protein concentration throughout this response. As the inhibition observed did not recover to basal levels over the duration of the experiments, secretin tests to verify the integrity of the preparation were carried out approximately 120 min after the initiation of the response. GLP-1, thus at lower and possibly more physiologically relevant doses exhibits an inhibitory effect on exocrine pancreatic secretion similar to that of glucagon (Shaw and Heath 1973).

It may, therefore, be postulated that GLP-1 appears to have three distinct dose ranges which are differentially capable of modulating exocrine pancreatic secretion. In a liberal classification, the first dose range of 0.1-1.0 μg (0.024-0.24 nmol) produces a glucagon-like inhibition. The second dose range of 5-20 μg (1.2-4.8 nmol) a CCK-like stimulation, whilst the third dose range of 40 μg (9.6 nmol) and possibly greater produces a secretin-like

response. The quite opposite effects observed between the low and high doses of GLP-1 are recognised features of the actions of certain other regulatory peptides. Such an example would include the high dose inhibition and low dose activation of adenylate cyclase activity within liver membranes, effected by both glucagon and secretin (England et al 1983), and perhaps more relevantly, by GLP-1 itself (Hoosein & Gurd 1984).

On the basis of the above evidence and the observation that both mammalian pancreas and gut produce a molecular form of GLP-1-like immunoreactivity, immunochemically and chromatographically indistinguishable from GLP-1-(1-37), (see Chapter 3), it may be postulated that GLP-1 has a role in regulating digestive function. Whether its stimulatory effects are mediated through the release of other pancreatic secretagogues is, however, yet to be determined. Although the inhibition produced by the lower doses may be considered to be of greater physiological relevance, local release of GLP-1 within the pancreas may well be able to mimic the concentrations achieved by intravenous boluses of up to 40 μ g (9.6 nmol). Its comparability to glucagon, CCK and secretin, together with its diversity in modulating exocrine pancreatic secretion, lends further support to an intra-pancreatic role. However, this hypothesis would be further strengthened, if the presence of receptors capable of interacting with GLP-1 were demonstrated on pancreatic acinar cell membranes.

CHAPTER 5

GENERAL CONCLUSION

The discovery that mammalian pre-proglucagon contains two additional glucagon-like peptide sequences (GLP-1 and GLP-2) led to two important questions. Firstly, were these peptides actually cleaved off and secreted by the glucagon producing cells of the gut and pancreas and, secondly, what might their biological activities be?

From the combined immunochemical and chromatographic studies described within this thesis, it is evident that the C-terminal portion, like that of the N-terminal portion of mammalian proglucagon is processed differently between the gut and pancreas. This results in the pancreas producing predominantly a cleavage product consistent in immunochemical and chromatographic character to that of the GLP-1 sequence linked to that of GLP-2 via the intervening "spacer" peptide II sequence. However, small amounts of free GLP-1-like immunoreactivity along with lesser amounts of free GLP-2-like immunoreactivity are also produced.

In contrast, the principal products of proglucagon processing within the gut are consistent in immunochemical and chromatographic character to free GLP-1 and an N-terminally extended form of the GLP-2 sequence likely to include that of the intervening "spacer" peptide II. Once again, the quantity of free GLP-2 LI recovered is small. It is, therefore, concluded that a difference between the gut and pancreas in the post-translational processing of the

C-terminal portion of mammalian proglucagon results primarily from the degree of cleavage that takes place at the C-terminal -Arg-Arg- residues of the GLP-1 sequence.

Although the recovered amounts of both GLP-1 and GLP-2 LI might be expected to be produced in equimolar quantities to that of glucagon (pancreas) and GLI (gut), these were in deficit. This implies that some degradation, derivatisation or post-translational modification of the GLP-1 and GLP-2 sequences occurs which significantly reduces their recovered immunoreactivities. However, the crossreactivities with the respective antisera, and the experimental processing losses incurred for the molecular forms other than those equivalent to the synthetic peptides are unknown. Therefore, although column recoveries were consistently in excess of 85%, it has to be taken into consideration that the tissue ratios quoted may not be an accurate representation of the tissue compositions of these peptide immunoreactivities.

The predominant form of GLP-1 and GLP-2 LI produced by the pancreas (Kav 0.15) is also the major circulating component in the plasma of normal healthy volunteers. Its production and secretion by the pancreas in greater quantities than free GLP-1 LI implies that it may serve an important biological role. Like its counterpart glucagon, it may serve as a classical hormone, functioning as a blood-borne messenger secreted by the pancreas acting on target tissues elsewhere. Further work is, therefore, required to isolate sufficient quantities of this component in a highly purified form so that its biological activity may be evaluated.

A similar molecular species deficient in GLP-2 LI is the major product of human pancreatic glucagonomas and is the likely origin of this material in the plasma of glucagonoma patients. At present, the diagnosis of the "glucagonoma syndrome" is valuably aided by the quantification of the elevated plasma levels of pancreatic glucagon LI secreted by the tumour. However, in each of these patients examined the plasma levels of GLP-1 and GLP-2 LI greatly exceeded that of GLI. Quantifying their levels may, therefore, provide a more efficient and robust marker for the presence of this type of tumour.

The complete conservation of the sequence of GLP-1 in the four mammalian species studied suggests that this peptide has an important biological role. This is further supported by the evidence that within these species its sequence is even more so conserved than that of glucagon. The work of Schmidt and colleagues (1985) points to an intra-pancreatic role for GLP-1. This is based on its glucose-dependent insulintropic action on isolated rat pancreatic islets. Although a significant elevation in the insulin response was achieved at only concentrations of GLP-1 above 1 µg/ml; local release of free GLP-1 LI within the pancreas may achieve these concentrations. The production of free GLP-1 LI by the mammalian gut, therefore designates this peptide as a candidate incretin, ie a hormone increasing the insulin response to oral nutrient. However, endocrine pancreatic receptors capable of interacting with GLP-1 are yet to be described and this combined with demonstrations of postprandial rises in plasma free GLP-1 LI are required to validate this hypothesis.

A chromatographic procedure capable of resolving GLP-1-(1-36) amide from that of GLP-1-(1-37) is required to ascertain whether the molecular forms of free GLP-1 LI produced by the gut and pancreas are identical. This may, apart from being able to identify the origin of the bioactive form, clarify the origin of normal human plasma free GLP-1 LI. The N-terminally extended form of intestinal GLP-2 LI, although representing the principal form of intestinal GLP-2 LI is also a major component of normal human plasma. It may, therefore, serve a hormonal role. Its isolation, purification and subsequent biological testing would thus be warranted.

In vivo studies in the rat, demonstrated GLP-1 but not GLP-2 to modulate pancreatic exocrine secretion. Over the dose range of 5-40 µg, GLP-1 stimulates pancreatic juice output, whilst an inhibition is observed at lower doses (0.1 and 1.0 µg). These stimulatory effects may be pharmacological in nature; particularly because the small amounts of free GLP-1 LI produced by the pancreas would seem unlikely to generate the high local concentrations required. However, with bolus doses, it is impossible to estimate the concentration of GLP-1 achieved in the vicinity of the target tissue and furthermore, free GLP-1 LI of intestinal origin may under physiological conditions, contribute to elevate these levels. It is also possible that this effect of GLP-1 may be mediated by inducing the release of other hormones which are pancreatic secretagogues. Further studies are, therefore, required to investigate the effect of intravenous doses of GLP-1 on plasma CCK and secretin levels.

The pancreatic concentration of free GLP-1 LI would seem sufficient to account for the inhibitory effects of GLP-1 on pancreatic juice

output. This effect may, therefore, be of greater physiological relevance. The status of GLP-1 as a modulator of pancreatic exocrine secretion would be further strengthened if a) specific receptors on pancreatic acinar cell membranes capable of interacting with GLP-1 are demonstrated, b) pancreatic and intestinal free GLP-1 LI are of identical molecular form and c) if this effect is not mediated by inducing the release of other pancreatic secretagogues.

The specific binding of both GLP-1 and GLP-2 to pituitary and hypothalamic membranes induces an increase in adenylate cyclase activity. However, within liver membranes this was not the case and neither GLP-1 nor GLP-2 were able to influence the binding of glucagon to its hepatic receptors nor modulate the resulting activation of adenylate cyclase (Hoosein & Gurd 1984). Studies described within this thesis have demonstrated that GLP-1 does not have specific high affinity receptors on rat liver membranes, does not displace glucagon from glucagon receptors on these membranes and does not stimulate the production of cyclic AMP in isolated rat hepatocytes. Moreover, it is observed not to have a glucagon-like effect on plasma glucose concentrations in cortisone pre-treated rabbits. Thus GLP-1, in agreement with the findings of Hoosein & Gurd (1984), is unlikely to have a direct action on hepatic carbohydrate metabolism.

There remained the possibility that the specific binding of both GLP-1 and GLP-2 to pituitary and hypothalamic membranes might realise in modulating secretory capacity. However, neither GLP-1 nor GLP-2 was observed to significantly alter the secretory capacity of the pituitary in relation to growth hormone, adrenocorticotrophic

hormone and prolactin release. It is possible that the release of hormones other than those examined was affected. Further studies are, therefore, required to verify the role(s) of these glucagon-like peptides within the central nervous system.

The likely co-release of both GLP-1 and GLP-2 with the enteroglucagons suggested that they might either be modulators or the causative agents responsible for the trophic effects attributed to the enteroglucagons. However, with GLP-1 this was not the case and it was found not to have any significant effects on parameters related to gut growth.

During the course of some of the early studies, the commercial inavailability of h-GLP-2-(1-34) precluded it from the biological testing programme. As a co-released peptide, it may well be capable of modulating the biological actions of other glucagon precursor-related peptides. Its testing within this context is, therefore, required. On the basis of their modest biological actions described, it would appear that the evolutionary constraints that have conserved the structure of the glucagon-like peptides to such extents must arise from physiological actions that remain to be discovered.

REFERENCES

Abraham G E

Solid phase radioimmunoassay of estradiol-17-B

J Clin Endocr Metab 1969; 29: 866-870.

Albano J D M, Ekins R P, Maritz G & Turner R C

A sensitive, precise radioimmunoassay of serum insulin relying on charcoal separation of bound and free hormone moities

Acta Endocrinol 1972; 70: 487-509.

Al-Mukhtar M Y T, Sagor G R, Ghatei M A et al

The relationship between endogenous gastrointestinal hormones and cell proliferation in models of adaptation

In: Robinson J W L, Dowling R H & Riecken E O (eds), Mechanisms of Intestinal Adaptation, Lancaster MTP Press, 1982; pp243-256.

Andrews P C & Ronner P

Isolation and structures of glucagon and glucagon-like peptide from catfish pancreas

J Biol Chem 1985; 260: 7: 3910-3914.

Arimura A, Culler M D, Turkelson C M, Luciano M G, Thomas C R, Obara N, Groot K, Rivier J & Vale W

In vitro pituitary hormone releasing activity of 40 residue human pancreatic tumour growth releasing factor

Peptides 1983; 4: 107-110.

Assan R & Slusher N

Structure/function and structure/immunoreactivity relationships of the glucagon molecule and related synthetic peptides

Diabetes 1972; 21: 843-855.

Atassi M Z

Precise determination of the entire antigenic structure of lysozyme

Immuno-chemistry 1978; 15: 909-933.

Baldissera F G A & Holst J J

Glucagon related peptides in the human gastrointestinal mucosa

Diabetologia 1984; 26: 223-228.

Bataille D, Gespach C, Coudray A M & Rosselin G

Enteroglucagon: a specific effect on gastric glands isolated from the rat fundus. Evidence for an "oxyntomodulin" action

Biosci Rep 1981; 1: 151-155.

Bataille D, Tatemoto K, Gespach C, Jornvall H, Rosselin G & Mutt V

Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum. Isolation of the peptide

Febs Lett 1982; 146: 79-85.

Baum J, Simon B E Jr, Unger R H & Madison L L

Localisation of glucagon in the alpha cells of the pancreatic islet by immunofluorescent techniques

Diabetes 1962; 11: 371-374.

Bell G I, Santerre R F & Mullenbach G T

Hamster pre-proglucagon contains the sequence of glucagon and two related peptides

Nature 1983a; 302: 716-718.

Bell G I, Sanchez-Pescador R, Laybourn P J & Najarian R C

Exon duplication and divergence in the human pre-proglucagon gene

Nature 1983b; 304: 368-371.

Bencosme S A, Liepa E & Lazarus S S

Glucagon content of pancreatic tissue devoid of alpha cells

Proc Soc Exp Biol Med 1955; 90: 387-392

Besterman H S, Bloom S R, Sarson D L et al

Gut hormone profile in coeliac disease

Lancet 1978; I: 785-788.

Besterman H S, Adrian T E, Mallinson C N et al

Gut hormone release after intestinal resection

Gut 1982; 23: 854-861.

Blobel G

Intracellular protein topogenesis

Proc Natl Acad Sci USA 1980; 77: 1496-1500.

Bloom S R

An enteroglucagon tumour

Gut 1972; 13: 520-523.

Bloom S R

Hormonal changes after jejuno-ileal bypass and their physiological significance

In: Maxwell J D, Gazet J C & Pilkington T R (eds), Surgical Management of Obesity, London: Academic Press 1980a; pp115-123.

Bloom S R

Gut and brain - endocrine connections

J R Coll Phys 1980b; 14: 51-57.

Bloom S R & Polak J M

The hormonal pattern of intestinal adaptation. A major role for enteroglucagon

Scand J Gastroenterol 1982; Suppl 72: 409-420.

Bomboy J D, Lewis S B, Sinclair Smith B C, Lacy W W & Liljenquist J

Insulin-glucagon interaction in controlling splanchnic glucose production in normal man

J Clin Endocrinol Metab 1977; 44: 474-480.

Bradbury A F, Finnie M D A & Smyth D G

Mechanism of C-terminal amide formation by pituitary enzymes

Nature 1982; 298: 686-688.

Bromer W W, Sinn L G, Staub A & Behrens O K

The amino acid sequence of glucagon

J Am Chem Soc 1956; 78: 3858-3860.

Chance R E, Ellis R M & Bromer W W

Porcine proinsulin - characterisation and amino acid sequence

Science 1968; 161: 165-167.

Christofides N D

Pancreatic glucagon

In: Bloom S R & Long R G (eds), Radioimmunoassay of gut regulatory peptides, W B Saunders & Co Ltd, London, Philadelphia, Toronto

1982; 11: 74-79.

Conlon J M, Samson W K, Dobbs R E, Orci L & Unger R H

Glucagon-like polypeptides in canine brain

Diabetes 1979; 28: 700-702.

DiMagno E P

Alterations and consequences of interdigestive and post-stimulatory human exocrine pancreatic secretion in health and disease

Scand J Gastroenterol 1983; 18: Suppl 87: 68-84.

Dockray G J

The action of secretin, cholecystokinin-pancreozymin and caerulein on pancreatic secretion in the rat

J Physiol 1972; 225: 679-692.

Dowling R H & Booth C C

Structural and functional changes following small intestinal resection in the rat

Clin Sci 1967; 32: 39-49.

Dubrasquet M, Bataille D & Gespach C

Oxyntomodulin (glucagon 37 or bioactive enteroglucagon) a powerful inhibitor of pentagastrin-stimulated acid secretion in the rat
Fourth International Symposium on Gastrointestinal Hormones,
Stockholm AB, Excelsior 1982; 56. (abstract)

Dunbar J C, Silverman H, Kirman E & Fon P P

Salivary gland and kidney glucagon
Fed Proc 1976; 35: 218-222.

England R D, Jenkins W T, Flanders K C & Gurd R S

Non-co-operative receptor interactions of glucagon and eleven analogues: inhibition of adenylate cyclase
Biochemistry 22; 1722-1728.

Felig P, Wharea J & Hendler R

Influence of physiologic hyperglucagonemia on basal and insulin-inhibited splanchnic glucose output in normal man
J Clin Invest 1976; 58: 761-765.

Gardener J D, Christophe J, Robberecht P et al

Membrane receptors for VIP and secretin in pancreatic acinar cells
In: Bonfils S (ed), Hormonal receptors in digestive tract physiology, Amsterdam, North Holland 1977; pp 227-235.

Gardner J D

Regulation of pancreatic exocrine function in vitro. Initial steps in the release of secretagogues
Ann Rev Physiol 1979; 41: 55-66.

Ghatei M A

Enteroglucagon

In: Bloom S R & Long R G (eds), Radioimmunoassay of Gut Regulatory Peptides, W B Saunders & Co Ltd, London, Philadelphia, Toronto, 1982; pp 80-91.

Ghatei M A, Uttenthal L O, Christofides N D, Bryant M G & Bloom S R
Molecular forms of human enteroglucagon in tissue and plasma.
Plasma responses to nutrient stimuli in health and disorders of the
upper gastrointestinal tract

J Clin Endocrinol Metab 1983; 57: 3: 488-495.

Gleeson M H, Bloom S R, Polak J M, Henry K & Dowling R H

An endocrine tumour in kidney affecting small bowel structure,
motility and absorptive function

Gut 1971; 12: 773-782.

Greenberg G R

Neurohormonal control of pancreatic function in man and its failure
Scand J Gastroenterol 1983; 18: Suppl 82: 101-109.

Heding L G, Frandsen E K & Jacobson H

Glucagon structure-function relationship: immunologic

Metabolism 1976; 25: 1327-1329.

Heinrich G, Gros P & Habener J F

Glucagon gene sequence:- Four of six exons encode separate
functional domains of rat pre-proglucagon

J Biol Chem 1984; 22: 14082-14087.

Holst J J

Extraction, gel filtration pattern and receptor binding of porcine
gastro-intestinal glucagon-like immunoreactivity
Diabetologia 1977; 13: 159-169.

Holst J J

Extrapancreatic glucagons
Digestion 1978; 17: 168-190.

Holst J J

Evidence that glicentin contains the entire sequence of glucagon
Biochem J 1980; 187: 337-343.

Holst J J

Evidence that peak II GLI or enteroglucagon is identical to the
C-terminal sequence (residues 33-69) of glicentin
Biochem J 1982a; 201: 381-388.

Holst J J, Knuhtsen S, Fahrenkrug J, Jensen S L, Swartz T W,

Nielsen O V & Rehfeld J F

Regulatory peptides in the neural control of the pancreas

Symposia Medica Hoechst 18

In: Systemic Role of Regulatory Peptides, Bloom S R, Polak J M &
Lindenlaub E (eds), F K Schattauer Verlag, Stuttgart-Newyork,
1982b; pp171-190.

Holst J J

Gut glucagon, enteroglucagon, gut glucagon-like immunoreactivity,
glicentin - current status
Gastroenterology 1983a; 84: 1602-1613.

Holst J J

Glucagon radioreceptor assays

In: Lefebvre P J (ed), Glucagon, Handbook of Experimental Pharmacology, Berlin: Springer Verlag, 1983b; 66: pp245-261.

Holst J J

Molecular heterogeneity of glucagon in normal subjects and in patients with glucagon-producing tumours

Diabetologia 1983c; 24: 359-365.

Hoosein N M & Gurd R S

Human glucagon-like peptide 1 and 2 activate rat brain adenylate cyclase

Febs Lett 1984; 178: 83-86.

Hunter W H & Greenwood F C

Preparation of ^{131}I -labelled human growth hormone of high specific activity

Nature 1962; 194: 495-496.

Jacobsen H, Demandt A, Moody A J & Sundby F

Sequence analysis of porcine GLI-1

Biochim Biophys Acta 1977; 493: 452-459.

Jensen R T, Collins S M, Pandoz S J & Gardner J D

Lessons from studies of secretagogue receptors on pancreatic acinar cells

In: Bloom S R & Polak J M (eds), Gut Hormones, 2nd edn, Churchill Livingstone, 1981; pp133-136.

Kaneko T, Cheng P Y, Toda G, Oka H, Yanaihara N, Yanaihara C, Mihara S, Nishida T, Kaise N, Shin S & Imagawa K

Biological and binding actions of synthetic possible C-terminal fragments of glicentin in rat liver plasma membrane

In: Miyoshi A (ed), Gut Peptides, Secretion, Function and Clinical Aspects, Elsevier, North Holland, Amsterdam, Oxford, New York, 1979; pp157-161.

Kirkegaard P, Moody A J, Holst J J, Loud F B, Skovolsen P & Christiansen J

Glicentin inhibits gastric acid secretion in the rat
Nature 1982; 297: 156-157.

Koranyi L, Peterfy F, Szabo J, Torok A, Guoth M & Tamas G Y

Evidence for transformation of glucagon-like immunoreactivity of gut into pancreatic glucagon in vivo
Diabetes 1981; 30: 792-794.

Krohn K A, Knight L C, Harwig J F & Welch M J

Differences in the sites of iodination of proteins following four methods of radioiodination

Biochim Biophys Acta 1977; 490: 497-505.

Laurent T C & Killander J

A theory of gel filtration and its experimental verification
J Chromatogr 1964; 14: 317-330.

Law G J, Keith R R & Wallis M

Effects of growth hormone-releasing factor, somatostatin and dopamine on growth hormone and prolactin secretion from cultured ovine pituitary cells

Febbs Lett 1984; 166: 1: 189-193.

Lefebvre P J

Commentary: glucagon and adipose tissue

Biochem Pharmacol 1975; 24: 1261-1266.

Lopez L C, Frazier L M, Chung-Jey S, Kumar A & Saunders G F

Mammalian pancreatic pre-proglucagon contains three glucagon-related peptides

Proc Natl Acad Sci USA 1983; 80: 5485-5489.

Love J W

A method for the assay of secretin using rats

Q Jl Exp Physiol 1957; 42: 279-284.

Lowry O H, Rosebrough N J, Farr A L & Randall R J

Protein measurement with folin phenol reagent

J Biol Chem 1951; 193: 265-275.

Lund P K, Goodman R H, Jacobs J W & Habener J F

Glucagon precursors identified by immunoprecipitation of products of cell-free translation of messenger RNA

Diabetes 1980; 29: 583-586.

Lund P K, Goodman R H & Habener J F

Pancreatic pre-proglucagons are encoded by two separate mRNAs

J Biol Chem 1981a; 256: 6515-6518.

Lund P K, Goodman R H & Habener J F

Intestinal glucagon mRNA identified by hybridisation to a cloned islet cDNA encoding a precursor

Biochem Biophys Res Commun 1981b; 100: 1659-1666.

Lund P K, Goodman R H, Dee P P C & Habener J F

Pancreatic pre-proglucagon cDNA contains two glucagon-related coding sequences arranged in tandem

Proc Nat Acad Sci USA 1982a; 79: 345-349.

Lund P K, Goodman R H, Mountminy M M & Hasoner J F

Pre-proglucagons polyproteins encoded by at least two different genes

Fourth International Symposium on Gastrointestinal Hormones - Stockholm, A B Excelsior - 1982b; 30: (abstract)

Lund P K, Goodman R H, Mountminy M R, Dee P C & Habener J F

Anglerfish islet pre-proglucagon II. Nucleotide and corresponding amino acid sequence of the cDNA

J Biochem 1983; 258: 3280-3284.

Matsuyama T & Foa P P

Plasma glucose, insulin, pancreatic and enteroglucagon levels in normal and depancreatized dogs

Proc Soc Exp Biol Med 1974; 147: 97-102.

Mallinson C N, Bloom S R, Warin A P, Salmon P R & Cox B

A glucagonoma syndrome

Lancet 1974; 2: 1.

McGarry J D, Robles-Valdes C & Foster D W

Role of carnitine in hepatic ketogenesis

Proc Natl Acad Sci USA 1975; 72: 4385-4388.

McGregor G P & Bloom S R

Radioimmunoassay of substance P and its stability in tissue

Life Sci 1983; 32: 655-662.

Moody A J, Jacobsen H, Sundby F, Frandsen E K, Baetens D & Orci L

Heterogeneity of gut-glucagon-like immunoreactants (GLIs)

In: Foa P P, Byg J S & Foa N L (eds), Glucagon: Its Role in

Physiology and Clinical Medicine, Springer, New York,

1977; pp129-135.

Moody A J, Jacobsen H & Sundby F

Gastric glucagon and gut glucagon-like immunoreactants

In: Bloom S R (ed), Gut Hormones, Churchill Livingstone, Edinburgh,

London 1978; pp369-378.

Moody A J, Holst J J, Thim L & Jensen S L

Relationship of glicentin to pro-glucagon and glucagon in the
porcine pancreas

Nature 1981a; 289: 514-516.

Moody A J & Thim L

The relationship between glucagon and gut glucagon-like immunoreactants (gut GLIs)

In: Bloom S R & Polak J M (eds), Gut Hormones, 2nd edn, Edinburgh, Churchill Livingstone, 1981b; pp312-319.

Munck A, Kervran A, Marie J C, Bataille D & Rosselin G

Glucagon-37 (oxyntomodulin) and glucagon-29 (pancreatic glucagon) in human bowel. Analysis by HPLC and radioreceptor assay

Peptides 1984; 5: 553-561.

Murlin J R, Clough H G, Gibbs C B F & Stokes A M

Aqueous extracts of the pancreas, influence on the carbohydrate metabolism of depancreatectomies

J Biol Chem 1923; 56: 253-296.

Mutt V

Chemistry of the gastrointestinal hormones and hormone-like peptides and a sketch of their physiology and pharmacology

In: Munson P L, Glover J, Diczfalusy E & Ison R E O (eds), Vitamins and Hormones, Academic Press Inc, NewYork, 1982; 39: pp231-427.

Noe B D & Bauer G E

Evidence for glucagon biosynthesis involving a protein intermediate in islets of the anglerfish (*lophicus americanus*)

Endocrinology 1971; 89: 642-681.

Noe B D & Bauer G E

Evidence for sequential metabolic cleavage of proglucagon to glucagon in glucagon biosynthesis

Endocrinology 1975; 97: 868-877.

Nottley J J & Rosselin G

Monoiodo glucagon: preparation, isolement, identification, controle radio-immunologique

C R Acad Sci (Paris) 1971; 273: 2218-2121.

Nygaard K

Resection of the small intestine rat, IV adaptation of gastrointestinal motility

Acta Chir Scand 1967; 113: 407-416.

Orci L, Forssmann W G, Forssmann W & Roullier C

Electron microscopy of the intestinal endocrine cells - Comparative Study

In: Bocchiarelli S D (ed), Electron Microscopy - 4th European Regional Conference, Rome 1968; pp369-370.

Park C R & Exton J H

Effect of glucagon on the liver

In: "Glucagon Molecular Physiology, Clinicial and Therapeutic Implications", Lefebvre P J & Unger R H (eds), Pergamon Press, Oxford 1972; pp77-92.

Patzelt C, Tager H S, Carroll R J & Steiner D F

Identification and processing of proglucagon in pancreatic islets

Nature 1979; 282: 260-266.

Patzelt C, Chan S J, Quinn P S, Carroll R J, Tager H S & Steiner D F
Biosynthetic precursors of glucagon and proglucagon
In: Waldhäusl W K (ed), Diabetes, Excerpta Medica, Amsterdam,
1980; pp119-127.

Patzelt C & Schiltz E
Conversion of proglucagon in pancreatic alpha cells: the major end
products are glucagon and a single peptide, the major proglucagon
fragment that contains two glucagon-like sequences
Proc Natl Acad Sci USA 1984; 81: 16: 5007-5011.

Perez Castillo A & Blazquez E
Synthesis and release of glucagon by human salivary glands
Diabetologia 1980a; 19: 12-29.

Perez Castillo A & Blazquez E
Tissue distribution of glucagon, glucagon-like immunoreactivity and
insulin in the rat
Am J Physiol 1980b; 238: E258-E266.

Pohl S L, Birnabaumer L & Rodbell M
The glucagon sensitive adenyl cyclase system in plasma membranes of
rat liver 1 properties
J Biol Chem 1971; 246: 1849-1856.

Powell R, White M C, Daniels M & Mashiter K
Domperidone directly stimulates TSH secretion in vitro
Febs Lett 1981; 129: 1: 97-99.

Rigopoulou D, Valverde I, Marco J, Faloona G & Unger R H

Large glucagon immunoreactivity in extracts of pancreas

J Biol Chem 1970; 245: 496-501.

Rodbard D

Statistical quality control and routine data processing for
radioimmunoassays and immunoradiometric assays

Clin Chem 1974; 20: 10: 1255-1270.

Rodbell M, Krans M J, Pohl S L & Birnbaumer L

The glucagon-sensitive adenyl cyclase system in plasma membranes of
rat liver III. Binding of glucagon: method of assay and specificity

J Biol Chem 1971; 246: 1861-1871.

Roulston J E

Validation of the self displacement technique for estimation of
specific radioactivity of radioimmunassay tracers

Ann Clin Biochem 1979; 16: 26-29.

Rubalclava B & Rodbell M

The role of acidic phospholipids in glucagon action on rat liver
adenylate cyclase

J Biol Chem 1973; 248: 3831-3837.

Sasaki H, Rubalclava B, Bacters D, Blazquez E, Srikant C B, Orci L &
Unger R H

Identification of glucagon in the gastrointestinal tract

J Clin Invest 1975; 56: 135-145.

Scatchard G

The attraction of proteins for small peptides

Ann N Y Acad Sci USA 1949; 51: 660-672.

Schmidt W E, Siegel E G & Creutzfeldt W

Glucagon-like peptide-1 but not glucagon-like peptide-2 stimulates
insulin release from isolated rat pancreatic islets

isolated rat pancreatic islets

Diabetologia 1985; 28: 704-707.

Sela M

Antigenicity: some molecular aspects

Science 1969; 166: 1365-1374.

Shaw H M & Heath T J

The effect of glucagon on the formation of pancreatic juice and bile
in the rat

Can J Physiol Pharmacol 1973; 51: 1-5.

Srikant C B, McCorkle K & Unger R H

Properties of immunoreactive glucagon fractions canine stomach and
pancreas

J Biol Chem 1977; 252: 1847-1851.

Staub F, Sinn L & Behrens O K

Purification and crystallisation of glucagon

J Biol Chem 1955; 214: 619-632.

Steiner D F, Quinn P S, Chan S J, Marsh J & Tager H S
Processing mechanisms in the biosynthesis of proteins
Ann N Y Acad Sci USA 1980; 343: 1-16.

Sundby F

Species variations in the primary structure of glucagon
Metabolism 1976a; 25: Suppl 1: 1319-1321.

Sundby F, Jacobsen H & Moody A J

Purification and characterisation of a protein from porcine gut with
glucagon-like immunoreactivity
Horm Metab Res 1976b; 8: 366-371.

Tager H S & Steiner D F

Isolation of a glucagon-containing peptide. Primary structure of a
possible fragment of proglucagon
Proc Natl Acad Sci USA 1973; 70: 2321-2325.

Tager H, Hohenboken M, Markese J & Dinerstein R J

Identification and localisation of glucagon-related peptides in rat
brain
Proc Natl Acad Sci USA 1980; 77: 6229-6233.

Tarding F, Nielsen P, Keiser-Neilsen B & Nielsen A V

Biological assay of glucagon in rabbits
Diabetologia 1969; 5: 146-154.

Thieden H, Holst J J, Dieh J, Moody A J & Sundby F

Effect of highly purified porcine gut glucagon-like immunoreactivity
(glicentin) on glucose release from isolated rat hepatocytes
Biochem Biophys Acta 1981; 675: 163-170.

Thim L & Moody A J

The primary structure of porcine glicentin (pro-glucagon)

Regul Pept 1981; 2: 139-141.

Thim L & Moody A J

Purification and chemical characterisation of a glicentin-related pancreatic [proglucagon fragment] from porcine pancreas

Biochem Biophys Acta 1982; 703: 134-141.

Trakatellis A C, Tada K, Yanagi K & Gardiki-Kouidou P

Isolation and partial characterisation of anglerfish proglucagon

Biochemistry 1975; 14: 1508-1512.

Turkelson C M, Arimura A, Caller M D, Fishback J B, Groor K, Kanda

M, Luciano M, Thomas C R, Chang D, Chang J K & Shimizu M

In vivo and in vitro release of ACTH by synthetic CRF

Peptides 1981; 2: 425-429.

Unger R H, Eisentraut A, McCall M S, Keller S, Lanz H C & Madison L

Glucagon antibodies and their use for immunoassay of glucagon

Proc Soc Exp Biol 1959; 102: 621-623.

Unger R H, Eisentraut A M, McCall M S & Madison L L

Sites of origin of glucagon in dogs and humans

Clin Res 1961; 9: 53.

Unger R H

The mullieu interior and the islets of Langerhans

Diabetologia 1981; 20: 1-11.

Uttenthal L O, Batt R M, Carter M W & Bloom S R

Stimulation of DNA synthesis in cultured small intestine by
partially purified enteroglucagon

Regul Pept 1982; 3: 84.

Vaitukaitis J, Robbins J B, Nieschlag E & Ross G T

A method for producing specific antisera with small doses of
immunogen

J Clin Endocrinol Metab 1971; 33: 988-991.

Valverde I, Rigopoulou D, Exton J, Ohneda A, Eisentraat A M

& Unger R H

Demonstration and characterisation of a second fraction of
glucagon-like immunoreactivity in jejunal extracts

Am J Med Sci 1968; 255: 415-420.

Valverde I, Rigopoulou D, Marco J, Faloona G R & Unger R H

Molecular size of extractable glucagon-like immunoreactivity (GLI)
in plasma

Diabetes 1970; 19: 624-629.

Wright N A, Watson A, Morley A, Appleton D, Marks J & Douglas A

The measurement of cell production rats in the crypts of Lieberkuhn

Virchows Arch [A] 1974; 364: 311-323.

Yalow R S & Berson S A

Immunoassay of endogenous plasma insulin in man

J Clin Invest 1960; 39: 1157-1161.

Yanaihara N, Yanaihara C, Nishida T, Hiraiwa T, Mihara S, Sakagami M, Ozaki J, Imagawa K & Shin S

Synthesis of glicentin and proglucagon related peptides and their immunologic properties

In: Rosselin G, Fromageut P, Bonfils S (eds), Hormone Receptors in Digestion and Nutrition, Amsterdam, Elsevier/North Holland 1979; pp65-68.

APPENDIX A

i) Source of Peptides

GLP-1-(1-19) was obtained custom-synthesized from Cambridge Research Biochemicals Ltd, Harston, UK. GLP-1-(1-37) was purchased from Bachem (Torrance, CA, USA) and both GLP-1-(1-36)-peptide amide and human GLP-2-(1-34) from Peninsula Laboratories Inc (Belmont, CA, USA). Crystalline glucagon was obtained from Novo (Novo Industries, Copenhagen, Denmark) and both synthetic hp GRF-(1-44) and CRF were generous gifts from the Salk Institute (USA).

ii) Chemicals

Acetonitrile, Rathburn Chemicals, Peebleshire, England.
Acetic acid (glacial), BDH Chemicals Ltd, Essex, England.
Ammonium hydrogen carbonate, Hopkins & Williams, Essex, England.
Ammonium sulphate, BDH Chemicals Ltd, Essex, England.
Arlacel A, Sigma Ltd, Poole, England.
Barbitone sodium, BDH Chemicals Ltd, Essex, England.
Bovine serum albumin (crystalline), Armour Pharmaceuticals, England.
Charcoal (Norit GSX), Hopkin & Williams, Essex, England.
Citric acid, BDH Chemicals Ltd, Essex, England.
L-Cysteine hydrochloride, BDH, Chemicals, Essex, England.
Dextran, clinical grade (MW 80,000), Sigma Ltd, Poole, England.
Dextran Blue, Sigma Ltd, Poole, England.
Glycine, BDH chemicals Ltd, Essex, England.
Hexadecane, puriss, Koch-Light, Suffolk, England.
Hydrochloric acid, BDH Chemicals Ltd, Essex, England.
Lactose, BDH Chemicals Ltd, Essex, England.

Potassium hydroxide, BDH Chemicals Ltd, Essex, England.

Potassium iodide, BDH, Chemicals Ltd, Essex, England.

Sephadex G-50 superfine, Pharmacia Ltd, Milton Keynes, England.

Sodium acetate (anhydrous), BDH Chemicals Ltd, Essex, England.

Sodium azide, BDH Chemicals Ltd, Essex, England.

Sodium chloride, Hopkin & Williams, Essex, England.

Di-sodium hydrogen orthophosphate dihydrate, BDH Chemicals Ltd,
Essex, England.

Sodium hydroxide, BDH Chemicals Ltd, Essex, England.

Sodium iodide (^{125}I), Amersham, England.

Trasylol (aprotinin), Bayer Co, W Germany.

Trifluoroacetic acid (TFA), BDH Chemicals Ltd, Essex, England.

M Tuberculosis (attenuated), MAFF, England.

iii) Equipment

Balances:	Cahn electrobalance, California, USA.
	Oertling balance, London, England.
	Sartorius analytical balance 1201 MP2, London.
Centrifuges:	Bench-top centrifuge, EBA 35, A R Horwell, London, England.
	Coolspin, MSE, Fisons, England.
Chromatography:	Fraction collector, FRAC 100, Pharmacia, England.
	HPLC, Waters system, Waters Associates, Cheshire, England.
	Pharmacia columns, K=.9/60 & K1.5/100.
	5 μ C-18 Techsil columns, HPLC Technology,
	Sep-pak-C-18 cartridges, Waters Associates, Cheshire, England.

Dispensers: Graduated syringe, A R Horwell, London, England.
 Holders/teats for volupettes, Medikent, St Albans, England.
 Hamilton syringe (repeater), V A Howe, London, England.
 Hamilton syringe (single shot), V A Howe, London, England.
 Volupettes, Medikent, London, England.

Gamma-counter: NE 1600, Nuclear Enterprises, Edinburgh, Scotland.

Homogeniser: Ultra-Turrax, Scientific Instruments, London, England.

Lyophiliser: Edwards High Vacuum, Sussex, England.

pH Meter: Corning pH meter, Corning, England.
 Portable photometer, Hypocount, Hypoguard, Suffolk, England.

Refrigeration: ESTA 601 freezer, Bejam, London, England.
 Osbourne bottle cooling trays, LEC, London.

Tubes: GW3 polystyrene tubes, Seward Labs, London.
 GW4 polystyrene tubes, Seward Labs, London.
 Freeze-drying glass vials, Anchor Glass, London, England.
 LH/10 lithium heparin containers, Seward Labs, London, England.
 LP3 polystyrene tubes, Luckham Ltd, Sussex, England.
 Universal containers, Sterilin, England.
 Z35 plastic blood containers, Seward Labs, London, England.

APPENDIX BPUBLICATIONSABSTRACTS

George S K, Ghigllione M, Uttenthal L O, Mulderry P K, Major J H, Yiangou Y and Bloom S R.

PHI and Bombesin-like immunoreactivity in isolated pancreatic islets of the rat.

Regul Pept 1983; 7: 286

Bloom S R, Mulderry P K, George S K, Bishop A E, Ghattei M A, and Polak J M.

Calcitonin-gene related peptide in the pancreas.

Diabetic Med 1984; 1: 160A

Uttenthal L O, Ghigllione M, George S K and Bloom S R.

Effect of glucagon-like peptide-1 (GLP-1) on pancreatic juice output in the rat.

Dig Dis Sci 1984; 29: 91S

Uttenthal L O, Ghigllione M, George S K and Bloom S R.

Radioimmunoassay for glucagon-like-peptide-1: presence in human pancreas and glucagonomas.

Diabetologia 1984; 27: 341A

Ghigllione M, Uttenthal L O, George S K and Bloom S R.

Glucagon-like peptide-1 has no glucagon-like effect on plasma glucose and insulin.

Diabetologia 1984; 27: 278A-279

Uttenthal L O, Ghiglione M, George S K and Bloom S R.
Post-translational processing of proglucagon in man.
Regul Pept 1984; 9: 351

Uttenthal L O, Ghiglione M, George S K and Bloom S R.
Presence of Glucagon-like peptide-1 in plasma of patients with
glucagon-secreting tumours.
Clin Sci 1985; 68: 69P

Uttenthal L O, Ghiglione M, George S K and Bloom S R.
A major cleavage product of human pancreatic proglucagon
contains glucagon-like peptide-1 and glucagon-like peptide-2
immunoreactivities.
Diabetic Med 1985; 2: 322A

PAPERS

Ghatei M A, George S K, Major J H, Carlei F, Polak J M and
Bloom S R.
Bombesin-like immunoreactivity in the pancreas of man and other
mammalian species.
Experientia 1984; 40: 884-886

Ghiglione M, Uttenthal L O, George S K and Bloom S R.
How glucagon-like is glucagon-like peptide-1.
Diabetologia 1984; 27: 599-600

Ghiglione M, Uttenthal L O, George S K and Bloom S R.

Effect of neuropeptide hpGRF on exocrine pancreatic secretion
in the rat.

Biomed Res 1985; 6: 107-109

Uttenthal L O, Ghiglione M, George S K, Bishop A E, Polak J M
and Bloom S R.

Molecular forms of glucagon-like peptide-1 in human pancreas
and glucagonomas.

J Clin Endocrinol Metab 1985; 61: 472-479

George S K, Uttenthal L O, Ghiglione M and Bloom S R.

Molecular forms of glucagon-like peptides in man.

Febbs Lett 1985; 192; 275-278

Ghiglione M, Blazquez E, Uttenthal L O, de Diego J G, Alvarez
E, George S K and Bloom S R.

Glucagon-like peptide-1 does not have a role in hepatic
carbohydrate metabolism.

Diabetologia 1985; 28: 920-921