A radioimmunoassay was developed and validated for human C-peptide in unextracted plasma, using a synthetic 31 amino acid human C-peptide for immunogen, standard and tracer. The sensitivity of the assay (10 pg/tube) enabled the measurement of both fasting and stimulated circulating C-peptide levels. Normal ranges were established in lean healthy volunteers after (a) fasting (b) stimulation of insulin secretion using oral and intravenous stimuli (c) suppression of endogenous insulin secretion using exogenous insulin. Human C-peptide measurements were used to investigate patients presenting with hypoglycaemia due to a number of clinical conditions and were found to be of especial use in the differential diagnosis of the factitious hypoglycaemia of insulin abuse.

A rat C-peptide radioimmunoassay was developed and validated to investigate the possibility that C-peptide, as well as insulin, inhibits fat stimulated GIP release. Both exogenous and endogenous C-peptide were shown to inhibit fat stimulated GIP release in rats fed normal laboratory food. However, neither insulin or C-peptide were effective in inhibiting fat stimulated GIP release in rats maintained on short-term high fat diets. Studies were, therefore, extended to investigate the feed-back inhibition of exogenous insulin on GIP release in humans maintained on low and high fat dietary regimens. Exogenous insulin was found to be ineffective in inhibiting fat stimulated GIP secretion in subjects maintained on a high fat diet. The control of GIP secretion with its consequent effect on insulin secretion via the enteroinsular axis therefore appears to be affected by dietary fat intake.
To

John
ACKNOWLEDGEMENTS

I would like to express my thanks to the following:-

Professor V. Marks for his encouragement, ideas and advice during this project.
Dr. L. Morgan and Ms. J. Tredger for reading the manuscript and their suggestions.
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All chemicals supplied by BDH Ltd., Poole, Dorset, unless otherwise stated.

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<td>Lactoperoxidase</td>
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<td>1,3,4,6,Tetrachloro 3a6a diphenylglycouril (iodogen)</td>
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<td>Hycal</td>
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<tr>
<td>MBq</td>
<td>megabecquerels</td>
</tr>
<tr>
<td>mCi</td>
<td>millicuries</td>
</tr>
<tr>
<td>NSS</td>
<td>Normal Sheep Serum</td>
</tr>
<tr>
<td>Dab</td>
<td>Double antibody</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<td>CSS</td>
<td>Charcoal Stripped Serum</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>HSA</td>
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<tr>
<td>LSI</td>
<td>Light scattering intensity</td>
</tr>
<tr>
<td>ID</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>MB</td>
<td>Maximum binding</td>
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<tr>
<td>NRS</td>
<td>Normal Rabbit Serum</td>
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<tr>
<td>%B</td>
<td>Percentage bound</td>
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<td>TFA</td>
<td>Fluoroacetic acid</td>
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<td>Normal Guinea Pig Serum</td>
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CHAPTER I

INTRODUCTION
1:1 General Introduction

The hormone insulin is synthesised in the B cells of the pancreatic islets from the prohormone proinsulin. Enzymatic cleavage of proinsulin results in the formation of equimolar amounts of insulin and C-peptide (See Figure 1:1). The role of C-peptide was initially thought to be only a structural one during their synthesis of holding the A and B chains of insulin in the correct conformation, but recent evidence has pointed to a possible physiological role for the peptide.

1:2 Morphology of the pancreatic islet cells

In man, the islets of Langerhans are composed of several distinct cell types which secrete different peptides. The cells all have a direct access to the vascular supply and are well innervated. The major islet cell types are the insulin (B cells), glucagon (A cells), somatostatin (D cells) and the pancreatic polypeptide (F cells) producing cells (Munger, 1981). Other cells have been identified but they have not as yet been completely elucidated. These islet cells appear in anastomosing cords, are cuboidal or columnar and the nucleus is present in the base of the cell. The endocrine cells are found in definite areas within the islets. In a cross-section of a human pancreatic islet the B cells occupy the central position with the A, D and F cells being mostly in the periphery (Orci and Unger, 1975, Orci et al. 1976, Fig 1:2). This peripheral area has been shown not to be homologous, since marked regional differences have been reported in the distribution of the A and F cells. In the mammalian pancreas the F cells are concentrated in small regions of the lower dorsal part of the head whilst areas rich in A cells are found in the body and tail (Orci et al. 1976). This difference in the distribution of the A and F cells could stem from the genesis of the pancreas which is formed by the fusion of two distinct pancreases, the so called dorsal and ventral pancreas (Pictet and Rutter, 1972).
Fig. 1:1 Schematic diagram of the biosynthesis of human insulin and C-peptide from preproinsulin

Preproregion → Proteolytic cleavage

Preproinsulin → Proinsulin

Insulin

C-peptide (31 amino acid in humans)
A or F cells Glucagon or pancreatic polypeptide
D cells Somatostatin
B cells Insulin

Fig. 1:2 Schematic representation of an islet of Langerhans shown distribution of glucagon, somatostatin, pancreatic polypeptide, and insulin containing cells. Islet-cell types for which no positive function has yet been established are omitted.

(Modified from functional subdivision of islets of Langerhans and possible role of D cells by Orci and Unger, 1975).
Junctions have been demonstrated between A and B cells as well as between individual B cells (Orci, 1974, 1975). These junctions are thought to enable the transfer of ions and small molecules between the cells and have been shown to be modulated according to the functional activity of the cells (Meda et al. 1978, Orci, 1976).

1:3 Evolutionary development of pancreatic islets

Studies using immunocytochemical techniques have enabled some information to be obtained on the evolution of pancreatic cells. As far as possible, information obtained using this technique, has been supplemented by radioimmunoassays of acid ethanolic extracts of the same tissues.

In the invertebrates there is neither islet organs or any exocrine pancreatic gland. Insulin-like immunoreactivity has been located in the cells of the gastrointestinal mucosa of invertebrates such as mussels and amphioxus (Falkmer et al. 1973, Fig 1:3). Somatostatin, glucagon and gastrin-like immunoreactivities have also been located in the gut cells of amphioxus.

A separate islet organ developed from the bile duct, but with no exocrine pancreas, appears for the first time in primitive vertebrates such as the lamprey and hag fish. Somatostatin and insulin occur in the bile duct mucosa and the islet parenchyma, the insulin producing cells being the most abundant in the islet organ. No glucagon producing cells occur in either location, they still remain exclusively in the gut mucosa (Falkmer and Osberg, 1977).

The first evidence of a discrete pancreas is observed in holocephalan fish e.g. the ratfish. It is intimately associated with the spleen and widely separated from the alimentary tract. A long slender duct connects the pancreas to the gut lumen. The islets contain insulin, somatostatin and glucagon cells. Pancreatic polypeptide cells are present in the gut mucosa and pancreatic duct epithelium.
MILLIONS OF YEARS AGO

RADIATA

CLASS | SUB-PHYLUM | PHYLUM

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<td>Chondrichthyes</td>
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<td>Osteichthyes</td>
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<td>Amphibia</td>
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<td>Vertebrata</td>
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<tr>
<td>Reptilia</td>
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<tr>
<td>Aves</td>
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<tr>
<td>Mammalia</td>
</tr>
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Fig. 1:3
A simple evolutionary tree, classifying on embryological grounds into two major evolutionary lines, protostomian and deuterostomian

(From Marks V and Morgan L M Gastrointestinal hormones In Molecular Aspects of Medicine, 1982)
At the next evolutionary level, that of the cartilaginous fish e.g. sharks and rays, the pancreas is close to the alimentary canal. All four islet hormone secreting cells are present in the islets of Langerhans. Somatostatin, pancreatic polypeptide and glucagon secreting cells are also found in the mucosa of the alimentary canal (Falkmer and Stefan, 1978). This pattern is similar to that found in higher vertebrates.

1:4 The B cells (beta cells)

As long ago as 1890 Von Mering and Minkowski demonstrated in dogs that diabetes could be caused by pancreatectomy. Later Banting and Best (1922) successfully extracted insulin from dog and beef pancreases. They demonstrated that insulin caused a rapid amelioration in the diabetic state of the animals. The chemical nature of insulin was a more elusive problem and not until 1951 was the amino acid sequence determined (Sanger and Tuppy, 1951). Ryle et al. (1955) established its two-chain structure. Lacy (1959) using electron microscopy and fluorescent antibody techniques demonstrated that insulin was stored and synthesised in the B cell. In 1967 Steiner and Oyer isolated a single polypeptide chain precursor of insulin which could be converted to insulin and C-peptide. They named the peptide proinsulin.

1:4:1 Biosynthesis of Insulin and C-peptide

Preproinsulin has since been identified as the initial insulin precursor which is processed to proinsulin and finally to insulin (Figure 1:1, Chan and Steiner, 1977, Patzelt et al. 1978). The 'pre'region on this protein varies in length between 20-30 amino acid residues depending on the species. A high proportion of these amino acids are hydrophobic. In humans, rats and chickens, this region consists of 24 amino acids. Conservation of the initial 14 residues of this region is observed in mammals and birds. Milstein et al.(1972) and Blobel and Doberstein (1975) proposed that the 'pre'region assists in the formation of the ribosome membrane junction leading to the release and segregation of the polypeptide and because of this, the secondary structure of the region is thought to be important for its function (Schecter et al. 1979, Fig 1:4).
Figure 1.4 Proposed mechanism for segregative transfer of presecretory proteins across the membrane of the rough endoplasmic reticulum (from Steiner D, Duguid JR, Patzel C, Chau S, Quim P; Labracheu A, Hasting R. In: Proceedings of the Symposium on Protein, Insulin, and C-peptide; Tokushima, 1978.)
Proinsulin is generated from preproinsulin by proteolytic elimination of the preregion. Proinsulin was discovered by Steiner and his colleagues (Steiner and Oyer, 1967, Steiner et al. 1969) using pancreatic islet cell tumour slices and radioactively labelled amino acids. It was initially isolated from crystalline pork insulin (Steiner and Oyer 1967) and the amino acid sequence determined by Nolan and Margoliash (1969).

Proinsulin is transported from the ribosomal site of biosynthesis on the rough endoplasmic reticulum to the golgi region. This energy dependent process takes approximately 15-20 minutes. Once the proinsulin has reached the golgi apparatus it is packaged into secretory granules where it undergoes proteolytic cleavage (Fig 1:5). This process has been reported as having a half-time of about one hour (Steiner, 1967). The protease enzymes responsible for the cleavage of proinsulin causes the release of arginine and lysine in the ratio 3:1. The arginine molecules originate from positions 31, 32 and 65 and the lysine from position 64 on the human proinsulin (Fig 1:1). The major products of this reaction are insulin and C-peptide. A small proportion, approximately 5 percent, remains as proinsulin. Insulin then undergoes crystallization with the zinc ions that are present in the granules. An additional cleavage of proinsulin has been reported to occur in rats due to chymotrypsin-like enzyme (Tager et al. 1973). It would appear unlikely that this chymotrypsin-like cleavage occurs in all animals due to the large variation in the primary sequence of C-peptide. The products of proteolysis are secreted by emiocytosis. This involves the migration of the granules to the B cell membrane, dissolution of the membrane at the points of contact and extrusion of the granule contents. This release process is modified by a number of factors such as oxygen and calcium ion concentration (Curry et al. 1968, Rubenstein, 1979). Oxygen consumption rises as insulin secretion is stimulated by exposing islets to high glucose concentration and ATP turnover is simultaneously increased. Studies have shown that calcium ions accumulate in the islets after stimulation with glucose (Malaisse et al. 1978) although the precise nature of its action remains to be elucidated.
Fig. 1.5 Schematic summary of the insulin biosynthetic machinery of the pancreatic beta cells. RER, rough endoplasmic reticulum; MV, microvesicles (From Steiner and Rubenstein, In: Proceeding of the Eighth Midwest Conference on Endocrinology and Metabolism, 1973)
1:4:2 The primary sequence of insulin and C-peptide

The amino acid sequence of insulin in a number of species has been determined. In man, insulin is made-up of a 30 amino acid B chain which is joined by two disulphide bridges to the A chain which contains 21 amino acids. The structure of the A and B chain is highly conserved in mammals and birds except in the rodent group which contains guinea pigs and coypus. The A chain has a small cone of amino acids in the middle of the chain which are divergent.

The primary structure of C-peptide is extremely diverse. It contains no aromatic residues and consequently does not absorb light at a wavelength of 276nm (Tager and Steiner, 1972). The majority of amino acids in the human C-peptide chain are neutral. Human C-peptides contain no basic residues and only 17 percent of the residues are acidic. The primary structure of C-peptide not only differs between species in the amino acid composition but also in the number of amino acids present. It has, however, maintained a length of between 26 and 35 residues throughout an evolutionary span of approximately 500 million years (Steiner et al. 1975, Steiner, 1978).

1:4:3 Biological function of C-peptide

During the biosynthesis of insulin the connecting peptide fulfills an important biological role by facilitating the formation of the correct secondary and tertiary structure of the hormone (Steiner et al. 1968). The peptide functions by aiding the folding of proinsulin so that the A and B chains are able to form the correct sulphydryl bridges in proinsulin. Studies on the folding of proinsulin have shown that a smaller peptide was sufficient to allow the formation of insulin in as much as the gap spanned by the connecting segment in the fully folded proinsulin is only 8-10 Ångströms (Blundell et al. 1972). Further studies supporting this conclusion came from the work of Blundell et al. (1978) who investigate insulin growth factors I and II (IGF I and II) which have an 11 amino acid region homologous to the proinsulin connecting segment. They showed that this 11 amino acid region was sufficient to span the gap between the ends of the
region corresponding to the insulin A and B chains. This would suggest that C-peptide, although required for the formation of insulin, may serve some other function unrelated to this structure-making role. Snell and Smyth (1975) have suggested that C-peptide covers and protects the biologically active site of proinsulin thus accounting for its low biological activity. A further suggestion has been proposed by Patzelt et al. (1978) that C-peptide functions as a spacer which makes the proinsulin molecule long enough to span the ribosome membrane junction.

Several other physiological roles have also been suggested. Wojcikowski et al. (1977) demonstrated that C-peptide had some inhibitory effect on glucose stimulated insulin release and Dryburgh et al. (1980) demonstrated that fat stimulated GIP release was inhibited by exogenous Gpeptide in the rat. These studies are described more fully in Chapter V where further work undertaken to elucidate the role of endogenous C-peptide on fat stimulated GIP release is described.

**1:4:4 Insulin secretion**

The most important stimulus to insulin secretion in man is glucose. Certain amino acids, e.g. leucine and arginine, also stimulate insulin release under physiological conditions independent of changes in blood glucose levels (Fajans and Floyd, 1972). Medium chain triglycerides and ketone bodies have been shown to stimulate insulin release in both man and animals. The pattern of insulin release from the B cell following glucose stimulation is biphasic (Grodsky et al. 1967), the first phase starting after 1 minute and lasting approximately 5 to 10 minutes. This is then followed by a sustained and more prolonged increase in insulin secretion over the next 30 to 60 minutes. A higher rise in plasma insulin occurs after oral than after intravenous administration of glucose. An incretin factor released by the gastrointestinal tract plays a major role in enhancing glucose-induced insulin secretion (McIntyre et al. 1964).
A number of hormones have been shown to stimulate insulin release. Excess circulating growth hormone and corticoids such as are present in acromegaly or Cushing's syndrome markedly enhance insulin secretion. Placental lactogen, progesterone and estrogen all play a role in enhancing insulin secretion in the latter half of pregnancy. Whether these hormones act indirectly by inducing a state of insulin resistance, or directly remains to be elucidated. Insulin release has been demonstrated by Samols et al. (1965) after an injection of glucagon.

Substances which react at the receptor level have been shown to affect insulin release. Adrenaline inhibits insulin release by acting on the alpha cell receptor (Porte, 1967). Adrenaline is both an alpha and beta adrenergic stimulator. When the adrenergic action is blocked with phenolamine the beta adrenergic stimulating action persists. Isoprenaline enhances insulin release by acting on the beta cell receptor.

1:4:5 Metabolism

Insulin is found not only in plasma but also in bile, lymph and urine in low concentrations. The half time disappearance of exogenous porcine insulin is 3 to 5 minutes in man (Samols and Marks, 1966, Orskov and Christensen, 1966, Turner et al. 1971). The metabolic clearance rate of bovine insulin in the rat has been calculated at 16.4±0.4 ml/min (Katz and Rubenstein, 1973). Many tissues accumulate insulin in small amounts but the major site of uptake and degradation is the liver (Samols and Ryder, 1961, Kanazawa et al. 1966) and kidney (Rubenstein et al. 1975). The liver removes approximately 20 to 50 percent of the insulin after a single transhepatic passage. This hepatic uptake mechanism for the extraction of insulin can be saturated (Rubenstein et al. 1972). In the kidney, insulin is filtered through the glomerulus and reabsorbed by the proximal tubular cell. The kidney also extracts insulin from the peritubular circulation (Rabkin et al. 1978). Of all the insulin removed by the kidney only five percent is excreted in the urine and the remainder is degraded by specific enzymes. Two
enzyme systems have been implicated in insulin degradation. Burgen et al. (1972) found an insulin specific protease in the cytosol of many tissues particularly in the liver, pancreas and kidney. A second enzyme, a glutathione-insulin transhydrogenase, catalyses the reductive cleavage of the insulin disulphide bonds by glutathione with the liberation of the intact A and B chain (Varandani, 1972). These chains are then rapidly degraded to small peptides. Proinsulin comprises only a small proportion of the secretory product of the B cell in normal subjects. No evidence has been found of the conversion of proinsulin to insulin and C-peptide in plasma. Starr and Rubenstein (1974) demonstrated in man that following the removal of an insulin producing adenoma proinsulin had a half-life of 17.2 minutes. Proinsulin is removed from the circulation by the kidney, hepatic extraction being almost negligible. (Katz and Rubenstein, 1973). The fractional urinary clearance of this peptide is 0.6 percent indicating that more than 99 percent of the amount filtered is sequestered and degraded in the kidney. Many workers have investigated the disappearance of C-peptide from the circulation both in man and rodents. The major site of removal of C-peptide from the body is the kidney (Katz and Rubenstein, 1973). The liver removes only a very minor amount (Canivet and Krebs, 1980). As with insulin and proinsulin, C-peptide is avidly absorbed from the lumen by the kidney cells where it is degraded (Katz and Rubenstein, 1973). The half-life of endogenous and exogenous C-peptide in man has been reported by a number of workers and values range from 11 to 33 minutes (Horwitz et al. 1973, Munemura et al. 1974, Kutuza and Maksuda, 1976, Krause, 1977, Faber et al. 1978). The wide range of values for the immunological half-life of C-peptide in plasma reported may be due to the different experimental conditions under which they were determined. Most C-peptide radioimmunoassays crossreact with proinsulin in varying degrees. Extraction of proinsulin from the serum prior to the
measurement of C-peptide would shorten the apparent half-life found when measuring disappearance rate using a C-peptide assay and should, therefore, be a more reliable estimate of actual circulating half-life. Additional studies in man with natural or biosynthetic human C-peptides are needed to resolve this problem. Studies in animals are of limited value for extrapolation to the human situation since many species of C-peptide are still unavailable in adequate amounts.

The urinary excretion of C-peptide is 5% of the total quantity secreted from the pancreas as compared with a value of 0.1% for insulin. The increased urinary clearance had been made use of by several workers to measure C-peptide levels in urine as an indicator of B cell activity over prolonged periods (Kuzuya et al. 1976, Horwitz et al. 1977, Meistas et al. 1981).

1:5 The A cells (alpha cells)
Glucagon, secreted by the pancreatic A cells, was not isolated until 1955 (Staub et al. 1955). Glucagon is a 29 amino acid peptide stored in the islets in an alpha helix form and like insulin it has been shown to be synthesised from a prohormone. This prohormone, glicentin, is believed to consist of 69 amino acids in pigs (Thim and Moody, 1981). Proglucagon has C and N terminal extensions which undergoes enzymic cleavage, similar to proinsulin, at sites with two basic amino acid residues (Tager and Markese, 1979). There is a remarkable degree of conservation of the amino acid sequence of this hormone in mammals and birds. Evidence of heterogenicity of circulating immunoreactive glucagon has been reported (Valverde et al. 1974, Weir et al. 1975). The biologically active hormone has a molecular weight of 3500 daltons. Other peptides with glucagon-like immunoreactivity have been identified in gut extracts with molecular weight ranging from 2000-160,000 daltons.
The D cells
The D cells of the pancreas secrete somatostatin. Somatostatin was initially isolated from ovine hypothalami (Burgus et al. 1973). Mammalian somatostatin contains 14 amino acids with a molecular weight of approximately 1642. It has been identified in various regions of the central nervous system, the gastrointestinal mucosa, several endocrine organs and the blood (Parsons et al. 1976). Prosomatostatin has been isolated from angler fish islets and consist of approximately 100 amino acids (Shield, 1980). As with the other prohormones prosomatostatin has areas which contain amino acids which are sensitive to enzyme cleavage.

The F cells (PP cells)
The F cells of the pancreas secrete pancreatic polypeptide. These cells are located at the periphery of the islets and are also present in the exocrine areas of the pancreas (Gersell et al. 1979). Mammalian pancreatic polypeptide is a 36 amino acid structure with an approximate molecular weight of 4200. It is also synthesised initially as a larger prohormone. The primary structure of various pancreatic polypeptides show very little homology with other pancreatic and gastrointestinal hormones.

Gastric inhibitory polypeptide (GIP)
A much greater insulin release is observed in response to oral as opposed to intravenous glucose (McIntyre et al. 1964). Unger and Eisentraut (1969) proposed the term enteroinsular axis to describe a regulatory system in which insulin secretion from the pancreatic islets was modified to some extent by gastrointestinal hormones. The gastrointestinal hormone, GIP, appears to be one of the factors involved in this regulatory system.

The pancreatic islet cells probably all originated in the gut mucosa and migrated to the pancreas in the course of evolution. The GIP cells present in the gut mucosa have also been identified in a number of early vertebrates. The absence of GIP cells in the sea squirt which has insulin and somatostatin cells (Falkmer et
GIP is an acronym for gastric inhibitory polypeptide initially named because of its ability to inhibit gastric acid secretion. However, GIP has subsequently been shown to stimulate insulin secretion (Brown et al. 1975). GIP appears to fulfill all the criteria for a physiological endocrine stimulator of insulin secretion, the so-called incretin effect. Creutzfeldt (1979) proposed several criteria that a substance had to meet before it could be called an incretin. It had to be an endocrine hormone, released in response to ingestion of nutrients in particular carbohydrates and stimulate insulin secretion in the hyperglycaemic state. The major physiological role of GIP appears to be that of stimulating insulin secretion. GIP levels are increased after ingestion of carbohydrate, fat and amino acids but insulin levels are increased only after carbohydrate and some amino acids. This would suggest that some other factor or factors are controlling the release of insulin by GIP. Primary among these factors that may determine the insulin secretory responsiveness of the B cell to GIP is the plasma glucose concentrations (Pederson et al. 1975, Andersen et al. 1978, Verdonk et al. 1980). Studies in man and rats have shown that the plasma glucose concentration must be above 5.5 mM before GIP is able to exert any insulinotropic activity. The release of GIP in response to nutrients has been shown to be affected by several factors. Nutrient absorption has been shown to be essential for the release of GIP. Studies with non-dietary fibres such as guar, which slows down the rate of nutrient absorption, have been shown to affect the GIP response to a meal (Morgan et al. 1979, Jenkins, 1980). The molecular configuration of sugars appears to affect GIP release (Sykes et al. 1980). Reiser et al. (1980) demonstrated that dietary adaptation affects GIP release. They maintained subjects on diets containing 30 percent starch or sucrose. An increased GIP
response to a sucrose load was observed in those subjects maintained on the sucrose diet. They suggested that this was due to induction of a specific intestinal enzyme e.g. sucrase and a consequent increase rate of absorption of glucose and fructose.

1:9 Estimation of C-peptide levels

The development of the C-peptide radioimmunoassay has enabled the measurement of this peptide to be made in a variety of biological fluids. The ability to measure circulating C-peptide levels has been extremely useful in the in vivo investigation of B cell function. However, difficulties have been encountered in the development and validation of C-peptide radioimmunoassays and these are discussed below.

1:9:1 Methodology

Radioimmunoassays are based on the principle of competitive inhibition whereby unlabelled hormone competes with a radiolabelled hormone for binding to a specific antibody (Yalow and Berson, 1959, Fig. 1:6). The amount of labelled hormone which is bound to the antibody is dependent on the concentration of unlabelled hormone. The higher the concentration of unlabelled hormone the less labelled hormone is bound to the antibody. The concentration of hormone in an unknown plasma or serum sample is determined from the degree of binding of labelled hormone observed in the sample by comparison with that observed in standard solutions, containing known concentration of the hormones. Many procedures are necessary in the validation of a radioimmunoassay. One of the essential procedures is the demonstration that the endogenous and standard hormone react with the antiserum identically under the conditions of the assay, and this has been a particular problem in the C-peptide radioimmunoassay.

1:9:2 The problems associated with measuring human C-peptide by radioimmunoassay

In 1970 Melani et al. described a double antibody radioimmunoassay for human C-peptide. However, several major problems have subsequently been
Fig. 1:6  Diagramatic representation of reactions occurring in a radioimmunoassay

Free labelled Antigen  Specific antibody  Labelled antigen antibody complex

Ag*  +  Ab  ⇌  Ag* - Ab

+  

Ag unlabelled antigen

↓

Ag-Ab unlabelled antigen-antibody complex
encountered specific to the radioimmunoassay of C-peptide.

The large degree of species variation in the primary structure of C-peptide has prevented the use of C-peptide obtained from other species for the development of human C-peptide radioimmunoassays. Human C-peptide has to be obtained either from natural sources or synthesised in order to develop the assays. Melani et al. (1970) and later Heding (1975) described assays using natural C-peptide extracted from human pancreases. However, Markussen et al. (1971) found that the amount of human C-peptide isolated from pancreases was extremely small and not practicable for any large scale production of the peptide. Human C-peptide, therefore, had to be synthesised chemically before it could become more readily available (Naithani, 1973, Yanaihara et al. 1974, 1978b, Igano et al. 1981). However, different forms of C-peptide of varying chain length have been synthesised. Several assays using antisera raised against synthetic human C-peptide have been reported in the literature (Kaneko et al. 1974, Beischer et al. 1976, Faber et al. 1976, Kuzuya et al. 1976). Although natural C-peptide has only 31 amino acids some peptides have been synthesised containing extra amino acids and functional groups (Naithani, 1973, Yanaihara et al. 1974). Crude human proinsulin components have also been used to develop a C-peptide radioimmunoassay (Heding et al. 1974).

The wide variation in fasting levels of C-peptide may, in part, be due to the different sources and varying purity of the peptides. Values of 0.9-3.5 µg/Litre have been reported in normal non-obese subjects (Block et al. 1972, Kaneko et al. 1974, Heding and Rasmussen, 1975, Heding, 1975). The development of an international standard should help reduce this variability. The first international standard, a formyl lysine 34 amino acid C-peptide, was unfortunately found to run non-parallel in most C-peptide assays (Carrick et al. 1980). These workers highlighted the need for an international standard by reporting the wide variety of C-peptide standards used by the participants in the study.

Difficulties have been encountered in raising an antiserum against C-peptide.
Human C-peptide has a molecular weight of 3025 and has to be coupled to a larger protein such as albumin in order to be immunogenic. Even when coupled to a carrier protein its immunogenicity is poor. Markussen (1971) suggested that the poor immunogenicity of C-peptide is due, in part, to its random coil conformation.

125I used for radiolabelling is incorporated into the tyrosine or histidine residues of a peptide using conventional iodination techniques. A further problem encountered with C-peptide radioimmunoassays is that human C-peptide possesses neither tyrosine or histidine residues and it is necessary to use a synthetic C-peptide into which a tyrosine residue has been incorporated.

Proinsulin is the major known crossreactant in the C-peptide assay because it contains the complete C-peptide molecule within its amino acid sequence. The cross-reactivity varies between 15-66 percent in C-peptide assays reported in the literature (Melani et al. 1970, Kaneko et al. 1974, Caygill et al. 1980). High proinsulin levels in plasma would affect the C-peptide result depending on the antiserum used in the assay. Basal proinsulin levels have been reported to range from undetectable to approximately 300 ng/Litre in normal non-obese subjects (Heding, 1977, Rainbow et al. 1979). Proinsulin levels are, therefore, normally less than 5 to 10 percent of those of C-peptide. However, because of differences in their half lives after oral glucose proinsulin levels rise slowly and peak later than insulin (Rubenstein et al. 1977). After 50g oral glucose load proinsulin levels increase by 8 fold from the fasting levels (Rainbow et al. 1979). Obese subjects with hyperinsulinaemia have raised fasting levels of proinsulin and a greater absolute increase after glucose compared with normal weight subjects (Melani et al. 1970). However, the proinsulin and insulin levels occur in relatively the same proportion in obese subjects as in normal volunteers (Gordon and Roth, 1969). High proinsulin levels have been reported in patients with insulin producing tumours (Sherman et al. 1972, Rubenstein et al. 1974, Rainbow et al. 1979). In this situation the proportion of insulin to proinsulin has been
shown to be extremely variable. However, by no means do all patients with insulin producing tumours have elevated percentages of proinsulin.

Proinsulin extraction and subsequent measurement of C-peptide levels in serum samples has been reported (Melani et al. 1970, Heding, 1975). Initially proinsulin was separated from C-peptide in sera by column chromatography (Melani et al. 1970). This technique is time consuming and unsuitable for handling large numbers of samples. Removal of proinsulin has also been accomplished by incubating patients' sera with sepharose-coupled insulin antibodies. These bound both insulin and proinsulin enabling C-peptide to be determined directly in the supernatant (Heding, 1975).

The heterogeneity of C-peptide in plasma has been investigated by a number of groups (Kuzuya et al. 1977, 1978; Kakita, 1980) Several workers have reported that different antisera used in the same assay system react to different degrees with the same plasma sample (Heding, 1975, Kuzuya et al. 1978). Variable cross-reactivities to various molecular forms of C-peptide has been suggested as a possible explanation of these differences. Kuzuya et al.(1977) reported the detection of a low molecular weight form of C-peptide which increased during storage of the samples. Recently Kakita 1980 detected a large molecular weight form of C-peptide which had the same immunoreactivity, in their assay, as the 3025 molecular weight C-peptide. Beck et al.(1981) suggested the existence of C-peptide in plasma which is bound to protein. This occurrence is relatively common and has been reported for insulin (Vallaume et al. 1981).

Difficulties have been encountered when measuring residual B cell activity in insulin treated diabetics. Insulin antibodies usually present in the sera from insulin treated diabetics bind not only insulin but proinsulin (Block et al. 1972). The half-life of proinsulin is increased when it is complexed with antibodies.
Finke et al. (1974) have reported that the proinsulin levels may accumulate in such diabetics far in excess of circulating C-peptide levels. The C-peptide levels measured in these diabetics, although indicating B cell activity, cannot therefore be compared with values obtained from normal subjects. Kuzuya et al. (1977a) precipitated the insulin antibodies in diabetic sera with polyethylene glycol and measured C-peptide levels in the supernatant. This method removed all the proinsulin bound to the antibodies but circulating unbound proinsulin remained. C-peptide levels ranged from 2.1-28.7 µg/Litre in the insulin treated diabetics, after polyethylene glycol, levels ranged from 0.5-4.3 µg/Litre.

1:9:3 C-peptide levels in normal non-obese subjects

Reported fasting C-peptide levels in healthy subjects range between 0.9 and 3.5 µg/Litre (Block et al. 1972, Kaneko et al. 1974, Heding, 1975, Heding and Rasmussen, 1975, Beischer et al. 1976, Beyer et al. 1977). Beyer et al. (1977) reported that C-peptide levels, like insulin, were dependent upon body weight. The larger the increase in body weight over ideal weight the higher the C-peptide levels.

Stimulation of the B cell results in a slower rise in circulating C-peptide levels in the peripheral circulation compared to insulin. Peak C-peptide levels occur one hour after oral glucose (Kuzuya et al. 1976) and peak levels are approximately 5 to 7 times the fasting values after a 100g glucose load (Block et al. 1972). C-peptide remains elevated for considerably longer than insulin because of its longer half-life.

Several workers have investigated the measurement of C-peptide in urine (Kuzuya et al. 1976, Horwitz et al. 1977, Meistas et al. 1981). Values for the twenty-four hour excretion of C-peptide vary between 36-81 µg (Kuzuya et al. 1976, Horwitz et al. 1977, Meistas et al. 1981). This variability could be attributed at least, in part, to intrinsic differences in the assay systems including the preparation of the standard C-peptide or antiserum. The urinary clearance of C-peptide is much higher than that of insulin. The 24 h urinary
clearance of C-peptide has been calculated to be between 4 and 20 percent (Kuzuya et al. 1976, Horwitz et al. 1977, Meistas et al. 1981). Except for patients with renal failure, urine C-peptide excretion reflects the daily B cell secretory activity fairly well. Weisinger et al. (1977) have investigated patients with varying degrees of renal function. They demonstrated a strong correlation between kidney function and the C-peptide excreted when the kidney function was less than 50 percent of a healthy kidney. They suggested that renal function should be assessed if the C-peptide levels in the circulation or urine were going to be used diagnostically.

C-peptide insulin ratios have been recently used as an indicator of hepatic extraction of insulin. Sando et al. (1980) found that hepatic insulin extraction was decreased in both obese and non-obese diabetics compared with normals. This ratio was also altered in subjects with moderate degrees of glucose tolerance (Malmquist et al. 1981).

1:9:4 The clinical importance of C-peptide measurements

Measurements of C-peptide levels have been useful in the evaluation of several clinical states.

Residual B cells activity has been assessed in diabetics by measuring C-peptide levels. In newly diagnosed insulin-requiring diabetics the highest C-peptide levels were associated with the lowest requirement for exogenous insulin (Faber and Binder, 1977). However, in long-term diabetics this relationship is not so clear and may partly be due to the presence of circulating insulin antibodies. Absence of measurable levels of C-peptide have been reported in some diabetics with good metabolic control (Rubenstein et al. 1981). The importance of good control in diabetics has been studied using the artificial pancreas and monitoring C-peptide levels. Mirouze et al. (1978) found that C-peptide levels increased and insulin doses were reduced compared with a control diabetic group.

Many clinical situations besides diabetes have made use of the measurement of C-peptide levels. An important use has been in the diagnosis of various
hypoglycaemic states notably the measurement of C-peptide levels with insulin
producing tumours. Turner and Heding (1977) and Sefvice et al. (1977)
demonstrated impaired suppression of the B cell by monitoring C-peptide levels
during hypoglycaemia induced by exogenous insulin in patients with insulinosmas.
The evaluation of results obtained during an insulin stress test is extremely
important in determining whether a patient has an insulin producing tumour
(Marks., 1981).

The clinical problem of factitious hypoglycaemia due to administration of insulin
can be clearly diagnosed with the measurement of low C-peptide and high insulin
levels. In the past the presence of insulin antibodies was diagnostic of insulin
administration. However, antibodies will only be present after repeated insulin
injections and also several cases of insulin autoantibodies have been reported
(Hirata et al. 1972, 1974) without previous insulin injections.

After removal of the pancreas due to nesidioblastosis or certain carcinomas,
C-peptide measurement has been useful to indicate the presence of any residual
pancreatic tissue. If an insulin producing tumour has been removed from the
pancreas long term C-peptide monitoring can be of use in detecting the
recurrence of the tumour or the presence of functioning metastases.

1:10 Project

A human C-peptide radioimmunoassay was developed using a synthetic thirty-one
amino acid C-peptide. Several problems were encountered when initially
developing the assay such as difficulties in producing an antiserum and a high
specific activity tracer. The lack of an international human C-peptide standard
made it essential to establish fasting and stimulated C-peptide values in normal
non-obese subjects in the assay system in order to assess clinical samples. The
diagnostic value of human C-peptide estimations has been evaluated in certain
clinical conditions.

Recent studies using exogenous C-peptide (Dryburgh et al. 1980) suggested that
C-peptide may have a physiological role and this was, therefore, investigated in
the rat. Due to the species variability in the primary sequence of C-peptide a specific rat C-peptide radioimmunoassay had to be developed. Endogenous C-peptide stimulated by glucose and tolbutamide was found to inhibit fat stimulated GIP release using the gut perfusion technique. However, this technique involves extensive handling of the gut which could have affected the results. The technique of orally dosing rats with fat was, therefore, investigated. Rats were orally dosed with fat for four days prior to investigation (pretreated) in order to increase their GIP response to fat. Endogenous C-peptide was found not to inhibit fat stimulated GIP release in these animals. A shortage of insulin antiserum prevented further investigation using this method in rats maintained on normal laboratory food (untreated). Studies using a known inhibitor of fat stimulated GIP release, insulin, in pretreated and untreated rats was therefore carried out. Fat pretreatment prevented exogenous insulin from inhibiting fat stimulated GIP release in the rat. Studies in man maintained on high and low fat dietary regimens also demonstrated that fat-stimulated GIP release was inhibited by exogenous insulin only when the subjects were on a low fat diet.
CHAPTER II

A RADIOIMMUNOASSAY FOR THE MEASUREMENT OF IMMUNOREACTIVE
HUMAN C-PEPTIDE
2:1 Introduction
Since the introduction of radioimmunoassay (RIA) in 1959 (Yalow and Berson, 1959) this analytical method has found a wide application in the quantitative determination of low concentrations of proteins in biological fluids (Hunter, 1973, Skelley et al. 1973). Two major advantages of radioimmunoassay over bioassay are sensitivity and specificity. However, in order to achieve this it is necessary to possess antibodies which have a high avidity and specificity. The production of antisera is determined by a number of factors. These include conjugation of the hapten to a carrier protein, the use of adjuvants, the choice of species and route of administration.

The immunogenicity of a substance is extremely important in the production of antisera. Immunogenicity is related to molecular weight and rigidity of structure of a substance. Low molecular weight substances, below 5000 daltons, have to be conjugated to large molecules before they will elicit an immune response. A variety of chemical procedures are available for the covalent linkage of such substances to large molecules (Nisonoff, 1967). Care must be taken when conjugating a large carrier protein not to distort or mask the smaller molecules or haptens, since the antibody produced will reflect this structure. The carrier protein itself does not have to be immunogenic. Immunogenic or non-immunogenic carrier proteins may be used with an equal expectation of success (Hum and Landon, 1971).

The use of an adjuvant, such as mineral oil, has been shown to increase the chances of an antibody response. The adjuvant is also mixed with an immunogen to form a stable emulsion. Adjuvants are believed to work by releasing immunogens slowly, thereby, preventing rapid uptake into the circulation and degradation of the immunogen by proteolytic enzymes. The adjuvant causes stimulation of the reticuloendothelial system by facilitating phagocytosis and causing formation of a local granulomatous lesion. This may act as a focus for antibody production. The use of adjuvants has allowed the
sustained release of antigens over a period of some weeks and careful monitoring of antibody production is, therefore, required to determine when to reimmunise an animal.

The immune response of an animal to a particular compound is genetically determined. Unless one is aware of a particular strain of animal which responds well to a particular immunogen it is probably best to choose randomly bred animals.

The route of administration is important. Evidence has indicated that routes affecting the lymph nodes may increase the effectiveness of immunisation (Hurn and Landon, 1971).

The sensitivity of a radioimmunoassay depends on a number of factors, the two most important being the avidity of the antibody and the specific activity of the tracer. The avidity of an antiserum can be regarded as synonymous with its sensitivity in the assay system. Construction of standard curves is an essential step in determining the suitability of an antiserum. Provided the specificity of the antiserum is suitable the best antiserum is usually that which provides the greatest sensitivity. Sensitivity in general terms can be determined as the least amount of unlabelled antigen that can be detected with confidence. The inherent sensitivity of any given antiserum is dependent on its equilibrium constant, $K$. The equilibrium constant characterises the reaction of the antigen with the predominant order of antibody binding sites, the higher the $K$ values the more sensitive is the assay. Having obtained an antiserum with suitable characteristics it is then necessary to employ a tracer. This is a labelled antigen that does not limit the sensitivity of the assay by its undesirable immunochemical properties or by its inappropriately high concentration (low specific activity).

The production of a high specific activity tracer is, therefore, also required if a radioimmunoassay is to have the ability to measure picogram quantities. Thus $^{125}$iodine is generally used in peptide RIA because of its relatively long half-life (60 days) compared with $^{131}$iodine (half-life 8 days).
The iodination reaction consists of a substitution of iodine, produced by oxidation of iodide, into an ionized tyrosine or histidine residue in the protein. The most common methods of oxidising the Na$^{125}$I involve using chloramine T (Hunter and Greenwood, 1962) as the oxidising agent or the enzyme lactoperoxidase (Thorell and Johansson, 1971). The enzymatic method does not directly expose the peptide to large amounts of oxidising agent and is, therefore, less likely to cause damage to the protein.

Many methods are available which separate the iodinated protein from the unreacted iodide and degradation products, the most commonly used being gel filtration and ion exchange systems. It is important to obtain a tracer free from all radioactive degradation products since this results in a higher specific activity. The shelf life of the tracer depends on three factors. Firstly, the radioactive decay of $^{125}$I; secondly, whether the peptide contains two or more iodinated tyrosine residues and finally, the slow loss of immunoreactivity which occurs during storage (Hunter, 1971).

To enable groups of workers measuring levels of a certain peptide to make quantitative comparisons between published results, it is useful to have a common standard. Unfortunately many such standards are not available and many laboratories have to use their own "in house" standard. One disadvantage of these standards is that usually their purity and homogenicity has not been investigated. The use of a common international standard is important. Production of such a standard takes a considerable time, firstly in obtaining sufficient supply of a suitable material and secondly in establishing its purity and stability. In addition, it is essential to ensure that in the radioimmunoassay system it reacts identically to the peptide in endogenous serum samples.

The widespread use of human C-peptide radioimmunoassay has been prevented by the scarcity of human C-peptide for standard and immunogen. Human C-peptide cannot be substituted by animal C-peptides because C-peptides are highly species specific in their primary structure. Human C-peptide, therefore, has to
be used for standard, iodinated tracer and as antigen for the production of antiserum. Natural human C-peptide can only be obtained from normal pancreas and because of the difficulty involved in obtaining this, human C-peptide has been synthesised by a number of workers (Yanaihara et al. 1974, 1978b, Igano et al. 1981). Human C-peptide has a low molecular weight (3025 daltons) and lacks a rigid secondary and tertiary structure (Frank and Veros, 1968; Markussen et al. 1971). These factors may explain its poor immunogenicity.

The production of antiserum to human C-peptide and its use in measuring circulating C-peptide concentrations was first described by Melani et al. (1970). They raised their antisera in guinea pigs by immunization with C-peptide extracted from human pancreases and measured C-peptide in extracted plasma samples. Block et al. (1972) then developed an assay that could measure C-peptide in unextracted plasma samples. Since then many workers (Faber et al. 1976, Heding et al. 1974, Kaneko et al. 1974, Yanaihara et al. 1974, Heding, 1975, Horwitz et al. 1975, Beischer et al. 1976) have developed their own radioimmunoassays using natural or synthetic C-peptide.

Natural human C-peptide has 31 amino acids (Kemmler and Steiner, 1970) but with the synthesis of C-peptide, it has been possible to synthesise the connecting peptide which contains the additional 4 basic amino acid residues which are split off during the conversion of proinsulin to C-peptide (Figure 2:1). A radioimmunoassay using a 35 amino acid connecting peptide for standard and hapten for immunization was developed by Kaneko et al. (1974). They showed that the natural C-peptide and synthetic connecting peptide gave similar crossreactivity with their antiserum. Workers have chosen a variety of animals including rabbits, goats and guinea pigs in which to raise their antisera (Kaneko et al. 1974, Heding, 1975, Beischer et al. 1976). Even though a number of different species and immunization protocols have been used, a high titre antiserum has not consistently resulted.
Figure 2: Human proinsulin covalent structure and cleavage to insulin and C-peptide.
Human proinsulin is the only substance, so far, shown to crossreact in a human C-peptide radioimmunoassay. Proinsulin plasma concentrations are much lower than those of C-peptide in normal subjects the contribution of proinsulin to serum C-peptide immunoreactivity is, therefore, usually very small. However, in patients with islet cell tumours, hypokalaemia or chronic renal failure, the plasma proinsulin levels are increased. To overcome this interference Melani et al. (1970) used an extraction procedure followed by gel filtration for measuring C-peptide levels in serum. The use of sepharose coupled insulin antibodies (Heding, 1975) has also enabled proinsulin to be removed from serum leaving behind the C-peptide.

The iodination of human C-peptide is complicated by the absence of tyrosine or histidine residues in the molecules in which to incorporate the $^{125}$iodine. Human C-peptide has one free amino group, namely that of the n-terminal glutamic acid to which it is possible to attach one molecule of tyrosine by a peptide bond.

The preparation of a suitable standard for the human C-peptide assay has depended upon the availability of materials. Workers developing human C-peptide radioimmunoassays have either used natural or synthetic C-peptides (Melani et al. 1970, Heding et al. 1974, Kaneko et al. 1974, Yanaihara et al. 1974, Heding, 1975, Horwitz et al. 1975, Beischer et al. 1976). Both natural and synthetic C-peptide may undergo some chemical changes during isolation and storage. Contaminants may be present in human C-peptides obtained from both sources. Increased purity can be achieved by use of high pressure liquid chromatography. No international reference standard is, as yet, available for use in the radioimmunoassay.

A radioimmunoassay for human C-peptide has been developed using a synthetic human C-peptide (31 amino acid) as standard and hapten. A detailed description of the development, validation and problems encountered are reported. This study resulted in a radioimmunoassay for human C-peptide which is capable of measuring C-peptide levels in unextracted plasma.
2:2 Materials and Methods

2:2:1 Antiserum Production

Human C-peptide antiserum was prepared using a synthetic human C-peptide (Dr. Geiger, Farbwerke, Hoechst A.G., Frankfurt, West Germany) (Fig. 2:2) as hapten.

(a) Preparation of Immunogen

A solution was made containing 2 mg of egg albumin and 1 mg of synthetic human C-peptide dissolved in sterile distilled water. To this was added 200 μl of 0.25M gluteraldehyde solution. The resulting conjugate was immediately mixed with two equal volumes of complete Freund's adjuvant (Freund, 1951), to give a final C-peptide concentration of 333 μg/ml. The solution was mixed vigorously until a stable emulsion formed.

(b) Immunisation Procedure

Attempts to produce antiserum in rabbits proved unsuccessful with this immunogen. Two Soay sheep were immunised with 500 μg of the synthetic C-peptide. They were immunised intradermally along the neck and back at fifteen separate injection sites and intramuscularly at four sites in the upper legs according to the method of Beischer et al. (1976). Both animals were reimmunised (boosted) at six week intervals with 250 μg of synthetic C-peptide until antiserum of sufficiently high titre was obtained. The antiserum currently used in the assay was obtained from one of these two sheep nine days after the first boost (batch no. MF/5/743-IB).

(c) Antibody Specificity

To determine antibody specificity the crossreactivity of the antiserum with human insulin (Wellcome Foundation Ltd., Beckenham), porcine insulin (Novo Research Institute, Denmark), porcine proinsulin (Novo Research Institute, Denmark), glucagon (WHO First International Standard 69/194), GIP (Dr. J. Brown, University of British Columbia, Canada) and human biosynthetic proinsulin (Eli Lilly and Company Ltd., Basingstoke, Hants) was assessed.
Figure 2:2

Ser - Ten - Gin 31

Molecular weight = 3025

Amino Acid Sequence of Human C-Peptide
A number of synthetic fragments of human C-peptide donated by Professor N. Yanaihara, were tested in an attempt to determine the antigenic determinants of the antiserum. The fragments were:

1) Batch No. NY-TN-8-62 - Fragments 20-31
2) Batch No. NY-TN-8-90 - Fragments 14-31
3) Batch No. NY-TN-8-94 - Fragments 10-31
4) Batch No. NY-TN-8-98 - Fragment 7-31

Synthetic C-peptides obtained from various sources were also investigated to determine their crossreactivity with antiserum batch no. MF/S/743-IB. The synthetic C-peptides were:

1) A 31 amino acid synthetic Human C-peptide - IRE-UK Ltd., High Wycombe
2) A 31 amino acid synthetic Human C-peptide - Novo Research Institute Denmark
3) A 31 amino acid synthetic Human C-peptide (batch no. NY.TN.8.102) Professor N. Yanaihara, Japan.
4) A 35 amino acid synthetic Human "C-peptide" - (batch no. NY.TN.8.86) Professor N. Yanaihara, Japan.
5) A 35 amino acid synthetic Human "C-peptide" - NIBSC Standard 76/561 which has a formyl lysine residue in position 34.
6) NIBSC standard 76/321 batch no. NY.TN.10.52. This C-peptide is synthetic, no other information is available.

2:2:2: Standard

(a) Preparation of Standard

The standard used in the C-peptide radioimmunoassay was the same 31 amino acid, synthetic human C-peptide (Hoechst Ltd., Germany) employed as antigen. The standard was weighed out on a Kahn balance, dissolved in a small quantity of 1M acetic acid and then diluted in 0.05M sodium phosphate buffer pH7.4 containing 0.1% human serum albumin. This was then aliquoted (20 ng/vial), freeze dried and stored at -20°C.
(b) Investigation of standard purity using High Pressure Liquid Chromatography (HPLC)

High pressure liquid chromatography was used to investigate the purity of the Hoechst and NY.TN.10.52 C-peptide standard. Chromatography was carried out at constant pressure using an Altex high pressure liquid chromatography machine equipped with a variable wavelength spectro photometer.

Organic solvents used were HPLC grade. Buffer and salt solutions were made up with Analar or Aristar reagents using double glass distilled water. Compounds for chromatography were freshly dissolved in 0.05M sodium phosphate buffer pH7.4. A 135 x 5 mm ID column was used with a guard column packed with Nucleoside 5-C8 and operated at ambient temperature. A 20 μl (20 μg) aliquot of standard was injected on to the column. Buffer A was 0.1 % trifluoroacetic acid (TFA) and Buffer B was 60 % acetonitrile with 40 % Buffer A. A linear gradient was supplied from 29 % -60 % acetonitrile at a wavelength of 220 nm. The chart speed was 200 mm hr⁻¹. 1 ml aliquots of elution buffer were collected using an automatic fraction collector. To each aliquot was added 1 mg bovine serum albumin (dry) and each was frozen. These aliquots were lyophilized and resuspended in 0.05M sodium phosphate buffer pH 7.4 containing 0.1% HSA. The concentration of human C-peptide in these aliquots was measured by radioimmunoassay.

Hoechst C-peptide (batch no. Op 25/26) was investigated further using an isocratic system. The compound for chromatography was freshly dissolved in 0.05M sodium phosphate buffer pH7.4. The HPLC column used was 150 x 4.6mm ID plus a guard column packed with Hypersil-ODS and operated at ambient temperature. A 10 μl (10 μg) aliquot of sample was injected into the column. The buffer was 27 % acetonitrile/75 % 0.1 % TFA. Absorbance was monitored at a wavelength of 215 nm. The flow rate was 1 ml/min and the chart speed was 200 mm/hr. 250 μl aliquots of the elution buffer were collected using a fraction collector. The samples were lyophilized with BSA, as before, and
resuspended in 0.05M sodium phosphate buffer pH7.4 containing 0.1% HSA. The concentration of human C-peptide in these samples was determined by radioimmunoassay.

2:2:3: Iodination of Tyrosylated human C-peptide

Since human C-peptide contains no tyrosine or histidine residues it is necessary to use a tyrosylated analogue for iodination. Three iodination and purification methods were investigated to obtain a stable iodinated product.

(a) Purification of tyrosylated human C-peptide

High pressure liquid chromatography was used to investigate the purity of the tyrosylated C-peptide (Dr. Geiger, Farbwerke, Hoechst, A.G., Frankfurt, West Germany). The machine and method were identical to that previously described for HPLC of human C-peptide standard.

(b) Iodination methods

(i) Chloramine T iodination

The method used was a modification of the Hunter and Greenwood (1963) method. Tyrosylated C-peptide was weighed out on a Kahn balance and dissolved in a small quantity of 1M acetic acid. The C-peptide was then diluted in a 0.05M sodium phosphate buffer pH7.4 to a concentration of 200 µg/ml. This solution was then aliquoted into plastic autoanalyzer cups. These cups contained 5µg of tyrosylated C-peptide and were stored at -20°C until required. Iodination procedure was as follows:- the reagents were added in the following order to a plastic autoanalyzer cup.

1) 5 µg tyrosylated C-peptide dissolved in 25 µl of 0.05M sodium phosphate buffer pH7.4
2) 25 µl of 0.5M sodium phosphate buffer
3) 18.5MBq (0.5 mCi) in a 5µl aliquot of Na$_{125}$I
4) 2 µg chloramine T dissolved in 10 µl of 0.05M sodium phosphate buffer pH7.4
5) 48 µg sodium metabisulphite dissolved in 200 µl of 0.05M sodium phosphate buffer pH7.4
6) 500 µg potassium iodide dissolved in 5 µl of 0.05M sodium phosphate buffer pH 7.4.

The reactants were mixed throughout this process with a small magnetic flea. The reactants were then loaded on to the purification column.

(ii) Lactoperoxidase Iodination

The method used was a modification of the Thorell and Johansson (1971) method. All reactions were carried out in a plastic autoanalyzer cup at ambient temperature. The reactants were mixed with a small magnetic flea. Iodination procedure was as follows: the reagents were added in the following order to a plastic autoanalyzer cup.

1) 5 µg tyrosylated C-peptide dissolved in 50 µl of 0.04M sodium acetate buffer pH 5.6
2) 50 µl 0.4M sodium acetate buffer pH 5.6
3) 18.5 MBq (0.5mCi) in a 5 µl aliquot of Na$_{125}^I$
4) 10 µl - 0.003% solution of hydrogen peroxide (100 vol) dissolved in 0.04M sodium acetate buffer pH 5.6
5) 10 µl - containing 500 ng of lactoperoxidase dissolved in 0.04M sodium acetate buffer pH 5.6

15 seconds

6) 240 µg sodium metabisulphite dissolved in 200 µl of 0.04M sodium acetate buffer pH 5.6

The reactants were then loaded on to the purification column.

(iii) Iodogen Iodination

The method used was a modification of the method of Salacinski et al. (1979). 20µg of Iodogen (1, 3, 4, 6 Tetrachloro 3α 6α diphenylglycouril) was dissolved in Analar dichloromethane in the bottom of a small polypropylene tube and allowed to dry. Iodination procedure was as follows:

1) 20 µg of Iodogen contained in a polypropylene tube.
2) 18.5 MBq (0.5 mCi) in a 5 μl aliquot of Na$_{125}$I

3) 5 μg tyrosylated human C-peptide dissolved in 0.1M sodium phosphate buffer pH7.4

The reactants were mixed by gentle shaking of the tube for 10 minutes.

4) 240 μg sodium metabisulphite dissolved in 200 μl of 0.05M sodium phosphate buffer pH7.4

The reactants were then loaded on to the purification column.

(c) **Purification of Iodinated C-peptide**

(i) **Purification on Sephadex G25**

The purification on Sephadex G25 was used for all three methods of iodination. A 25 x 1.1cm column of Sephadex G25 was equilibrated with 0.05M sodium phosphate buffer pH7.4 containing 0.1% HSA. The iodination mixture was applied to the column and the iodinated C-peptide was eluted from the column with 0.05M sodium phosphate buffer pH7.4 containing 0.1% HSA. The unreacted iodide peak was eluted after the protein peak. The purified tracer was aliquoted and stored at -20°C.

(ii) **Purification on Sephadex QAE A25**

This method of purification has been used only for the lactoperoxidase iodination products. A 10 x 1.1 cm column of Sephadex QAE A25 was equilibrated with 0.1M histidine/0.05M HCL pH6.1. The iodination mixture was applied to the column. Iodinated lactoperoxidase followed by unreacted iodide were eluted with 0.1M histidine/0.05M HCL pH6.1. The iodinated tracer was eluted with 0.02M HCL containing 1% HSA. This tracer was then aliquoted and stored at -20°C.

(iii) **Purification on Octadecasilyl (ODS) silica**

This is a modification of the method of Bennett et al. (1977). A 1 x 0.5 cm column of Spherisorb (10 micron ODS silica) was washed with 80 v/v methanol in 1 v/v TFA (Trifluoroacetic acid). After equilibration with 1% TFA in water the iodination mixture was loaded onto the column and washed with increasing
concentrations of methanol containing 1% TFA to elute the $^{125}$I Tyr-C peptide. This purified tracer was stored at -20°C in 0.05M phosphate buffer pH7.4 in 20 µl aliquots.

2:2:4: Assay procedure

(a) Human C-peptide assay (Table 2:1)

The diluent buffer used in the assay was 0.05M sodium phosphate buffer pH7.4 containing 1g HSA per litre.

Standard curves were constructed using human C-peptide in the range 0.125-8 µg/Litre diluted in charcoal stripped serum (see section 2:2:4:(b)).

To a series of polystyrene LP3 tubes were added either 100 µl of C-peptide standard or 100 µl of plasma sample and 100 µl antiserum diluted in assay buffer to a final dilution 1:4500. Standards and samples were set up in duplicate. A set of tubes were set up without antiserum to measure non-specific binding of the samples and standard curve. The counts in these tubes were subtracted from the total $^{125}$I C-peptide bound when calculating the amount of immunologically active C-peptide in unknown samples. All tubes were mixed on a vortex mixer and incubated at 4°C for 48 hours. Tracer was diluted to give approximately 6000 cpm/100 µl (8550 dpm/100 µl). Tubes were then mixed on a Vortex mixture and incubated for a further 24 hours at 4°C. Separation of bound from free $^{125}$I Tyr-C-peptide was carried out by double antibody precipitation. The antisheep γ globulin (Batch no. HP/43-IC) was used at a final dilution of 1:200. Double antibody precipitation time can be reduced by the addition of polyethylene glycol 6000 to the tubes. Polyethylene glycol, at a final concentration of 2%, was added to all tubes except totals, mixed on a vortex mixture and incubated at 4°C for 2 hours. The tubes were then spun in a refrigerated centrifuge for 25 minutes at 2000g. The supernatant was then aspirated. The protein precipitate which contained the antibody bound $^{125}$I C-peptide was counted in an automatic gamma counter. The amount of immunologically active C-peptide in unknowns and standards was determined by subtracting the number of counts in the non-specific tubes. These corrected values were used in the construction of a standard curve and in calculating results.
## Protocol Table

<table>
<thead>
<tr>
<th>Tube</th>
<th>DAY 1</th>
<th>DAY 3</th>
<th>DAY 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer µl</td>
<td>Antiserum µl</td>
<td>Standard Sample or CSS µl</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reagent NSB</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Zero</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Test/Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSB</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Test/Serum</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
(b) **Preparation of charcoal stripped serum**

Pooled serum was obtained from volunteers after an overnight fast. The serum was mixed with activated charcoal 20 g per 100 ml serum overnight at 4°C. The plasma was then centrifuged at 2000 g for 15 min and the supernatant filtered through a Zeitz filter Grade HP/EKS. The serum obtained was then aliquoted and stored at -20°C until required.

(c) **Collection of samples**

Venous blood was collected into lithium heparin tubes. The blood was centrifuged within 15 minutes of collection and the plasma was frozen immediately and stored at -20°C.

(d) **Storage of samples**

The effect on C-peptide levels of storing samples at three different temperatures (4°C, -20°C and -80°C) was investigated.

(e) **Validation of C-peptide assay**

(i) **Recoveries**

625, 1250, 2500 and 5000 pg of human C-peptide standard were each added to one ml of serum. The serum was obtained from a normal volunteer after an overnight fast.

(ii) **Parallelism of standard curve with circulating endogenous C-peptide**

A plasma sample, which had a high endogenous C-peptide level, was serially diluted with charcoal stripped plasma.

2:2:5: **Correlation of plasma levels using two Human C-peptide antisera**

Goat human C-peptide antiserum was received from Dr. E.F. Pfeiffer, Germany. This antiserum was prepared using a synthetic human C-peptide supplied by Hoechst. The antiserum was produced using a carbodiimide method of conjugating C-peptide to rabbit albumin. Immunoreactive C-peptide levels in 45 samples were assessed using the C-peptide assay previously described. Samples were measured using both the Pfeiffer and MF/S/743-IB C-peptide antiserum. The correlation between the two values of the samples was ascertained.
2:3:Results

2:3:1:Antibody Specificity

The antiserum showed no crossreactivity with human insulin, porcine insulin, porcine proinsulin, glucagon and GIP over the concentration range tested (Figure 2:3). The antiserum had a 10% crossreactivity with biosynthetic human proinsulin (Figure 2:4).

Crossreactivity with the C-peptide fragments (Figures 2:5) showed slight displacement with those fragments 7-31 and 10-31. The crossreactivity of C-peptide antiserum (MF/S/743-IB) with 1-35 and 1-31 synthetic C-peptide obtained from Professor N. Yanaihara was 4.1% and 33% respectively. The NIBSC standard 76/561 has a 35 amino acid chain with a formyl lysine on residue 34. This only crossreacted 15% in the assay.

Standard curves with Hoechst, Novo, NIBSC standard 76/321, and IRE synthetic C-peptide were obtained (Figure 2:6). The NIBSC standard 76/321 (NY.TN.10.52) is a synthetic C-peptide of unknown chain length. All the other synthetic C-peptide standards have 31 amino acids.

2:3:2:Purity of Standard

The purity of Hoechst (Figure 2:7) and NIBSC 76/321 (Figure 2:8) C-peptide standard were investigated using HPLC. The Hoechst synthetic C-peptide contains two compounds which can be separated on an isocratic HPLC system (Figure 2:9). Each peak can then be measured by radioimmunoassay. Fractions collected from HPLC runs were measured in the C-peptide radioimmunoassay (Figures 2:7, 2:8, 2:9).

2:3:3:Assay Conditions

(a) Assay Conditions

The effect of increasing the preincubation time on human C-peptide standard curves (Figure 2:10) demonstrated that the greatest displacement was obtained after 48 hours.
Fig. 2: Crossreactivity studies with human C-peptide antiserum MF/S/743-18

Key
- Insulin Human
- Insulin Porcine
- Proinsulin Porcine
- GIP Porcine
- Glucagon Porcine
- Human C-peptide
Figure 2: Human C-peptide standard curve showing cross-reactivity with human proinsulin.

10% Crossreactivity with biosynthetic human proinsulin (Eli Lilly lot 759-0B6-201)
Human C-peptide cross-reactivity with synthetic C-peptide and fragments

Key:
- △ Fragment 20-31
- ▽ Fragment 14-31
- ▼ Fragment 10-31
- ○ Fragment 7 -31 amino acids
- ■ Yanaihara Synthetic C-peptide 35 amino acids
- ▲ C-peptide Standard 76/561 from NIBSC
- ● Hoechst Synthetic C-peptide 31 amino acid
- □ Yanaihara Synthetic C-peptide 31 amino acid

%Bound ¹²⁵I C-peptide of Zero

Log concentration (µg/Litre)
Fig. 2 : 6

**Immunoreactivity of different synthetic C-peptides**

- NY-TN-10-52 - A synthetic C-peptide 80% pure (MRC 76/321)
- Hoechst C-peptide - A 31 amino acid C-peptide
- Novo C-peptide - A 31 amino acid C-peptide
- IRE UK Ltd - A 31 amino acid C-peptide
Fig. 2:7
Hoechst synthetic human C-peptide HPLC trace and immunoreactivity of fractions on a gradient system.

Human C-peptide batch no. 0p 25/26

HPLC gradient
(A) 0.1% TFA
(B) 60% CH₃CN
40% A

Injected sample (20 µg)

Fractions collected at 0.1 min intervals
Chart speed 200 mm hr⁻¹
Flow rate 1 ml min⁻¹
Wavelength 220 nm

Human C-peptide immunoreactivity of HPLC fractions
Fig. 2: Human C-peptide batch no. NY-TN-10-52 HPLC trace and immunoreactivity of fractions on a gradient system.

HPLC trace

HPLC gradient (A) 0.1% TFA Chart speed 200 mm hr⁻¹
(B) 60% CH₃CN Flow rate 1 ml min⁻¹
40% A Wavelength 220 nm
Fraction collected at 0.1 mins intervals

Human C-peptide immunoreactivity of HPLC fractions
Figure 2: 9 Hoechst human C-peptide HPLC trace and immunoreactivity of fraction of an isocratic system.

Key
Buffer 27% Acetonitrile/73% 0.1% TFA (v/v)
Column 150 x 4.6 mm OD hypersil
Wavelength 215 nm
Chart speed 200 mm/hr
Flow rate 1 ml min⁻¹

Injected sample (10 µg)

C-peptide immunoreactivity of fractions obtained from HPLC run of Hoechst C-peptide (Op 25/26)
Figure 2:10

Optimisation of Human C-peptide standard curves

Key
- ○ 24h Preincubation/24h Tracer/24h Separation
- △ 30h " " "
- ▲ 48h " " "

% Bound 1125 C-peptide of Zero

C-peptide (μg/Litre)
(b) **Standard curve**

Under the conditions described for the C-peptide radioimmunoassays increasing quantities of unlabelled C-peptide displaced $^{125}$I tyrosylated C-peptide in the range 0.11 - 8μg/Litre (Figure 2:11). The non-specific binding varied with the age of the tracer but was always less than 10%.

(c) **Sensitivity**

The limit of sensitivity of the assay defined as two standard deviations from the zero standard (Feldman and Rodbard 1971) was 10 pg.

(d) **Scatchard Plot**

The binding affinity and equilibrium constant were determined by Scatchard (1949) analysis (Figure 2:12). The method of Walker (1977) was used to calculate the amount of tracer added to each assay tube. The binding affinity of the antiserum was calculated to be $7.75 \times 10^{-9}$ Litre/mole.

2:3:4: **Iodination**

(a) **Purity of Tyrosylated Human C-peptide**

Figure 2:13 shows the HPLC trace of tyrosylated C-peptide which appears as two peaks.

(b) **Assessment of iodination methods**

Chloramine T iodination resulted in a tracer which maximally bound only 54% of C-peptide tracer under conditions of antibody excess. The binding of the tracer to the antiserum deteriorated rapidly on storage. Figure 2:14 shows the antibody dilution curves produced with the iodogen and lactoperoxidase produced tracer. The iodogen tracer was separated on G25 Sephadex while the lactoperoxidase tracer was purified on Sephadex QAE A25. The highest affinity tracer was produced when tyrosylated C-peptide was iodinated with iodogen. However, attempts to obtain a standard curve using this tracer resulted in a hooked shaped curve. This effect disappeared after the tracer had been stored for two weeks (Figure 2:15) but the tracer also showed a considerable loss of binding after storage for 1 month.
Human C-peptide standard curve showing the intra-assay precision for each point on the standard curve.

$\bar{I} \pm SD$

$N = 20$
Figure 2.12
Scatchard plot of human C-peptide antiserum (batch no. MF/S/743-IB)

\[ k = \text{Equilibrium constant} \]
\[ n = \text{Antibody binding capacity} \]
\[ k_1 = 7.75 \times 10^9 \text{ Litre/mol} \]
\[ n_1 = 87 \text{ mmol/Litre} \]
\[ k_2 = 2.35 \times 10^9 \text{ Litre/mol} \]
\[ n_2 = 164 \text{ mmol/Litre} \]
Figure 2:13 HPLC trace of Hoechst tyrosylated C-peptide

HPLC gradient (A) 0.1% TFA
(B) 40% CH₂CN
60% CH₂CN

Chart speed 200 mm hr⁻¹
Flow Rate 1 ml min⁻¹
Wavelength 200 nm
Fractions - 0.1 interval fractions collected

Sample injected
Figure 2: Comparison of tracers produced using the Iodogen and lactoperoxidase methods of iodination in antisera dilution curves.
Figure 2.15 Comparison of Human C-peptide standard curves using tracers produced by lactoperoxidase and Iodogen methods.

Final concentration of antiserum 1:4500

- Lactoperoxidase method
- Iodogen Method

[Graph showing comparison between lactoperoxidase and Iodogen methods for Human C-peptide standard curves]
(c) Purification of tracer

Figure 2:16 shows the result of separating lactoperoxidase iodinated tyr-C-peptide on a Sephadex QAE A25 column using a two buffer system. The tracer obtained was aliquoted and stored at -20°C. The tracer remained stable for up to eight to ten weeks. This tracer was far more stable when separated using Sephadex QAE A25 than when using G25 Sephadex as a separation method. The elution profile obtained from separating iodinated tyr-C-peptide using an ODS silica system is shown in Figure 2:17. The antibody dilution curves and standard curves produced with this tracer and the QAE A25 Sephadex tracer (Figure 2:18 and 2:19) were almost identical. However, the ODS silica separated tracer was less stable and could be used for only four weeks before loss of antigen binding occurred.

Specific activity could not be calculated using the QAE A25 Sephadex separation method, as a large amount of free iodide remained on the column. However, the specific activity was calculated to be 172 mCi/mg by determining the amount of tracer added to each tube (Walker, 1977).

2:3:5: Validation of Human C-peptide assay

(a) Precision

The intraassay coefficient of variation at a mean plasma level of 1.3 μg/Litre was 8.6% (n = 7) and at 6.2 μg/Litre was 10.7% (n = 7).

The calculated interassay coefficient of variation of four serum samples is shown in Table 2:1.

(b) Recovery

The mean recovery of exogenous human C-peptide when added to fasted serum was 97% (Table 2:2).

(c) Parallelism

Plasma samples containing a high concentration of endogenous C-peptide serially diluted in charcoal extracted plasma were superimposable upon the human C-peptide standard curve (Fig. 2:20).
Figure 2:16 Profile of lactoperoxidase iodinated Tyr-C-peptide separated on QAE A25 Sephadex (Peak A - iodinated lactoperoxidase peak B - unreacted iodide; Peak C - iodinated C-peptide (approx. 80% maximum binding) eluted with 0.02 M HCl in 1% HSA).
Figure 2.17: Profile of lactoperoxidase-iodination Tyr-C-peptide separated on ODS silica

- Peak A: Iodinated Tyr-C-peptide
- Peak B: Unreacted iodide
Figure 2: Comparison of antibody dilution curves using lactoperoxidase iodinated tracer purified on Sephadex QAE A25 and ODS silica

- ODS silica purified tracer
- QAE A25 purified tracer
Figure 2:19 Comparison of Human C-peptide standard curves using lactoperoxidase iodinated tracer purified in Sephadex QAE A25 and ODS silica

△ Sephadex QAE25 purified tracer
△ ODS silica purified tracer
Table 2:1  **Interassay precision of plasma samples containing a range of endogenous C-peptide levels.**

<table>
<thead>
<tr>
<th>Mean C-peptide Value (µg/Litre)</th>
<th>No. of Observations</th>
<th>SD</th>
<th>Co-eff. of Variation (%)</th>
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<tbody>
<tr>
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<tr>
<td>6.17</td>
<td>7</td>
<td>0.39</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Table 2:2  **Recovery data - addition of varying amounts of C-peptide standard to normal fasted serum (mean of duplicate estimation)**

<table>
<thead>
<tr>
<th>Exogenous C-peptide added pg/tube</th>
<th>C-peptide measured pg/tube</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>-</td>
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<tr>
<td>25</td>
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<td>94</td>
</tr>
<tr>
<td>375</td>
<td>495</td>
<td>105</td>
</tr>
</tbody>
</table>
Human C-peptide (μg/Litre)

% Bound 125I C-peptide of Total

- Human C-peptide standard diluted in charcoal-extracted serum
- Serum samples diluted in charcoal-extracted serum

Human C-peptide of Total serum upon a synthetic Human C-peptide standard similarly diluted.
(d) **Stability of C-peptide samples**

The stability of endogenous C-peptide was only affected when stored at 4°C. Levels remained similar at both -20°C and -80°C (Figure 2:21). No deterioration in the endogenous C-peptide was apparent after seven months.

(e) **Normal Range of human C-peptide levels**

Twenty blood samples were taken from non-obese healthy volunteers after an overnight fast. The mean C-peptide level was 1.38 µg/Litre ± 0.61 (x ± SD), the observed range being from undetectable to 2.45 µg/Litre.

(f) **Correlation of plasma levels using two human C-peptide antisera**

Blood samples were taken and measured in the assay system described using two different antisera. The correlation of values obtained from both assays was 0.97 (Fig. 2:22).

2:4: **Discussion**

A radioimmunoassay for the detection of immunoreactive human C-peptide has been developed. All previously published C-peptide radioimmunoassays have used antisera either raised by using crude β insulin component or conjugated C-peptide using the carbodiimide method. Carbodiimide reagent reacts with many functional groups and it is difficult to forecast where conjugation has taken place. In this study, antiserum was raised by conjugating C-peptide to egg albumin using the gluteraldehyde method. This method is quick, simple and the place of conjugation is less variable. Gluteraldehyde is thought to react with the epsilon amino group of lysine (Quiocho and Richards, 1966) and possibly the amino group at the N-terminal end of the protein. Human C-peptide, which has 31 amino acids, has no lysine residues therefore the conjugation was presumed to have occurred on the N-terminal glutamic acid.

Antiserum to human C-peptide has been produced in a variety of animal species. Rabbits, goats and guinea pigs have all been used by other workers (Melani et al. 1970; Kaneko et al. 1974; Heding, 1975; Beischer et al. 1976, Faber et al. 1976.) Initial attempts to produce antiserum in rabbits resulted in only one
Figure 2:21 Stability of C-peptide plasma samples at various temperatures

- $4^\circ C$
- $-20^\circ C$
- $-80^\circ C$

Days after storage
Correlation of plasma levels using two Human C-peptide antiserum

\[ r = 0.968 \]
\[ p < 0.001 \]
\[ y = 1.08 x - 0.23 \]
animal responding. This animal produced an antiserum which had a low titre and avidity. The immunogenicity of compounds varies according to the species of animals used. A primitive breed of sheep was therefore immunized with the conjugated human C-peptide. These animals responded and a usable antiserum was obtained after the first reimmunisation.

The crossreactivity of the antiserum was assessed with synthetic C-peptide fragments. Only slight displacement was shown with fragments 10-31 and 7-31. No fragments were available containing the amino acid sequence at the amino end of the molecule. It is possible that the antigenic determinants of the human C-peptide are at the amino end of the molecule for this antiserum. It cannot be established from the data available whether or not there is more than one determinant. It is known that in the case of peptide hormones each antigenic determinant consists of a sequence of some 2-8 amino acids, having a diameter of less than 15 angstrom units. It has been shown that these amino acids have a unique three dimensional configuration. A difference of only one amino acid is sufficient to distort this arrangement and impair the binding to specific antibodies.

Studies with various synthetic standards showed varying degrees of displacement compared with the Hoechst standard. The need, therefore, for an International standard which could be used by all laboratories measuring C-peptide is required. Unfortunately the NIBSC C-peptide standard 75/561 only crossreacted 15% in the assay. Another sample of synthetic standard (NY-TN-10-8) from NIBSC crossreacted 52.4% in the assay. High pressure liquid chromatography of this standard showed more than one component. This could explain the low crossreactivity in the assay. The chain length is also important in recognition of a standard, since the 1-35 C-peptide crossreacted by 4.1% while the 1-31 C-peptide crossreacted by 33%. Synthetic C-peptide standards obtained from Novo and IRE-UK Ltd. produced similar standard curves in the assay when compared with the Hoechst standard. Both these standards are synthetic and have 31 amino acids.
The high pressure liquid chromatography trace of the Hoechst C-peptide showed that it contains two components. When these components were separated using an isocratic HPLC method, each component displaced tracer in the assay. The antiserum used in the assay was raised against a previous batch of synthetic C-peptide. Previous batches may also have contained two components. It can be postulated that of these two components, one has the correct C-peptide sequence while the other component is an analogue, varying by one or more amino acids. It would appear that because of the close elution of these two components that they vary in only one or two amino acids. Investigating the amino acid sequence of this second component would take considerable time, but it is possible to conjecture that the amino acids which differ are the glutamine residues as these residues can be easily hydrolysed to glutamic acid.

Tyrosylated C-peptide eluted in two peaks from the HPLC column. The minor component could be human synthetic C-peptide without the tyrosine moiety. Iodination of human C-peptide proved to be extremely difficult despite following previously published methods for C-peptide iodinations (Melani et al. 1970, Heding, 1975, Beischer et al. 1976). Initial iodinations using the chloramine T method produced a tracer which only maximally bound 40% under condition of antibody excess. This method of iodination is fairly harsh since it directly exposes the peptide to the oxidizing agent. The lactoperoxidase method was, therefore, investigated. Previous workers (Beischer et al. 1976) have used this method and left the reaction mixture for twenty minutes before terminating the reaction. After varying the reaction time it was found that terminating the reaction after only 15 seconds produced a high specific activity tracer. The tracer produced by iodination using the iodogen method gave a higher maximum binding than with the lactoperoxidase trace in the assay. However, it was observed that an anomalous rise occurred in the percentage binding of the labelled antigen to the antibody upon the addition of low concentration of the unlabelled antigen. This observation has been described in the literature a
number of times (Cresto, 1974; Beischer et al. 1976). Although this effect disappeared after the tracer had been stored for two to three weeks iodination of C-peptide using iodogen was not considered suitable for routine assay without a considerable amount of further investigation into this effect and the alternative lactoperoxidase method was therefore used.

The shelf life of the tracer can be increased by some four weeks by selecting the optimum purification procedure using an ion-exchange chromatography method, first described by Heding et al. (1974). A lactoperoxidase tracer was obtained which remained stable for up to 8-10 weeks after the iodination. This improved separation of iodinated from non-iodinated C-peptide and iodinated lactoperoxidase resulted in a highly purified tracer of consequently high specific activity being added to the assay system. The sensitivity and reliability of the assay was greatly improved by this method of purification of the tracer.

The stability of the human C-peptide in plasma samples was investigated. Samples stored at \(-20^\circ\text{C}\) and \(-80^\circ\text{C}\) had similar values when measured in a radioimmunoassay after 20 weeks' storage. If stored at \(4^\circ\text{C}\), however, there was a 24 % decrease of immunoreactivity after only 22 days. These results do not substantiate those reported by Garcia-Webb et al. (1979). They reported that there was a marked reduction of C-peptide in samples stored at \(-18^\circ\text{C}\) for longer than three weeks but found that no change occurred in samples stored at \(-70^\circ\text{C}\) for up to 16 weeks.

The values obtained in the assay system for fasting C-peptide levels in normal subjects, compared closely with other published ranges (Block et al. 1972; Beischer et al. 1976). Heding et al. (1974) reported levels which were lower than the values reported in this chapter. Their assay, however, involved the extraction of proinsulin and proinsulin-like components before measurement of C-peptide.

The human C-peptide assay developed crossreacted 10% with human biosynthetic proinsulin. This compares favourably with other workers who have reported
crossreactivity with proinsulin in their C-peptide assays ranging from 15-66% (Melani et al. 1970; Casygill et al. 1980). C-peptide values obtained in unextracted plasma by this assay method are the sum of crossreactivity of C-peptide, proinsulin and proinsulin-like components. The levels of proinsulin and proinsulin-like components have been reported to be very low in normal subjects (Heding, 1977, Rainbow et al. 1979) but raised in certain clinical situations e.g. insulin producing tumours.

The sensitivity of an assay is determined by a number of factors. It is related to the slope of the dose response curve and is affected by the precision with which the percent bound can be measured in replicate tubes containing 'O' standard. The sensitivity of the assay may be affected by physiological fluids. Plasma and serum samples have been shown to cause both incubation damage and interference in the separation system. It has been shown that charcoal stripped serum from different individuals has lower binding and a greater variation than that of the 'O' standard in diluent. This effect is defined by Hunter (1971) as 'noise' and it has been suggested that the sensitivity of an assay could be defined as the minimum amount of hormone that will produce a fall, in percent bound, significantly below that arising from noise. The sensitivity of the C-peptide assay described was 10 pg, sensitivity being defined as two standard deviations from zero. No account has been taken in the calculation of the effect of plasma and serum in the assay.

Other workers have shown variations in the level of circulating C-peptide immunoreactivity measured in human subjects depending on the antiserum used. In particular Heding (1975) has shown that there was considerable variation in displacement of the tracer from different antisera by serum from long-term insulin treated diabetics. The correlation of C-peptide immunoreactivity in samples measured using two different antiserum was extremely close. However, the majority of the samples assessed were obtained from normal subjects and this may explain why no differences were apparent.
CHAPTER III

HUMAN C-PEPTIDE LEVELS IN NORMAL NON-OBESE SUBJECTS
IN THE FASTED AND STIMULATED STATES
Radioimmunoassays for the measurement of human C-peptide have been developed by a number of workers (Melani et al. 1970, Kaneko et al. 1974, Heding 1975, Faber et al. 1976, Beischer et al. 1976). There are many clinical conditions in which C-peptide measurement is useful in diagnosis. Its greatest use has been in the diagnosis of various hypoglycaemic disorders. The establishment of a normal range of C-peptide values in normal non-obese subjects in the fasted and fed states is important. Since there is no international standard available each laboratory has to use its own standard which may vary in amino acid chain length and amount of impurities (see Chapter II). Fasting C-peptide levels in healthy subjects vary depending on the assay (Block et al. 1972, Kaneko et al. 1974, Heding, 1975, Heding and Rasmussen, 1975, Beischer et al. 1976, Beyer et al. 1976.)

Evidence to date regarding diurnal rhythms observed in blood levels of insulin and glucose are not well understood. It is not certain whether truly endogenous circadian rhythms exist or whether the phenomena are secondary to feeding and activity patterns.

A variety of observations have been made on the effect of fasting on plasma glucose levels. Fairman and Moorhouse (1967), reported that a diurnal rhythm in blood glucose levels was observed in maturity onset diabetics during three days of starvation. They could not, however, demonstrate a rhythm in normal non-obese volunteers. Merimee and Tyson (1974), reported lower fasting blood glucose levels in women than in men. The difference is noticed after twenty four hours fasting and is striking after thirty six hours. The variation in the blood glucose levels in men and women may be due to differences in muscle mass, the effect of sex hormones on the uptake of glucose into the tissues or ketosis which may inhibit the release of precursors of gluconeogenesis.

A circadian rhythm in plasma insulin levels has been shown in fasted rats and mice (Bellinger et al. 1975, Pessaq et al. 1976) but evidence in man is less convincing. Fairman and Moorhouse (1967), have reported no significant change in fasting
insulin levels in normal non-obese subjects either within one day or from day to
day. Freinkel et al. (1968), however, have shown that in a group of normal non-
obese volunteers, fasted for three days, consistently lower plasma insulin levels
were recorded in the afternoon (1500-1600h) than in the early morning (0700-
0800h). This lower plasma insulin level in the afternoon was not associated with
any changes in blood glucose. A diurnal change in plasma insulin and glucagon
levels has been reported in a patient with a malignant insulinoma (Ohneda et al.
1979). The glucagon level peaked between 0600-0730h while the insulin level
peaked at approximately 0730h.

Insulin estimations in the fasted state are very near the detection limit of the
conventional insulin radioimmunoassay. When measuring these low insulin levels,
the coefficient of variation of the assay is large and may mask any small changes
which occur in insulin concentration. C-peptide is present in much higher
concentrations than insulin in the peripheral circulation and fasting levels in
most subjects are therefore well above the detection limit of the assay in most
subjects and can be measured with greater precision. However, the long half life
of C-peptide would prevent short term changes in B cell activity being detected.

Evidence for a diurnal rhythm in post-prandial plasma insulin and glucose levels
is conflicting (SchlieTf and Raetzer, 1972, Hautecouverture et al. 1974, Ahmed et
al. 1976). SchlietT and Raetzer (1972), reported a study carried out in normal
non-obese young subjects in which either six small meals or three large meals
were given on two separate occasions to each volunteer. The same total energy
intake was maintained on both regimens. These workers found improved glucose
tolerance during the morning and high blood glucose values in the afternoon. The
integrated insulin areas for 24 h were identical after both regimens. Cohn et al.
(1968) reported that the size of the insulin response was a function of the energy
load. Ahmed et al. (1976) using a three meal schedule found a diurnal rhythm in
plasma glucose responses but only in women. The integrated glucose area
increased significantly through the day, although integrated insulin area did not
change. Hautecouvare et al. (1974) using a two-meal schedule, showed no difference in glucose response between sexes, but a significantly higher insulin response in the morning compared with the afternoon. Obviously it is difficult to compare findings when different meal times and groups of subjects are used.

Diurnal variation in glucose tolerance in man, with reduced tolerance to both intravenous and oral glucose in the afternoon has been reported by a number of workers (Roberts 1964, 1967, Bowen & Reeves 1967, Jarrett and Keen, 1969, 1970). Carroll and Neeté (1973) found that after an evening oral glucose tolerance test the insulin response was delayed and the insulin response to an intravenous glucose load was diminished in the evening compared with the morning. These workers suggested that this reduced glucose tolerance in the evening may be due to the impairment of the early response. Other workers (Gibson and Jarrett, 1972, Gagliardino et al. 1976) have suggested that the diurnal difference in glucose tolerance might be attributed to a diurnal variation in sensitivity to endogenous insulin. Gibson and Jarrett (1972) showed that exogenous insulin was less effective in the afternoon than in the morning. Schulz et al. (1980) showed that normal subjects who were infused with a constant amount of glucose required varying amounts of exogenous insulin to maintain normoglycaemia at different times during the day. Human C-peptide levels were monitored to show any changes in endogenous insulin activity. The highest insulin requirement occurred between 1400-1800h and lowest between 0200-0600h.

Small seasonal variations in fasting blood glucose levels and good glucose tolerance with a low insulin secretion during the summer months have also been observed in a group of men suffering from myocardial infarction (Fahlen et al. 1971). It is not known whether normal subjects have a seasonal variation in insulin and glucose levels.

The disposal of a glucose load is progressively impaired in subjects over the age of 60 years (McKendry, 1980, Davidson, 1979). The reason for this impairment remains unclear but poor diet, lower physical activity, decrease in lean body
mass in which to store the carbohydrate load, decreased insulin secretion and insulin antagonism may all be contributory factors. Care must, therefore, be taken to age match subjects as much as possible when investigating changes in glucose tolerance.

Studies have been carried out in order to obtain a fasting range of C-peptide levels in normal, non-obese volunteers. Human C-peptide levels have been studied in fasting subjects and in response to oral and intravenous stimulation in normal volunteers. These investigations provide background information in which to assess C-peptide levels found in hypoglycaemic subjects. These are discussed in a later chapter.

3:2 Materials and Methods

3:2:1 Study 1

Twenty four hour profiles of C-peptide, insulin and glucose levels in normal subjects following a meal and subsequent fasting

Ten normal healthy volunteers, four men and six women, aged between 20-39 years (27.1 ±1.78 (x ± SEM)) and within 20% of their ideal body weight (Metropolitan Life Insurance Company Tables) took part in the study. Relevant physical characteristics are summarised in Tables 3:1. During the study the volunteers were confined to the hospital unit. A test meal (Table 3:2) was eaten between 0710-0730 h after an overnight fast. Two jam doughnuts and tea or coffee were taken at 0945 h. A 10 ml blood sample was taken at hourly intervals for 24 h starting at 1000 h by means of an indwelling catheter inserted into an antecubital vein, kept patent with 0.12M sodium citrate. After each sample a 100 μl aliquot of whole blood was deproteinised for glucose analysis. The remaining blood was collected into a lithium heparin tube mixed and centrifuged. Plasma was aliquoted and stored frozen at -20°C until assayed.

3:2:2 Study 2

C-peptide, insulin and glucose levels after intravenous glucose and a test meal at two different times within twenty four hours
Table 3:1  

**Physical Characteristics of subjects in 24 hour study**

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<th>Subject (code)</th>
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<th>Height (cm) in shoes</th>
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<td>B</td>
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Table 3:3  

**Physical Characteristics of subjects in two meal study**

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Table 3:4  

**Physical Characteristics of subject in intravenous glucose study**

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Table 3:2

TEST MEAL

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<tr>
<td>Total</td>
<td>17.1</td>
<td>36.7</td>
<td>100.9</td>
<td>779</td>
<td></td>
</tr>
</tbody>
</table>
(a) A study on the effect of intravenous glucose administration at two different times during twenty four hours

Five normal healthy volunteers, two men and three women, aged between 22-33 years (27.4 ± 1.96) and within 20% of their ideal body weight (Metropolitan Life Insurance Company Tables) were studied. The physical characteristics of the subjects are shown in Table 3:4. During the study the volunteers were confined to the hospital unit. All subjects fasted overnight for 15 hours prior to the start of the study. The basal blood samples were taken by means of an indwelling catheter inserted into an antecubital vein, kept patent with 0.12M sodium citrate. At 1030 h the volunteers were intravenously given 25g of glucose, made up in 50 ml sterile saline, by means of an indwelling catheter inserted into the antecubital vein in the other arm. Samples were taken at 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 minutes after the intravenous administration of the glucose. The volunteers were then allowed to carry on with their normal activities and were allowed to drink water if they wished. A second 25g intravenous glucose load was given at 0130 h, 15 h after the first load. Blood samples were taken at the same time points as before. After each blood sample 100 μl aliquots of whole blood were deproteinised for glucose analysis. The remaining 10 ml of blood was mixed in a lithium heparin tube. The tube was spun and plasma aliquoted, stored and frozen at -20°C until assayed.

The rate of disappearance of glucose (K value) was calculated, according to the formula of Conard (1955), from the regression time of the blood glucose levels against time between 15 and 60 minutes after glucose injection.

(b) A study on the effect of a test meal eaten at two different times in twenty-four hours on glucose, insulin and C-peptide levels

Five normal healthy volunteers, four men and one woman, aged 23-28 years (27.4 ± 1.96) were studied. Their weights did not exceed ideal body weight by more than 10% (Metropolitan Life Insurance Company Tables). The physical characteristics of the subjects are shown in Table 3:3. During the study the
volunteers were confined to the hospital unit. All subjects fasted overnight for 15 hours prior to the start of the experiment. Blood samples were taken by means of an indwelling catheter inserted into an antecubital vein, kept patent with 0.12M sodium citrate. At 1030 h, after a basal blood sample had been taken, the volunteers were given a test meal (see Table 2). Blood samples were taken at hourly intervals for 24 h starting at 1045 h. An identical test meal (see Table 2) was given 15 h after the first test meal. Subjects were allowed to drink water as and when they wished. After each blood sample a 100 μl aliquot of whole blood was deproteinised for glucose analysis. The remaining blood was collected into lithium heparin tubes, mixed and centrifuged. Plasma was aliquoted and stored at -20°C until assayed.

3:2:3 Study 3

Diurnal rhythms in C-peptide, insulin and glucose levels in response to a test meal given at different times over twenty four hours

Six normal healthy volunteers, three men and three women, aged between 23-33 years (27.8 ± 1.47) and within 20% of their ideal body weight (Metropolitan Life Insurance Company Tables) were studied. The physical characteristics of the volunteers are shown in Table 3:5 All subjects fasted for 10-12 h before the start of each experiment. Test meals (see Table 3:2) were eaten at the following times, 0200, 0600, 1000, 1400, 1800, 2200 h on six separate occasions. At least a week elapsed between each experiment. The volunteers were divided into two groups. Each group were given a test meal at a different time on the same day. Two basal blood samples were taken by means of an indwelling catheter inserted into an antecubital vein, kept patent with 0.12M sodium citrate. The test meal was eaten within 30 minutes of the start of the experiment. Blood samples were taken 30, 60, 90, 120, 180, 240, 300 and 360 minutes after the meal. During the sampling the volunteers were confined to the hospital unit. Blood samples were collected into lithium heparin tubes, mixed and centrifuged. Plasma was aliquoted and stored frozen at -20°C until assayed.
Table 3:5

Physical Characteristics of subjects on diurnal rhythms study.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Weight (Kg)</th>
<th>Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>F</td>
<td>29</td>
<td>57</td>
<td>173</td>
</tr>
<tr>
<td>B</td>
<td>F</td>
<td>30</td>
<td>60</td>
<td>175</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>25</td>
<td>80</td>
<td>173</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>27</td>
<td>75</td>
<td>180</td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>23</td>
<td>61</td>
<td>183</td>
</tr>
<tr>
<td>F</td>
<td>F</td>
<td>23</td>
<td>72</td>
<td>170</td>
</tr>
</tbody>
</table>

Table 3:6

K values obtained after intravenous glucose study

K value = per cent removal of glucose per minute between 15 and 60 minutes

<table>
<thead>
<tr>
<th>Subject</th>
<th>K values after 1st infusion</th>
<th>K values after 2nd Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>3.46</td>
<td>1.6</td>
</tr>
<tr>
<td>W</td>
<td>2.8</td>
<td>0.87</td>
</tr>
<tr>
<td>X</td>
<td>2.04</td>
<td>1.1</td>
</tr>
<tr>
<td>Y</td>
<td>1.1</td>
<td>0.61</td>
</tr>
<tr>
<td>Z</td>
<td>1.7</td>
<td>1.46</td>
</tr>
</tbody>
</table>

t = 3.15  p < 0.025
3:3 Assays

3:3:1 Glucose analysis

Blood glucose was determined in duplicate by a glucose oxidase method (Trinder, 1969) in Study 1 and 2. Plasma glucose determination in Study 3 involved the use of hexokinase enzyme in an automatic glucose analyser.

3:3:2 Insulin Radioimmunoassay

Plasma immunoreactive insulin was measured by a double antibody radioimmunoassay developed at St. Luke’s Hospital, using guinea-pig antiserum. The standard used was human insulin standard and the $^{125}$I insulin tracer was obtained from the Radiochemical Centre, Amersham. The detection limit of the assay was 2.5 mU/Litre. The interassay coefficient of variation was 15% at 65 mU/Litre and 7.4% at 46 mU/Litre.

3:3:3 C-peptide Radioimmunoassay

Plasma immunoreactive C-peptide levels were measured in duplicate by the method described in Chapter II. The assay has a detection limit of 10 pg/tube (100 ng/Litre). The interassay coefficient of variation was 24.5% at 0.44 µg/Litre and 9% at 3.7 µg/Litre.

3:3:4 Statistical analyses

Statistical analyses of the results were carried out using a student 't' test for paired data. Correlation coefficients were determined by simple linear regression. Integrated areas were calculated by plotting the graph, cutting out and weighing the paper representing the area under the curve.

3:4 Results

3:4:1 Normal Range of Human C-peptide levels

Blood samples were taken from healthy non-obese volunteers after an overnight fast. Fasting immunoreactive C-peptide levels correlated with fasting immunoreactive insulin levels in seventeen subjects (correlation 0.78) (Fig. 3:1). When all twenty subjects were included the correlation was reduced to 0.387. The three subjects who were excluded from the initial correlation had fasting
Figure 3:1

Correlation of basal insulin with C-peptide levels in healthy volunteers.

N = 17
Slope 3.609 Corr Coeff 0.78
Intercept 1.6 p < 0.001
immunoreactive insulin levels of 4, 5, 6 mU/Litre yet fasting immunoreactive C-peptide values of 2.55, 2.45, 2.4 µg/Litre. One of these subjects had a renal function test in order to determine creatinine clearance. The value obtained was 80 ml/min, the normal range being 95-105 ml/min. The renal function of the other two subjects was not investigated.

Fasting immunoreactive C-peptide values were found to correlate significantly with body weight (correlation 0.89) and body mass index (correlation 0.78) (Fig. 3:2 and 3:3). The weight and height of the healthy volunteers ranged from 50-79 kg (63 ± 2.4) and 165-188 cm (174.7 ± 1.87).

Twenty four hour profiles of immunoreactive C-peptide, insulin and blood glucose in normal volunteers following a meal and subsequent fasting.

The mean blood glucose levels throughout the sampling period are shown in Figure 3:4. The mean blood glucose levels showed very little variation throughout the 24 h sampling period. There was no significant difference between males and females when plotted separately.

The mean plasma immunoreactive insulin levels throughout the study are shown in Figure 3:5. Plasma immunoreactive insulin peaked one hour after ingestion of the meal (40.1 ± 5.51 mU/Litre (x ± SEM)) and returned to fasting levels after approximately 5 hours. The mean fasting levels defined as 6 hours after the start of sampling until the end of the study was 5.27 ± 1.7 mU/Litre.

The profile of mean plasma immunoreactive C-peptide levels in the study are shown in Fig. 3:6. Plasma immunoreactive C-peptide levels peaked after one hour at 5.86 ± 0.76 µg/Litre and returned to basal levels after 11 hours. The initial fall in immunoreactive C-peptide levels was sharp, from 5.86 µg/Litre to 2.4 µg/Litre, in three hours. The mean fasting immunoreactive C-peptide, defined as 12 hours after the start of sampling until the end of the study was 1.7 ± 0.2 µg/Litre.

The peak values of the blood glucose and hormones in the study would not necessarily reflect the actual peak values obtained in view of the sampling
Fig. 3:2

Correlation of fasting C-peptide levels and body weight of healthy volunteers.

N = 15
Correlation 0.89
Intercept 4.14
Slope 16.06
Degree of Freedom 13
p < 0.001

Figure 3:3

Correlation of fasting C-peptide levels and Body mass index (BMI)

N = 15
Correlation 0.78
Intercept 15.8
Slope 3.56
Degree of Freedom 13
p < 0.001
Figure 3:4
Twenty four hour profile of blood glucose, insulin and C-peptide in normal non-obese volunteers following a meal and subsequent fasting.

Blood Glucose mmol/Litre

Figure 3:5
Insulin mU/Litre

Figure 3:6
C-peptide µg/Litre
times. In order to obtain an accurate value more frequent sampling would
initially be necessary because of the rapid post-prandial rise.

3.4.3 Study 2

Immunoreactive C-peptide, insulin and blood glucose levels after intravenous
glucose and test meals at two different times in twenty four hours.

(a) Intravenous glucose tolerance tests

The mean blood glucose levels throughout the sampling period are shown in
Fig. 3:7. Blood glucose levels peaked 5 minutes after the start of the infusion
and similar levels were reached after both infusions. Blood glucose levels after
the 1000 h intravenous infusion took 90 minutes to fall to fasting levels. After
the 0200 h infusion blood glucose levels reached basal values 150 minutes after
the glucose had been infused. Statistical analysis of the area under the curves
showed a significant difference (p < 0.0125) using student paired 't' test.
Estimation of the k values, the rate of disappearance of glucose against time
(15-60 minutes) showed that all volunteers had smaller values after the second
infusion (Table 3:8).

The mean plasma immunoreactive insulin levels throughout the study are shown
in Fig. 3:8. The mean fasting immunoreactive insulin level at the start of the
study were 63 ± 0.73 mU/Litre. The levels increased sharply reaching a peak 5
minutes after the glucose load and similar levels were reached after each
infusion. Plasma insulin levels took longer to return to basal levels after the
second infusion. Calculation of the area under the curve of individuals followed
by statistical analysis by paired 't' test showed no significant difference between
the two responses. Significantly higher mean insulin levels were reached 90, 120
and 180 minutes after the second infusion compared with the first infusion.

The mean level of immunoreactive C-peptide in the two intravenous glucose
tolerance tests are shown in Fig. 3:9. In this study mean fasting immunoreactive
C-peptide levels were 1.22 ± 0.11 µg/Litre. Levels increased sharply after the
glucose load, reaching a peak 5 minutes after the start of both infusions. The
Blood glucose levels after intravenous glucose infusion at two different times in 24 hours in healthy volunteers.

○ 0200h
● 1000h
** p < 0.005

Figure 3:7
Figure 3:8

Plasma insulin levels after an intravenous glucose infusion at two different times in 24 hours in healthy volunteers.

- 0200 h  * p < 0.0125
- 1000 h  ** p < 0.005

Time (min)

Insulin ml/Litre

180 150 120 90 60 30 15 0 -15

* SEM
Figure 3:9

Plasma C-peptide levels after an intravenous glucose infusion at two different times in 24 hours in healthy volunteers.

\[ I \pm \text{SEM} \quad * \quad p < 0.05 \]

○ 0200h \quad ** \quad p < 0.025

● 1000h \quad *** \quad p < 0.0025
peak levels after the first and second infusion were $4.06 \pm 0.87 \mu g/Litre$ and $4.44 \pm 1.2 \mu g/Litre$ respectively. Immunoreactive C-peptide levels returned to basal values 180 minutes after the first infusion. After the second infusion immunoreactive C-peptide levels had not returned to basal levels by the end of the experiment. Calculation of the area under the curve of individuals, followed by statistical analysis by paired 't' test showed a significant difference ($p < 0.01$) between the two responses.

The molar ratio of immunoreactive C-peptide to immunoreactive insulin in the fasted samples was $9.75 \pm 1.42$. This level decreased to $3.17 \pm 0.56$ 5 minutes after the 1000 h glucose infusion (Fig.3:10).

(b) A study on the effect of a test meal at two different times in twenty four hours on immunoreactive C-peptide, insulin and blood glucose levels

The mean blood glucose levels throughout the sampling period are shown in Fig. 3:11. Blood glucose levels peaked at $5.12 \pm 0.4 \text{ mmol/Litre}$ approximately 3.5 hours after food ingestion. The peak response in blood glucose levels was $9.56 \pm 1.2 \text{ mmol/Litre}$ which occurred 30 minutes after the second test meal was ingested. Blood glucose levels took approximately 5.5 hours to return to basal levels.

The plasma immunoreactive insulin levels after the two test meals are shown in Fig. 3:12. The peak level was $48.2 \pm 4.7 \text{ mU/Litre}$ and fasting levels were regained 4.5 hours after ingestion of the morning meal. The second meal peak levels were $63 \pm 5.1 \text{ mU/Litre}$ and levels were still elevated at the end of the study. Statistical analysis showed that the area under the curve was significantly larger after the second test meal ($p < 0.01$) (Table 3:7).

The plasma immunoreactive C-peptide levels after the two meals are shown in Fig. 3:13. The maximum plasma C-peptide response was $5.95 \pm 1.2 \mu g/Litre$ following the first meal. Fasting levels were $1.44 \pm 0.23 \mu g/Litre$. Levels did not return to fasting values until 5.5 hours after food ingestion. After the second meal levels peaked at $7.92 \pm 1.5 \mu g/Litre$ and had not returned to basal
Figure 3:10

Molar ratio of CP/Insulin after morning intravenous glucose tolerance test.

± SEM
Figure 3:11
Blood glucose levels in non-obese volunteers given a meal at two different times in 24 hours ± SEM
N = 5
Figure 3:12
Insulin levels in non-obese volunteers given a meal at two different times in 24 hours.

= Mean ± SEM
N = 5

mU/Litre Insulin

Meal

1000 1900 0100
Time (h)
Table 3:7 Statistical analysis of insulin results obtained during two meal study

Units mU/Litre.h

<table>
<thead>
<tr>
<th>Subject</th>
<th>Area under curve 1st meal</th>
<th>Area under curve 2nd meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>133</td>
<td>163</td>
</tr>
<tr>
<td>M</td>
<td>96</td>
<td>213</td>
</tr>
<tr>
<td>N</td>
<td>73</td>
<td>169</td>
</tr>
<tr>
<td>P</td>
<td>101</td>
<td>218</td>
</tr>
<tr>
<td>S</td>
<td>133</td>
<td>197</td>
</tr>
<tr>
<td>Mean</td>
<td>107.2</td>
<td>192</td>
</tr>
<tr>
<td>SD</td>
<td>25.8</td>
<td>25.1</td>
</tr>
<tr>
<td>SEM</td>
<td>11.5</td>
<td>11.2</td>
</tr>
</tbody>
</table>

t value 5.05  p < 0.005
Figure 3.13: C-peptide levels in non-obese volunteers given a meal at two different times in 24 hours.
the curve was significantly greater after the second meal (p < 0.0005) (Table 3:8). The peak value of blood glucose and hormones measured in this study would not necessarily reflect the true peak values because of the infrequent sampling times.

3:4:4 Study 3

Diurnal rhythms in response to test meals given at different times over twenty four hours

The mean plasma glucose levels at all six time points in the study are shown in Fig. 3:14. Peak plasma glucose levels varied with the time the meal was ingested. The maximum mean peak level reached was 9.03 ± 0.26 mmol/Litre after the 0200 h meal. The minimum mean response occurred after the 1800 h meal. The glucose levels returned to fasting levels 1.5 hours after the 1000 h test meal. After all other meals the levels took considerably longer to return to fasting levels. After the 0200 h meal levels took approximately 4.5 hours to return to basal values. Statistical analysis of the area under the curves showed that the glucose response was less at 1000 h compared with all other time, the maximum response being at 2200 h (Fig. 3:15).

The mean immunoreactive plasma insulin levels for all six meals are shown in Fig. 3:16. The mean peak level was 106.4 ± mU/Litre which was reached 90 minutes after the 2200 h meal. The mean nadir plasma insulin level occurred 60 minutes after ingestion of the 0600 h meal. The integrated response to food ingestion, defined as the period from 0 minutes until the levels returned to basal values, showed a significant increase at 2200 h compared with 1000 h (p <0.025) (Fig. 3:15). It is interesting to note that the shape of the mean insulin response curve was altered considerably depending on the time the meal was eaten.

The mean immunoreactive plasma C-peptide levels for all six meals are shown in Fig. 3:17. Calculation of the integrated ratio of immunoreactive C-peptide to insulin after each meal showed no statistical difference between the responses.

Integrated plasma immunoreactive GIP (gastric inhibitory polypeptide) responses showed a significant increase after 1400h compared with 2200h (Fig. 3:15).
Table 3:8

Statistical analysis of immunoreactive C-peptide results obtained after the meal study
Units ng/Litre.h

<table>
<thead>
<tr>
<th>Subject</th>
<th>Area under curve 1st meal</th>
<th>Area under curve 2nd meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>147</td>
<td>295</td>
</tr>
<tr>
<td>M</td>
<td>112</td>
<td>198</td>
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<tr>
<td>N</td>
<td>97</td>
<td>221</td>
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<tr>
<td>P</td>
<td>138</td>
<td>269</td>
</tr>
<tr>
<td>S</td>
<td>79</td>
<td>197</td>
</tr>
<tr>
<td>Mean</td>
<td>115</td>
<td>236</td>
</tr>
<tr>
<td>SD</td>
<td>28.2</td>
<td>44</td>
</tr>
<tr>
<td>SEM</td>
<td>12.6</td>
<td>19.7</td>
</tr>
</tbody>
</table>

$t = 11.93 \quad p < 0.0005$
Variation in plasma glucose response to a test meal given at 6 different times over a 24 hour period.
The integrated responses to a test meal at various times during 24 hour period.

<table>
<thead>
<tr>
<th>Plasma Glucose (mmol/Litre.h)</th>
<th>C-peptide (µg/Litre.h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

- **p < 0.05
- ***p < 0.02
- ****p < 0.01
- *****p < 0.001

<table>
<thead>
<tr>
<th>Insulin (mU/Litre.h)</th>
<th>GIP (ng/Litre.h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

- **p < 0.05
- ****p < 0.001
At 8 different times over 24 hours.
3:5 Discussion

Very little variation in fasting blood glucose, immunoreactive insulin and immunoreactive C-peptide levels occurred in the twenty four hour study. Fairman and Moorhouse (1967) reported no diurnal cycle in blood glucose and insulin levels in normal non-obese subjects. Their study involved blood sampling every four hours over a three day period of starvation. Other workers (Merimee and Tyson, 1974) have shown that women had lower blood glucose levels after fasting for twenty four hours. The volunteers in Merimee and Tyson's study (1974) were only sampled once in the morning and again in the afternoon. In the 24 hour study no significant difference was found when treating data from men and women separately.

Oscillations in blood glucose and B cell activity have been reported by a number of workers (Hansen and Johansen 1970, Ohneda et al. 1979, Lang et al. 1979). Lang et al. (1979) working with normal non-obese volunteers sampled every minute and found that basal insulin levels oscillated regularly at 15 minute intervals. Goodner et al. (1977) showed that, in monkeys, insulin and C-peptide levels oscillate in phase within a period of nine minutes. They demonstrated that these oscillations occurred spontaneously and disappeared with prolonged fasting. Since this oscillation is due to cyclical secretion of insulin rather than an oscillating pattern of hormone metabolism any oscillation occurring in B cell activity would, therefore, not have been detected in the twenty four hour study due to the interval between samples. The measurement of fasting insulin levels in normal non-obese subjects is very near the detection limit of most insulin radioimmunoassays. In the present study the C-peptide levels are well above the detection limit of the radioimmunoassay. One disadvantage, however, in measuring C-peptide is that short and rapid changes in B cell activity cannot be detected since the long circulating half life of C-peptide tends to mask such changes.
Fasting immunoreactive C-peptide values compare very closely with fasting C-peptide values obtained by other workers (Block et al. 1972, Kaneko et al. 1974, Heding, 1975, Beischer et al. 1976).

Obesity in man and animals is often associated with glucose intolerance (Smith and Levine, 1964) and the degree of intolerance is exaggerated by weight gain (Sims et al. 1968) and ameliorated by weight reduction (Olefsky, Reaven and Farquhar, 1974). This intolerance is almost invariably accompanied by hyperinsulinaemia (Karam, Grodsky and Forsham, 1963). Other workers (Bagdade, Bierman and Porte, 1967) reported a correlation between the degree of obesity and basal insulin levels. In the twenty four hour study fasting C-peptide levels correlated with weight and surface area in the normal subjects. Correlation was increased when fasting C-peptide levels were plotted against body weight. A positive correlation would have been expected in view of the evidence obtained from investigating insulin levels and obesity.

Peripheral fasting insulin and C-peptide levels correlated very closely, although three subjects had to be excluded from the data. High C-peptide levels and low insulin values were observed in these three subjects. Substantial amounts of insulin are removed from the blood in its initial transit through the liver, while the hepatic extraction of C-peptide is very low or negligible (Kuhl et al. 1978), the major site for the degradation of C-peptide being the kidney (Katz and Rubenstein, 1973). It has been reported (Katz and Rubenstein, 1973) that in animals, insulin, C-peptide and proinsulin are all metabolised by the kidney to some extent and elevated fasting C-peptide levels have been reported in patients with renal insufficiency (Kuzuya et al. 1976). Defective degradation and removal of C-peptide from the body by the kidneys could, therefore, explain the high levels of one of these volunteers whose creatinine clearance was found to be low. However, the other volunteers were not assessed. Other workers (Horwitz et al. 1975) have observed that insulin and C-peptide levels correlate well in the fasting and fed states. This close correlation has enabled C-peptide
concentration to be used as an indicator of B cell secretion in peripheral blood in situations where it is difficult to measure insulin itself.

The intravenous glucose tolerance study has confirmed that impairment of glucose tolerance occurs in the latter part of the day (Roberts, 1964, 1967, Bowen and Reeve, 1967, Jarrett and Keen, 1969, 1970, Abrams, 1968). In this study the k values were reduced in all subjects after the second infusion. The values obtained were within normally accepted criteria for diagnosis of diabetes mellitus based on glucose disposal rates in the early part of the day. Thus normal volunteers reacted as mild diabetics after the evening glucose infusion. Other workers (Carroll and Nestel, 1973) have also reported that after a glucose tolerance test the k values are reduced in the evening.

In the intravenous glucose tolerance study, the effect of glucose infusions in the morning and late evening on B cell activity was investigated. There was no significant difference in the integrated insulin responses after the two test meals but the C-peptide response was significantly increased after the second test. Carroll and Nestel (1973) reported significantly higher insulin levels when comparing evening and morning glucose tolerance tests. These insulin levels remained significantly higher up to 50 minutes after the start of the infusion. Blood samples were taken at two minute intervals up to 10 minutes into their study. The short half life of insulin (Samols and Marks, 1966) and the long half life of C-peptide could, therefore, explain the difference in the insulin and C-peptide results in the glucose infusion study since the time intervals between sampling in the study were considerably longer than that reported by Carroll and Nestel (1973).

The molar concentration of C-peptide is substantially greater than insulin in fasting healthy subjects (Melani et al., 1970, Block et al., 1972). The molar ratio of C-peptide to insulin at time of peak secretion was nearly equimolar in the peripheral circulation. Portal concentrations of insulin and C-peptide after stimulation by eating reach a value which closely approximates to equimolar secretion (Horwitz et al., 1975).
The six meal study was designed to determine B cell activity and plasma glucose levels following a test meal at six different times in a 24 hour period. This study has several advantages over previous reported work (Ahmed et al. 1976, Malherbe et al. 1969, Hansen and Johansen 1970, Molnar et al. 1968, 1972, Schlierf and Raetzer, 1972). The meals were identical on all six occasions and they were randomly given to each group of subjects at different times. In addition, the subjects fasted for ten to twelve hours prior to the meal and C-peptide levels as well as insulin concentrations were measured after each meal.

In this study plasma glucose responses were considerably altered depending on the time of the day the meal was ingested. Both the peak size and the shape of the postprandial response of plasma glucose were affected.

The mean integrated plasma glucose response to food showed a diurnal change. A delayed postprandial return of plasma glucose to basal levels was observed from the afternoon onwards. Other workers (Ahmed et al. 1976, Carroll and Nestel, 1973) have also observed this delayed return to fasting values in the afternoon.

Absorption of nutrients from the alimentary canal was altered during the day. The postprandial peak response of glucose was earlier in the morning compared with the early and late evening. The response of gastrointestinal hormone GIP has been reported to be dependent upon absorption of nutrients from the gut since in certain diseases with reduced nutrient absorption, for example, untreated coeliac disease or in cases of gross malabsorption there is a reduction in GIP response to oral stimuli (Creutzfeldt et al. 1976, Besterman et al. 1979). The integrated GIP response was only significantly increased in this study at 1400 h. The failure to observe any changes in this response at other times may be due to only small changes in the rate of absorption after each meal.

The similar B cell activity and increased glucose response during the six meal study suggests a progressive state of insulin insensitivity. There are a number of degrees of insulin resistance one being insulin insensitivity. Insulin resistance
may be defined as a state where a normal concentration of insulin produces a less than normal biological response. Gibson and Jarrett (1972) and Schultz et al. (1980) have reported a diurnal rhythm in insulin sensitivity. Normal subjects require more insulin to maintain normoglycaemia between 0200-0600 h (Schultz et al 1980). Beck-Nielsen et al. (1978) reported that in isolated monocytes taken from fasted young healthy subjects, insulin binding was low in the late afternoon but increased during the evening reaching a maximum about 0800 h. The decrease in insulin binding to isolated monocytes (Beck-Nielsen et al. 1978) and insulin insensitivity (Gibson and Jarrett, 1972) during the afternoon can be interpreted as evidence that the known diurnal change in insulin insensitivity might be due in part to changes in insulin binding. Insulin action at the level of the target tissue consists of a complex sequence of events the details of which are still incompletely understood. Thus a defect at any point along the cascade of events involved in the insulin action sequence could result in an insulin resistant state (Olefsky, 1976b).

The impairment of the glucose response to a meal in the evening, therefore, was probably partly due to an alteration in the alimentary tract absorption of nutrients and partly to a change in insulin sensitivity causing an impairment of glucose utilization by the tissues.

The studies have shown that there are several advantages to measuring C-peptide levels. Fasting C-peptide levels are well above the detection limit of the radioimmunoassay. Because of its slow metabolic clearance, plasma C-peptide levels remain elevated when insulin levels have returned to basal values. Blood samples can, therefore, be taken less frequently without effecting the shape of the response curve. Circulating levels of C-peptide reflect B cell secretory activity and because neither insulin nor insulin antibodies interfere with the radioimmunoassay its measurement can be of use in studying B cell function in a variety of physiological and pathological circumstances.
CHAPTER IV

CLINICAL SIGNIFICANCE OF IMMUNOREACTIVE C-PEPTIDE MEASUREMENTS
4:1 Introduction

The development of immunoassays for the measurement of serum immunoreactive C-peptide has been of great value in the investigation and diagnosis of certain clinical problems. Serum C-peptide levels correlate well with those of insulin, as the two peptides are secreted from the pancreas in equimolar amounts. In the peripheral circulation the relationship of C-peptide to insulin is approximately seven to one due to their different metabolic clearance rates. Substantial amounts of insulin are removed in the initial transit through the liver whilst the hepatic extraction of C-peptide is very low (Samols and Marks, 1966, Stoll et al. 1970). C-peptide levels are a reliable measure of B cell secretory activity after stimulation of these cells and can, therefore, be used as an indicator of endogenous insulin in the presence of exogenous insulin. The human C-peptide radioimmunoassay because of its species specificity, is unaffected by non-human C-peptide components which may be present in exogenous insulin.

4:1:1 Hyperglycaemic states

In the hyperglycaemic state, C-peptide estimations are clinically useful in the evaluation of pancreatic status after pancreatectomy, pancreatic tumour removal and in assessing residual B cell activity in diabetics. After an operation for removal of the pancreas due to nesidioblastosis or insulinoma, monitoring of C-peptide levels can indicate the presence of any residual pancreatic tissue. Removal of the tumour and long term monitoring of the C-peptide levels will indicate whether the tumour has been completely removed and the recurrence of the tumour or the presence of functional metastases. Serum insulin levels cannot always be assessed in these situations since insulin may have to be administered to control blood glucose levels.

One of the earliest uses of the C-peptide radioimmunoassay has been in monitoring B cell function in insulin dependent diabetics. The C-peptide radioimmunoassay, unlike the insulin radioimmunoassay, being unaffected by
exogenous insulin. The presence of circulating insulin antibodies in insulin
dependent diabetic interferes with the measurement of insulin levels using the
insulin radioimmunoassay (Moxmess et al. 1971). Heding (1978) showed that in
insulin treated diabetics proinsulin was bound to circulating insulin antibodies.
This binding slows the removal of proinsulin from the circulation and thus
increases its concentration in serum. The C-peptide and insulin
radioimmunoassay would both give artificially high results when assessing this
serum. The high results obtained would be a reflection of the crossreactivity of
both the antisera with proinsulin. Kuzuya et al. (1977a) have used polyethylene
glycol to precipitate the antibody bound insulin and proinsulin, thus leaving the
C-peptide measurement as a direct reflection of endogenous B cell activity.

4:1:2 Hypoglycaemic states

Human C-peptide measurements have been found to be most useful in the
evaluation of hypoglycaemic states. There are three major hypoglycaemic
situations where C-peptide measurements are of special value. Firstly, patients
may become hypoglycaemic due to surreptitious injections of insulin. Before the
development of the C-peptide assay these patients were diagnosed by detecting
the presence of insulin antibodies. This interpretation may have been incorrect
in some cases, since it has been reported (Folling et al. 1972, Hirata et al. 1972,
1974) that some subjects, that have spontaneous hypoglycaemia, have insulin
autoantibodies. Secondly C-peptide levels are useful in the detection of an
insulinoma in a diabetic in whom insulin measurements are affected by the
presence of circulating insulin antibodies. The incidence of insulinoma of the
pancreas in patients with diabetes mellitus appears to be about twice that of the
general population. Thirdly C-peptide measurement is used in the diagnosis of
insulinomas in non-diabetic patients.

There are three different types of carcinoma of the pancreas, namely
adenocarcinoma arising from ductal or acinar cell, cystadenocarcinoma and
carcinoma arising from the islet cell.
Pancreatic islets are composed of a number of distinct cell types each synthesising and secreting a specific hormone. Neoplasms may arise from any of these distinct cell types and may contain one or more different secretory cell types. The most common islet cell tumour are those which secrete gastrin (Zollinger-Ellison syndrome). The incidence of malignancy of these tumours has been reported as between 50-70 per cent. The second, most common type of tumour, is the insulinoma, which is usually benign. The incidence of this type of tumour is extremely rare. Kavlie and White (1972) reported an incidence of 1 case per million persons. It has been reported that 5-10% of these insulinomas have been found to be malignant (Van Heirden et al. 1979 Hsein-Chin et al. 1980). Other islet cell tumours such as the glucagonoma (Montenegro et al. 1980) the somatostatinoma (Ganda et al, 1977) and the vasoactive intestinal polypeptide tumours are extremely rare.

(a) Insulinomas

Insulinoma, the insulin secreting neoplasm of the beta cell was the first islet cell tumour to be recognised. The tumours are found evenly distributed throughout the pancreas as solitary or multiple, spherical to ovoid shaped nodules ranging in size from a few millimetres to several centimetres. Tumours in the body and tail account for two-thirds of the insulinomas removed at operation. On the surface of the pancreas the tumours are brown, dark blue or blue-black in appearance due to their vascularity. Ectopic insulinomas are extremely rare. Creutzfeldt et al. (1973, 1976b, 1977) have classified insulinomas by electron microscopy and histochemical studies into four types. The majority of insulinomas are type I comprised almost entirely of cells containing typical B granules. Type II tumours have some cells which contain B cell granules. The have types II and IV, a-typical granules and carcinomas with virtually agranular appearance respectively.

(i) Presenting symptoms of insulinoma patients

The presenting symptom of insulinoma is usually but not always hypoglycaemia.
The clinical manifestations are, however, frequently bizarre and may go undiagnosed for a number of years. The major symptoms are due to the effect of low blood sugar levels on the central nervous functions. The lack of the brain's major energy supply, glucose, causes it to utilize ketone bodies. A number of hours are required, in man, for this change to be effected and therefore the brain is not protected against acute hypoglycaemia. This results in symptoms which are frequently mistaken for a primary nervous disorder. The symptoms which are only transient include dizziness, coma, seizure, abrupt behavioural changes and diplopia.

The second group of symptoms encountered in patients with insulinomas are associated with the adrenaline discharge that occurs in the face of hypoglycaemia. When exogenous insulin is given to healthy volunteers the symptoms resulting include profuse sweating, nervousness, tremulousness, palpitation, hunger, and pallor all of which are relieved after glucose administration. However, these symptoms are relatively uncommon in patients with insulinomas, the reason for this being that the glucose levels decrease gradually in these individuals thus avoiding any counterregulatory response. Hypoglycaemic coma occurs in a small percentage of these patients for a short duration. Between these hypoglycaemic attacks patients are usually normal.

(ii) Localisation

As pancreatic islet cell tumours are often small lesions that become manifest, not by their tumour mass, but by the hormones they secrete, their localisation can be difficult.

The non-invasive methods in use for the detection of a tumour are computed tomography and sonography. Invasive techniques include angiography and arteriography. Angiography involves the use of a radioopaque dye which is injected into a branch of the coeliac artery. This method is capable of detecting tumours of 1.5 cm in diameter, precisely localising where the tumour is situated.
and may also detect the presence of metastases in the liver. This technique has several drawbacks in that it is complex, expensive and time consuming. Coeliac and superior mesenteric arteriography was a major advance in the localisation of islet cell tumours. The arteriograph method has a success rate of some 40-70 per cent in localisation of tumours. This technique together with measuring the hormone levels can localise various cell tumours (Ingemanssen et al. 1975, 1976, Lunderquist et al. 1978).

(iii) Dynamic function tests

The diagnosis of insulinoma requires the demonstration of a serum insulin concentration which is inappropriately high for the existing level of blood glucose. In patients suspected of having B cell tumours, two methods of detection are available. The first depends on the development in these patients of hypoglycaemia. A prolonged fast for up to 72 hours is sometimes used in order to provoke a hypoglycaemic attack. Blood samples if obtained when the patient is undergoing a hypoglycaemic attack are extremely important. The measurement of serum insulin, C-peptide and glucose can establish an initial diagnosis. Further tests can then be carried out in order to confirm this diagnosis. If an insulinoma is suspected in a patient and it is not possible to provoke fasting hypoglycaemia, a variety of tests can be used in order to obtain a diagnosis. The most useful of these is the insulin suppression test. In normal subjects, insulin secretion is inhibited in the presences of hypoglycaemia, but patients with B cell tumours do not show this inhibition. The infusion of insulin and the monitoring of plasma glucose and C-peptide levels demonstrates whether or not the patient's endogenous insulin is being suppressed. If this test gives an inconclusive result, a number of other insulin provocation tests can be carried out. However, in general provocation tests are less reliable in the diagnosis of all tumours (Marks, 1981)

Patients with islet cell tumours have a decreased storage capacity for insulin, resulting in uncontrolled release (Floyd et al. 1964). This results in increased
insulin and proinsulin levels. Proinsulin levels have been found to vary widely between patients with islet cell tumours (Rubenstein et al. 1974). The fasting plasma proinsulin concentration varied between 0.23-17.48 ng/ml. The cross-reactivity of proinsulin in both insulin and C-peptide radioimmunoassays varies considerably depending on the antisera. Care must, therefore, be taken when assessing samples from an insulinoma patient to know the degree of cross-reactivity of the antisera with proinsulin.

The work described in this chapter attempts to evaluate with specific examples the usefulness of human C-peptide estimations in the diagnosis of various diseases.

4:2 Materials and Methods

4:2:1 Plasma glucose

Plasma glucose levels were measured using hexokinase enzyme and an automatic glucose analysis.

4:2:2 Insulin Radioimmunoassay

Plasma immunoreactive insulin levels were measured in duplicate by the method described in Chapter III. The detection limit of the assay was 2.5 mU/Litre and the interassay coefficient of variation was 15% at 6.5 mU/Litre and 7.4% at 46 mU/Litres.

4:2:3 C-peptide Radioimmunoassay

Plasma immunoreactive C-peptide levels were measured in duplicate by the method described in Chapter II. The assay has a sensitivity of 10 pg/tube (0.1 μg/Litre). The interassay coefficient of variation was 24.5% at 0.44 μg/Litre and 9% at 37 μg/Litre.

4:3 Clinical Studies

4:3:1 Factitious hypoglycaemia

Four cases of factitious hypoglycaemia are described.

Patient (A) The first was a woman, 23 years, who was a nurse. This patient was investigated because of a history of unexplained fits. She displayed biochemical
hypoglycaemia on fasting associated with neuroglycopenic symptoms. Samples were taken during fasting and throughout the day and plasma immunoreactive insulin, C-peptide and glucose levels were measured (Figure 4.1). The demonstration of low plasma C-peptide with high circulating insulin and hypoglycaemia after 16 hours of fasting confirmed the diagnosis of insulin induced hypoglycaemia. For comparison, plasma glucose, immunoreactive C-peptide and insulin levels measured in ten healthy, non-obese, volunteers after a 12 hour fast are also shown in Figure 4.1.

Patient (B) The second was a 35 year old man, who was admitted into hospital in a deeply unconscious state. He was reported to have been well all day and went to bed rather early claiming he was tired. Unable to rouse him, his wife called for an ambulance. On examination patient (B) was found to be deeply unconscious and unrouseable. Investigation revealed that his plasma glucose was 1.5 mmol/Litre. He was immediately put on a glucose drip and regained consciousness within a few hours. Extensive tests were carried out including prolonged fasting with heavy exercise in order to provoke a hypoglycaemic attack. Hypoglycaemia in no instance could be established.

He was then discharged from hospital only to be re-admitted complaining of vomiting, intermittent diplopia, feeling weak and drowsy, having slurred speech and bouts of sweating. He was discharged again from hospital.

He was readmitted into hospital in a coma when his plasma glucose level was found to be 0.27 mmol/Litre. Thirty-six hours after the start of glucose infusion he regained consciousness. A further 72 h fast was instituted and blood samples were taken for glucose estimation, none of which fell below 3.6 mmol/Litre. A sample for immunoreactive C-peptide and insulin estimation was obtained from this patient when in the second coma. Immunoreactive C-peptide level was undetectable with insulin estimation of 109 mU/Litre. These results together with the low plasma glucose levels are diagnostic of insulin induced hypoglycaemia.
Plasma immunoreactive C-peptide, insulin and glucose levels in a patient (patient A ●●●) with factitious hypoglycaemia undergoing a 16 hour fast.

\[ \overline{x} \pm SD \text{ in 10 normal subjects after 12 h fasting} \]

**C-Peptide (ug/l)**

- Time (h): 12, 14, 16
- Values: 1.0, 1.2, 1.4, 1.6

**Insulin (mU/l)**

- Time (h): 12, 14, 16
- Values: 60, 60, 60

**Glucose (mmol/l)**

- Time (h): 12, 14, 16
- Values: 4.0, 2.0, 4.0
It was subsequently discovered that the patient obtained insulin from his brother-in-law who was a diabetic.

Patient (C) This patient was a 19 year old woman, whose insulin requirements had steadily increased until she was taking a total of 120 units daily when she was admitted into hospital. She was changed to a monocomponent insulin and her requirement fell to 3 units of Actrapid per day. This large decrease in insulin requirement when changing to monocomponent insulin is unusual. She was admitted into hospital for investigation. On two occasions she had hypoglycaemic attacks with plasma glucose levels of 2 and 1.5 mmol/Litre. The immunoreactive insulin levels were greater than 200 mU/Litre with undetectable immunoreactive C-peptide levels on both occasions. No insulin had been given to the patient for twelve days. These results suggest that the patient was causing these hypoglycaemic attacks by insulin administration. However, no evidence of such administration could be obtained from the patient.

She was admitted into hospital on a subsequent occasion in a hypoglycaemic coma and at that time an ampoule of insulin was found hidden in one of her stockings.

Patient (D) A 24 year old man was investigated. He had a history of blackouts which usually occurred before meals when he was sitting down. These blackouts only lasted a few seconds but when regaining consciousness, he felt cold and sweaty. There was no family history of diabetes and no previous illnesses. His general health was good although he did complain of increased thirst. On admission into hospital he had several faints in the ward but plasma glucose estimation on a blood sample taken during one of these attacks was found to be 6.4 mmol/Litre. He was discharged from hospital without medication.

The patient was reported to be injecting himself with insulin and also taking dextrose tablets, lots of lucozade and behaving in an odd manner. He stated in a letter to his doctor that he had been prescribed insulin at the diabetic clinic. At no time had anyone in the clinic prescribed insulin for him. The patient was seen
at the diabetic clinic again and it was emphasised that he should not continue to inject himself with insulin as he was not a diabetic.

On the next occasion at the clinic a plasma glucose estimation of 6 mmol/Litre was recorded, the patient collapsed 30 minutes after this sample was taken. A blood sample was taken for plasma glucose, immunoreactive C-peptide and insulin estimation. The plasma glucose level was 2 mmol/Litre with an immunoreactive insulin level of greater than 200 mU/Litre and 1.1 µg/Litre immunoreactive C-peptide level. These results suggest that the patient was still continuing to self-administer insulin.

4:3:2 Insulinomas

There are generally three distinct stages in the diagnosis of an insulinoma:

(i) Investigation of the patient's clinical history
(ii) Confirmation of the suspected diagnosis by clinical investigation.
(iii) Localization of the tumour (Marks, 1981).

The following case studies demonstrate the usefulness of C-peptide measurement in insulinoma diagnosis.

Patient (E) A 32 year old woman was admitted into hospital for investigation. Eighteen months previously she began to lose consciousness for short periods, this being associated with dieting. The unconsciousness could be prevented by drinking sweet coffee. The patient had no allergies and was not receiving any medication. She appeared normal except for a six to seven stone increase in weight since the birth of her child. On admission to hospital a series of tests were carried out to investigate her condition. Blood samples were taken throughout the tests and assessed for plasma glucose, immunoreactive insulin and C-peptide levels.

(1) Insulin Stress test (0.15 U/kg body weight)

Plasma glucose levels were low (1.0 mmol/Litre) at the start of the insulin stress test (Fig. 4:2). The plasma glucose levels altered only slightly throughout the study. Immunoreactive C-peptide levels fell from a basal level of 1.9 µg/Litre
Glucose mmol/l

0.15U/kg bw insulin

C-Peptide ug/l

0.15U/kg bw insulin

$x \pm SD$ in normal subjects
to 0.9 µg/Litre 100 minutes from the start of the test. In ten normal, non-obese, volunteers the mean basal level of immunoreactive C-peptide was $1.85 \pm 0.6$ µg/Litre ($\bar{x} \pm SD$), which fell to below 0.9 µg/Litre after 60 minutes and $0.63 \pm 0.34$ µg/Litre after ninety minutes. The mean plasma glucose level in the normal non-obese volunteers reached $1.37 \pm 0.5 \text{ mmol/Litre}$ after 30 minutes and returned to 75 per cent of the basal level 120 minutes after the insulin injection.

(2) Calcium infusion test (10 mg Ca²⁺/kg infused 0-10 minutes)

The data from the calcium infusion test (Patient E) is shown in Figure 4:3. The plasma glucose level fell from the basal value of 1.95 mmol/Litre to 1.1 mmol/Litre 45 minutes from the start of the infusion. The basal immunoreactive insulin level was 9 mU/Litre but rose to 21 mU/Litre 45 minutes after the start of the infusion. Rises in immunoreactive C-peptide levels were also recorded.

(3) Oral glucose tolerance test (100 g Hycal)

The data from the oral glucose tolerance test is shown in Figure 4:4. Basal plasma glucose level in the patient was 1.15 mmol/Litre. After the glucose load plasma glucose levels reached a maximum of 5.3 mmol/Litre and remained elevated up to 210 minutes after the oral glucose. Levels returned to basal 300 minutes after the start of the test. Basal immunoreactive C-peptide level was 1.95 µg/L and fasting values were not reached by the end of the study.

The plasma glucose and immunoreactive insulin levels after a 100 g oral glucose tolerance in normal non-obese volunteers are shown in Fig. 4:5. The mean fasting plasma insulin levels was $6.64 \pm 1.15 \text{ mU/Litre}$ ($\bar{x} \pm \text{SEM}$). The plasma insulin levels did not return to fasting values until 300 minutes. Glucose levels returned to fasting values only 120 minutes after the start of the test.

(4) Diazoxide infusion test (300 mg I.V. 0-30min)

A diazoxide infusion test was carried out in this patient. This test was carried out to investigate whether the patient could be controlled by diazoxide if the tumour could not be removed. The results showed that immunoreactive insulin levels fell from a basal level of 15 mU/Litre to <1 mU/Litre after 30 minutes.
A Calcium Infusion Test carried out in a patient (patient E) suspected of having an insulinoma.

Graph showing changes in glucose, C-peptide, and insulin concentrations over time (0-110 min) after infusion of 10 mg Ca²⁺/kg.

Figure 4:4
Oral Glucose Tolerance test in normal non-obese volunteers

Figure 4:5
Oral Glucose Tolerance test in normal non-obese volunteers
Immunoreactive C-peptide levels fell while plasma glucose rose during the infusion. The data from these tests indicated that the patient had a B cell tumour. She underwent surgery and a 2 cm tumour in the tail of the pancreas was removed. Patient (F) this patient was a 43 year old man, who had an eighteen month history of episodes of feeling drunk and unsteady. These attacks were usually precipitated by physical exertion or anxiety but were not related to meal-times or alcohol. These attacks occurred about four times a week. Each episode began with numbness of the lips and tongue, followed by blurred vision and was relieved by taking sweets. No sweating, palpitation, loss of consciousness or fits had occurred in the last six months. He used to drink heavily until three months prior to hospital admission when he found that abstinence decreased the frequency of these attacks. A prolonged fast was carried out with the measurement of B cell hormones and plasma glucose levels. (Fig. 4:7) Plasma glucose levels fell from a basal level of 4.4 mmol/Litre to 1.3 mmol/Litre after 24 hours. Immunoreactive plasma insulin levels, initially was 23 mU/Litre fell to 4 mU/Litre before rising to 13 mU/Litre after 22 hours. Immunoreactive plasma C-peptide levels fell to a level of 1.7 µg/Litre after 34 hours of fasting. In view of this data the patient was diagnosed as having an islet cell tumour.

An angiogram revealed a 2 cm vascular mass at the junction of the body and the head of the pancreas. The tumour was removed and the patient recovered.

Patient (G) A 76 year old woman was admitted into hospital after hypoglycaemic attacks which occurred two or three times per week. These attacks seem to be preceded by headaches and could be prevented by sitting down. The patient underwent a full physical examination and was found to have lost three stones in weight in one year although she had a good appetite. When a random blood sample was taken the plasma glucose level was 1.3 mmol/Litre with immunoreactive insulin and C-peptide levels of 19 mU/Litre and 5.9 µg/Litre respectively. A prolonged fast was initiated with two-hourly blood
Figure 4:6

An Diazoxide Infusion test in a patient (patient E) suspected of having an insulinoma.

Diazoxide 300 mg infused 0–30 min

- Glucose
- Insulin
- C-Peptide

Glucose mmol/l

Insulin mU/l

C-Peptide ug/l

0 10

0

0 2

60 (min)

120
A prolonged fast in a patient suspected of having an insulinoma (Patient F)

C-Peptide μg/l

Insulin mU/l

Glucose mmol/l

x±SD in 10 normal subjects after 12h fasting
sampling (Fig. 4:8). The plasma glucose level fell to below 2 mmol/Litre after two hours and remained low throughout the study. Immunoreactive plasma insulin and C-peptide levels remained elevated throughout the study. This data indicated the presence of a B cell tumour. An EMI scan was carried out but no tumour was visible. Plasma glucose levels were found to be low and she was prescribed diazoxide and chlorothiazide. Her plasma glucose levels stabilized and she was discharged from hospital. Three months later she was readmitted because of hypoglycaemic episodes at home which resulted in loss of consciousness. The patient underwent surgery and a small nodule was removed from the tail of the pancreas. After the operation problems arose due to variable blood pressure and elevated plasma glucose levels. The patient died two days later and a post mortem report stated that the cause of death was intraduodenal blood loss following partial pancreatectomy.

Patient (H) The patient was a 43 year old male who complained of episodes of suddenly feeling intoxicated while driving. These attacks had recently been accompanied by impairment of consciousness, hallucination, odd behaviour and poor recollection of events. These attacks could be alleviated by food. A blood sample taken during one of these attacks revealed a plasma glucose level of 1.6 mmol/Litre. Plasma immunoreactive insulin and C-peptide levels were 22 mU/Litre and 2.8 μg/Litre respectively. These results indicated that this patient had a B cell tumour. A coeliac angiogram showed a tumour in the left lobe of the liver. A tumour was subsequently removed from the tail of the pancreas and a haemangioma removed from the liver. The tumour from the pancreas was histologically examined confirming the diagnosis of an islet cell tumour.

Patient (J) This patient was a 79 year old man who had been having attacks of spontaneous hypoglycaemia for the last two to three years. He had previously been aggressive and had lost consciousness in the morning prior to admission into hospital. A blood sample was taken and the plasma glucose level was found to be 1.5 mmol/Litre. He was immediately given glucose and after a few hours
C-Peptide ug/l

Insulin mU/l

Glucose mmol/l

\[ x \pm SD \text{ in 10 subjects normal fasting after 12 h} \]
regained consciousness. Fasting plasma glucose levels were monitored for the next three days and found to be above 2.5 mmol/Litre. He was discharged from hospital and was given no medication.

He was readmitted into hospital after another attack. A blood sample was taken, plasma glucose level was 1.8 mmol/Litre with immunoreactive C-peptide and insulin levels of 1.6 µg/Litre and 22 mU/Litre respectively. This data indicated that the patient hypoglycaemia was due to a B cell tumour. Due to the age of the patient it was decided to control the hypoglycaemia with diazoxide. The patient remained stabilised with fasting plasma glucose levels of 3.5 mmol/Litre.

Patient (K) A 74 year old man with a history of hypoglycaemic attacks over a two-year period was admitted into hospital. During these attacks the patient felt intoxicated, confused and suffered dizzy spells. He had lost consciousness several times during these attacks. The patient was in good health apart from angina two years previously. In hospital he had an unsteady gait and slurred speech. His plasma glucose level was 1.6 mmol/Litre on a random blood sample. He was, therefore, kept in hospital for further investigations.

(1) Prolonged oral glucose tolerance test (75 g Hycal)

The basal plasma glucose levels was 3.2 mmol/Litre and was not regained until 300 minutes after glucose ingestion (Figure 4.9). Basal immunoreactive insulin and C-peptide levels were 6 mU/Litre and 3.5 µg/Litre respectively. Immunoreactive insulin levels did not return to basal levels until 300 minutes had elapsed. Immunoreactive C-peptide levels were elevated throughout the test.

(2) Diazoxide infusion test (300 ng/h)

A diazoxide infusion test was carried out on this patient. The results showed that immunoreactive insulin levels fell from a basal level 8 mU/Litre to 2.5 mU/Litre in 30 minutes (Fig. 4:10). Immunoreactive C-peptide levels fell while plasma glucose levels rose from 0.9 mmol/Litre to 3.6 mmol/Litre by the end of the infusion.
Figure 4:9 A prolonged Oral Glucose Tolerance Test (75g Hycal) in a patient (Patient K) suspected of having an insulinoma.

Glucose mol/l

Insulin mU/l

C-Peptide ug/l
Fig 4.10  A Diazoxide Infusion test in a patient (Patient K) suspected of having an insulinoma

Diazoxide infusion 300 mg/h

- Glucose mmol/l
- Insulin mU/l
- C-Peptide μg/l
The data from this test indicated that the patient had a B cell tumour that could be controlled by diazoxide. In view of his age he was given diazoxide and chlorothiazide and discharged from hospital.

He was soon readmitted during a hypoglycaemic attack with a plasma glucose level of 2 mmol/Litre. He was maintained on a high carbohydrate diet and given dextrose tablets. He underwent surgery and a tumour was removed from the tail of the pancreas. The patient, however, died 5 days later in cardiac failure due to ischaemic heart disease. Histological examination of the tumour showed that it was mainly composed of B cells.

Patient (L) This patient, a 69 year old woman, had a two year history of episodes of feeling unwell. During these episodes the patient became confused and had impairment of consciousness, she was unable to recollect events and walked as if intoxicated. The patient had lost consciousness completely on two occasions. These attacks became more frequent occurring every one to three weeks. The patient had not tried eating to relieve the symptoms. She had also been suffering from frequent headaches, recurrent attacks of diarrhoea with resulting weight loss. The patient was investigated in hospital.

1. **Prolonged oral glucose tolerance test (75 g Hycal)**
   A prolonged oral glucose tolerance test was carried out (Figure 4:11). The basal plasma glucose levels was 2.5 mmol/Litre and was regained after 150 minutes. Basal immunoreactive insulin and C-peptide levels were 2.2 mU/Litre and 4 μg/Litre respectively. Peak immunoreactive insulin and C-peptide levels occurred 90 minutes after the oral glucose load.

2. **Insulin stress test (0.15 U/kg insulin)**
   The basal immunoreactive C-peptide level prior to the insulin stress test was 4.5 μg/Litre (Fig. 4:12) and fell to 1.7 μg/Litre 120 minutes after the start of the test with a fall in plasma glucose level to 1.6 mmol/Litre. Immunoreactive C-peptide did not fall below 1.6 μg/Litre during the test.
A prolonged oral glucose tolerance test (OGTT) in a patient (Patient L) suspected of having an insulinoma.
Glucose mmol/l x ±SD in 10 normal subjects

C-Peptide ug/l

Time (min)
(3) Prolonged fast followed by exercise

Immunoreactive C-peptide and insulin levels were initially high and remained so throughout the fasting and exercise period (Fig. 4:13). The plasma glucose level after two hours was 2.5 mmol/Litre and continued to fall to 2.1 mmol/Litre after 6 hours.

This data indicated that the patient had a B cell tumour which was subsequently removed from the head of the pancreas.

4:3:3 Renal Failure

The patient was a 34 year old male with chronic renal failure presumably from childhood glomerulonephritis. This renal failure became clinically apparent at the beginning of 1978 when regular haemodialysis was commenced.

The patient presented with symptoms of hunger, sweating and dizziness. These symptoms were relieved by eating, especially carbohydrates. These attacks occurred when the patient was undergoing dialysis when hypertension also occurred. The patient's drug regimen consisted of triazolan, diazepam and alucaps. He was suspected of having a B cell tumour and an insulin stress test was performed.

(1) Insulin stress test (0.1 U/kg body weight)

Blood samples were taken at half-hourly intervals over a three-hour period following administration of 0.1 U/kg body weight of insulin (Fig. 4:14). The plasma glucose level fell to 1.5 mmol/Litre after 30 minutes and a level of 3.6 mmol/Litre was reached 120 minutes after the insulin injection. In ten normal volunteers mean plasma glucose levels fell to 1.4 ± 0.5 mmol/Litre ($\overline{x} \pm SD$) and rose to a level of 2.9 ± 1.04 mmol/Litre after 120 minutes. The basal C-peptide level in this patient was 8.7 $\mu$g/Litre considerably higher than in the normal volunteers (1.64 ± 0.6 $\mu$g/Litre). The patients levels fell throughout the test to 4.9 $\mu$g/Litre.

It was concluded that in patients with renal failure who are suspected of having an insulinoma, monitoring C-peptide levels has a limited value.
Fig 4:13  A prolonged fast followed by exercise in a patient (Patient L) suspected of having an insulinoma.
C-Peptide ug/l

\[ \bar{x} \pm SD \] in 10 normal subjects: 0.15 U/kg bw

Glucose mmol/l

Time (min)
Drug Interference

The patient, a 67 year old woman was admitted unconscious into hospital with a plasma glucose level of 1.4 mmol/Litre. An intravenous dextrose infusion was immediately started and the patient regained consciousness within thirty minutes.

The patient had previously been undergoing treatment for tuberculosis of the spine which had developed 5 years earlier. At the time of admission she was receiving rifinah which contains rifampicin and isoniazid.

A 48 hour fast was initiated after which a blood sample was taken. Plasma glucose level was 1.4 mmol/Litre and immunoreactive C-peptide and insulin levels were undetectable and <1 mU/Litre respectively. These results indicated that although the plasma glucose level was low, the plasma insulin and C-peptide levels were depressed making the diagnosis of an insulinoma unlikely. It was concluded that her hypoglycaemic episodes were probably caused by isoniazid medication which has been reported to depress circulating glucose levels (Marks 1981a).

Comparison of fasting insulin, C-peptide and glucose levels in factitious hypoglycaemia, insulinoma patients and normal subjects

Fasting immunoreactive C-peptide, insulin and glucose levels were measured in 14 insulinoma patients, 1 child with nesidioblastosis, 6 factitious hypoglycaemic patients and 28 normal subjects. Figure 4:15 shows the comparison of plasma immunoreactive C-peptide levels versus plasma glucose levels in these subjects. A significant correlation was observed in normal subjects (p < 0.05) but not, however, between glucose and insulin levels. The molar ratio of insulin/C-peptide levels is shown in Figure 4:16. Similar ratios were observed in insulinoma patients and normal subjects except the malignant insulinoma patient who had an elevated ratio. The ratio was considerably elevated in patients with factitious hypoglycaemia.
Fig 4.15  Comparison of fasting plasma immunoreactive C-peptide and plasma glucose levels in fasted normal subjects and patients

- Insulinoma patients
- Factitious hypoglycaemic patients
- Normal subjects
- Nesidioblastosis after pancreatomy

Corr 0.45
Intercept 0.53
Slope 0.236
N=27
- Disseminated insulinoma
- Nesidioblastosis after pancreatectomy.

Insulinomas  Factitious hypoglycaemia  Normal subjects
Discussion

The symptoms of hypoglycaemia can occur in a wide variety of diseases. They can be categorized into fasting or stimulated (reactive) hypoglycaemia. In reactive hypoglycaemia a pathologically low glucose concentration occurs in response to food whilst in order to precipitate fasting hypoglycaemia, starvation for a period of a few hours or longer is necessary. Patients with fasting hypoglycaemia may also exhibit a stimulated component, whereas patients with reactive hypoglycaemia never have symptoms when food is withdrawn. There are two causes of hypoglycaemia in the fasted state. Either the liver is not producing enough glucose to meet ordinary demands or peripheral glucose utilization becomes so great that maximal hepatic production is insufficient to match glucose egress from the plasma compartment.

Fasting hypoglycaemia is usually investigated with the recognition and biochemical demonstration of hypoglycaemia and then the diagnosis of the cause of the symptoms. A doctor very rarely observes a patient when undergoing such an attack and the initial diagnosis is usually made on the patient's clinical history. Care must be taken to obtain details of hypoglycaemic attacks from relations since the patients are often incorrect regarding the nature, severity and duration of symptoms because of amnesia. A number of provocative tests are carried out in order to substantiate the initial diagnosis or to reduce its likelihood. The order in which these tests are carried out are based on the previous history of the patient.

1. Factitious hypoglycaemia

One of the commonest causes of hypoglycaemia in practice is therapeutically administrated antidiabetic agents, insulin and the sulphonylureas (Goodman, 1953, Marble, 1959). Factitious hypoglycaemia due to surreptitious injection of insulin (Ibbertson and Doak, 1961, Couropmitree et al. 1975, Safrit and Young, 1978) and sulphonylureas (Walfish et al. 1975, Jordan et al. 1977) is uncommon but is an important cause of spontaneous hypoglycaemia. This syndrome is almost as uncommon as insulinomas (Jordan et al. 1977) and the purpose is
usually attention seeking. One of the patients (patient D) reported in this chapter suffered from delusions about his diabetic status and similar cases have been reported in the literature (Odei 1968). Since the advent of the C-peptide measurement the diagnosis of such patients is relatively simple but the management of such a syndrome involves psychiatric and social readjustment. This syndrome is commonly associated with women between the ages of twenty and forty who know the side effect of insulin. The few men discussed with this syndrome have been diabetics, tramps and down and outs (Creutzfeldt et al. 1969, Jordan et al. 1977) although one of the men (patient D) reported in this chapter did not fall into any of these categories. In the four cases reported all subjects had high insulin levels with inappropriately low or undetectable C-peptide levels in the presence of hypoglycaemia. Similar results have been reported in the literature (Service et al. 1974, Couropmitree et al. 1975). Ibbertson and Doak (1961) reported that such patients have a delayed insulin response to intravenous glucose, glucagon and tolbutamide due to surreptitious self-administration of insulin.

Factitious hypoglycaemia can also be caused by surreptitious administration of sulphonylureas or other oral anti-diabetic agents. In this situation C-peptide measurement is not as helpful in diagnosis as hypoglycaemia occurs associated with elevated levels of both insulin and C-peptide. Provocation tests, in these patients, such as intravenous infusion of glucose, glucagon and L-leucine usually result in an excessive and rapid rise in plasma insulin levels (Jordan et al. 1977, Alberti et al. 1972, Walfish et al. 1975). Harrop et al. (1982) have reported a patient with factitious hypoglycaemia due to chronic glibenclamide ingestion whose C-peptide level failed to be suppressed after an insulin stress test. The response to insulin was similar in this patient compared with a patient with an islet cell tumour. It is, therefore, essential, in order to confirm a diagnosis, to take a blood sample from such patients to monitor for any antidiabetic agents.
(2) Drug induced hypoglycaemia

A number of other drugs apart from therapeutic agents used in diabetes, have the ability to cause hypoglycaemia in patients. Care must, therefore, be taken to record a clinical history noting all drugs the patient has been prescribed.

Another hypoglycaemic agent besides those used for therapeutic effect is alcohol. Salicylates have a hypoglycaemic effect which is increased when taken with sulphonylureas (Setzer, 1972). Care must therefore be taken to note the drug combination that patients are prescribed. Other drugs which are known to cause hypoglycaemia under various conditions include paracetamol (Ruvalcada et al. 1966) propranolol (Skinner & Misbin, 1976) and barbiturates (Hunter and Greenberg, 1954). A patient with drug induced hypoglycaemia has been reported in this chapter. The patient had been prescribed rifinah which contains isoniazid. Isoniazid has been reported by Marks (1981a) to cause spontaneous hypoglycaemia in patients. Rifinah was therefore withdrawn from this patient.

(3) Insulinomas

Hypoglycaemia caused by an insulin secreting tumour is rare but following its natural course is a pernicious clinical entity. The diagnosis of an insulinoma is suspected on the basis of previous clinical history and results from biochemical tests. The commonest problem in differential diagnosis experienced by clinicians unfamiliar with spontaneous hypoglycaemia is the difference between insulinoma patients and patients with reactive hypoglycaemia (Marks, 1981). Tumours of the islets of Langerhans produce hypoglycaemia as their major clinical manifestation because of their ability to secrete insulin inappropriately rather than in large amounts.

The neuroglycopenic symptoms experienced by the insulinoma patients cover a wide range of deranged central nervous functions, ranging from impairment or loss of co-ordination of various autonomic, sensory or motor activities to changes in behaviour patterns, personality and intellectual qualities. Many workers (Marks, 1965, Laurent et al. 1971, Gressner et al. 1973, Marks
and Samols, 1974) have reported similar symptoms in patients with islet cell tumours. These symptoms in the patients can be alleviated in some cases by eating frequently and can be aggravated by abstinence from food. Weight gain was associated with the occurrence of the symptoms in patient E, reported in this chapter, who had previously associated her attacks with lack of food. These symptoms may go unrecognised for periods of greater than five years (Marks, 1981). It has been reported by Stefanini et al. (1974) that only one-third of 1067 patients were diagnosed in the first year of onset of their symptoms.

Neuroglycopenic symptoms in a patient may be due to an insulin producing tumour and therefore the first aim is to demonstrate an association of the patient’s symptoms with fasting hypoglycaemia. The demonstration of hypoglycaemia due to fasting and of relief of symptoms by glucose remains the keystone of the diagnosis of an insulinoma as first proposed by Wipple (1944). Hypoglycaemia may only be slight or insufficient to cause neuroglycopenic symptoms after an overnight fast in some subjects due to frequent occurrence of low plasma glucose levels. A prolonged fast of up to 72 hours or more, accompanied by moderate exercise may be necessary to precipitate neuroglycopenic symptoms and is usually carried out in patients suspected of having an islet cell tumour. Johansen (1979) reported that patients with fasting hypoglycaemia usually developed neuroglycopenic symptoms as well as chemical hypoglycaemia during the first twelve to thirty six hours of the prolonged fast. Hypoglycaemia due to an insulin producing tumour is distinguished from other diseases causing hypoglycaemia by the inappropriately high plasma insulin and C-peptide levels. Insulinomas secrete insulin in short spurts and frequent measurement of insulin levels are required to demonstrate inappropriately high levels in peripheral blood. C-peptide with its long half-life and larger pool size is less likely to show such marked changes in plasma concentration over a short period and is probably the B cell product of choice to measure in cases of suspected insulinomas. The prolonged fast has now been superceded, in most
cases, by the insulin stress test. The prolonged fast has the disadvantages of being tedious and expensive although in some cases as in patient G it may be the only test that is available.

In the absence of fasting hypoglycaemia, if a patient is suspected of having an insulinoma a suppression test such as the insulin stress test can be advocated in which plasma glucose and immunoreactive C-peptide levels are monitored. (Turner and Johnston, 1973, Turner and Heding, 1977, Service et al. 1977.) The insulin stress test is simple to carry out and can be accomplished without hospitalization of the patient. In the normal subject insulin produces marked suppression in C-peptide levels together with decreased plasma glucose level, C-peptide levels remaining low for approximately sixty minutes. Patients E and L underwent insulin stress tests and impaired suppression of immunoreactive C-peptide was observed compared with normal subjects confirming the diagnosis of islet cell tumours in these patients. This impairment of suppression is due to the lack of feedback inhibition of insulin on the B cells of the tumour. The regulation of insulin secretion is normally mediated by stimulatory and inhibitory signals depending on changes of glucose concentration and modulating effects of various hormonal and non-hormonal factors. In insulinomas the secretion is inappropriate to the stimulus and the ensuing hypoglycaemia fails to turn off further insulin secretion.

Measurement of immunoreactive C-peptide and insulin levels prior to the tests are important since some insulinomas are histologically undifferentiated and secrete proinsulin rather than C-peptide and insulin (Melani et al. 1970, Lazarus et al. 1972, Creutzfeldt et al. 1973, Rubenstein et al. 1974). Gutman et al. (1973) reported a patient with a malignant tumour which consisted of 89% proinsulin. Turner and Heding (1977) reported that following an insulin suppression test, monitoring C-peptide levels is sufficient to diagnose most insulinomas but that a proinsulin assay is necessary to detect most undifferentiated adenomas. Raised plasma proinsulin levels after an overnight
fast have been reported by a number of workers (Turner and Heding 1977, Service et al. 1977) in patients with insulin producing tumours. A low C-peptide level, therefore, does not exclude the possibility of an insulinoma because of the low crossreactivity of human proinsulin in most C-peptide assays. However, crossreactivity of proinsulin in the C-peptide assays has been observed to vary (Cargill et al. 1980). The commonest cause of false positive C-peptide suppression tests is the failure to achieve hypoglycaemia of a sufficient degree and duration to permit the plasma C-peptide levels to fall (Marks, 1981).

There are two other suppression tests which are sometimes used to diagnose an islet cell tumour. The diazoxide test has been found to be useful (Le Quesne et al. 1979, Marks, 1981.) in assessing, prior to removal of an insulinoma, whether the patient can be controlled by diazoxide, so that if the surgeon is unable to resect the tumour he may not have to resort to a blind subtotal pancreatectomy. In both patients (patients E and K) reported in this chapter the infusion of diazoxide produced a decline in insulin levels with a corresponding fall in C-peptide values and a rise in plasma glucose levels. Both patients could therefore be controlled by diazoxide if the tumours were not found during surgery. Diazoxide is a hyperglycaemic thiazide which inhibits insulin release from the B cells of the pancreatic islets by incompletely inhibiting insulin synthesis and/or storage. (Creutzfeldt et al. 1969). Creutzfeldt et al. (1969) have shown that as a result of diazoxide the granules containing the insulin in the B cell undergo intracellular digestion.

The infusion of somatostatin as a diagnostic suppression test has been investigated in a few insulinoma patients (Christensen et al. 1975, Kerner et al. 1980) but results have proved unpredictable.

Provocation or stimulation tests of insulin secretion are less reliable in the diagnosis of islet cell tumours than suppression tests and prolonged fasts. Provocative agents include tolbutamide, leucine, glucagon, arginine and glucose.
They are used, nowadays only to investigate difficult cases in which they may provide additional information. A minority of malignant tumours do not respond to the provocation tests. The benign and better differentiated the tumour the more likely it is to respond favourably to suppression by diazoxide and somatostatin (Marks, 1981.)

The calcium infusion test detects inappropriate insulin release due to a defect in the secretory mechanism control in patients with islet cell tumours. Calcium infusion has been known for some years to stimulate the release of gastrin, calcitonin and at times 5 hydroxy tryptamine in patients with certain types of endocrine tumours (Passaro et al. 1972). An infusion of calcium gluconate causes insulin release and a fall in blood glucose concentration in patients with insulin secreting tumours (Kaplan et al. 1972, Frerichs and Creutzfeldt 1976, 1980) but no changes were observed in healthy volunteers (Gaeke et al. 1975). This has led to its use as a test for insulinomas either alone (Ohneda et al. 1975) or with intravenous glucose (Frerichs and Creutzfeldt 1976, 1980). Frerichs and Creutzfeldt (1976) reported that calcium given intravenously with glucose augmented the insulinaemic response to hyperglycaemia and was positive in approximately 65 per cent of insulinoma cases. However, this test does not produce consistent results and its value in the diagnosis of insulin-secreting tumours of the pancreas remains in question. In the series of patients described in this chapter this test was used in a single case. Patient E demonstrated elevated insulin and C-peptide levels and a fall in plasma glucose level due to the calcium infusion. Due to the positive result in this test together with the results from other tests it was possible to diagnose that the patient had an islet cell tumour.

The oral glucose tolerance test is indispensible for the differentiation of the common functional reactive (post absorptive) hypoglycaemia, but is of no use in the diagnosis or differential diagnosis of fasting hypoglycaemia (Marks, 1981). Misinterpretation of results from extended oral glucose tolerance tests
has lead to an epidemic of "non-hypoglycaemia" which is prevalent in the U.S.A. (Yager and Young, 1974). Reactive hypoglycaemia occurring during an extended oral glucose test is not at all unusual in conditions characterised by fasting hypoglycaemia in the case of an insulinoma patient. In the two cases reported in this chapter plasma glucose levels of below 2 mmol/Litre occurred during the oral glucose test. Neither the shape, magnitude nor duration of the initial hyperglycaemic phase provide help in determining the aetiology of the reactive hypoglycaemia. Insulinoma cells are generally relatively insensitive to a glucose stimulus, the sensitivity decreasing with the degree of functional dedifferentiation.

Glucagon is a potent stimulator of insulin release but also one of the most powerful glycogenolytic agents known and it can be used to test insulin secretion and glycogen reserves (Marks, 1960, Marks and Samols, 1968, Kumar et al. 1974). The glucagon provocation test can be carried out in two ways; either after an overnight fast or in an attempt to terminate a spontaneous hypoglycaemic attack. The interpretation of results is different after the two tests. In the case reported in this chapter, reactive hypoglycaemia in the patient was immediately terminated by intravenous glucagon. The glucagon caused an immediate rise in plasma glucose and insulin levels. Kumar et al. (1974) have reported a similar exaggerated rise in plasma insulin levels within 5-10 minutes in 70 per cent of the insulinoma patients, after which time insulin levels fell very rapidly. After an overnight fast insulinoma patients given glucagon, experience symptomatic rebound hypoglycaemia after an initial exaggerated rise in plasma insulin levels. Exaggerated insulinaemic responses to glucagon are shown in a number of other conditions, such as obesity, acromegaly and Cushing's syndrome, but none of these conditions can be clinically confused with insulinomas. Fajans and Floyd (1975) reported that 58% of patients with proven islet cell disease had abnormal glucagon responses.
The remaining provocation tests such as the L-leucine and tolbutamide test are very rarely used due to the significant percentage of false negatives reported (Wolfsdorf and Senior, 1979, Fajans and Floyd, 1975.)

The major site of removal of C-peptide from the body is the kidneys (Katz and Rubenstein, 1973, Horwitz et al. 1977). Patients with hypoglycaemia with chronic renal failure cannot be investigated by the measurement of C-peptide levels. Care must be taken to investigate the clinical history of the patient in order to exclude tests which would be of no practical use. In the case reported, in this chapter, the insulin suppression test resulted in abnormally high C-peptide levels yet plasma glucose levels were within the normal range. Metabolism and removal of C-peptide was presumably impaired during this test due to the patients poor renal function. Other tests which allow the measurement of insulin should be used to investigate such a patient and establish a diagnosis.

Removal of the insulinoma by surgery is the first and obvious choice of treatment for most patients. In some cases, either because of the inability to localise the tumour or the age of the patient, medical treatment is preferred. A number of workers (Marks, 1971, Marks and Samols, 1974, Le Quesne et al. 1979) have recommended that patients undergo a diazoxide test, in order, to determine whether hypoglycaemia can be controlled with this treatment. Failure to find the tumour at operation should not, therefore, result in blind distal pancreatectomy but closure of the abdomen when the patient can then be controlled with diazoxide. Mengoli and Le Quesne (1967) and Stefanini et al. (1974) showed that the majority of occult tumours are found in the head of the pancreas. Diazoxide, is a potent hyperglycaemic agent especially when given in combination with a diuretic benzothiadiazine such as chlorothiazide (Marks and Samols 1968a, Brunetti et al. 1968). Chlorothiazide is also given to abolish the discomfort due to electrolyte and water retention. Diazoxide is not cytotoxic or teratogenic nor does it produce permanent or irreversible damage to the B cell.
Other hyperglycaemics have been tried without success and have adverse side effects. They include propranolol (Scandellari et al. 1976, 1978) and phenytoin (Ginsberg-Fellner, 1980). Cytotoxic drugs have been used in the treatment of malignant tumours of the pancreas and these include streptozotocin (Border and Carter, 1974), adriamycin (Eastman et al. 1977) and 5 flourouracil (Kraybill et al. 1976).

In normal subjects a significant correlation was observed between fasting C-peptide and plasma glucose levels, no correlation could be obtained, however, between fasting insulin and plasma glucose levels although increasing the number of normal subjects may enable correlation of fasting insulin and plasma glucose levels. Plotting fasting C-peptide level against plasma glucose values from insulinoma patients clearly demonstrated a difference between these patients and normal subjects.

In conclusion, this series of patients has emphasised that the clinical history of the patient is extremely important in the diagnosis of most insulinomas. Insulinomas in a majority of cases in this series were easily detected by either prolonged fasting or insulin stress tests. Some patients, however, require further investigation in order to demonstrate the existence of tumours. The most useful test which is carried out in patients is the insulin stress test with the measurement of glucose and C-peptide levels.
CHAPTER V

A POSSIBLE PHYSIOLOGICAL ROLE FOR C-PEPTIDE
5:1 Introduction
A high degree of species specificity exists in the primary structure of C-peptide. Considerable variation in both the amino acid sequence and the chain length is observed between species (Figure 5:1). This variation in the primary structure of C-peptide is much greater than that which has been observed in insulin structure. In addition, in some species, for example the rat, two different proinsulins are synthesised and two insulins and C-peptides are released equimolarly. The two rat C-peptides only differ in the amino acids at positions 8 and 17. The two rat proinsulins maintain the same net charge at neutral pH. The negative charge due to the glutamic acid at position 17 in rat C-peptide I is off-set by the positive charge at lysine B29 in the corresponding rat insulin. Neutral amino acid residues appear at position 17 and B29 of rat C-peptide II and rat insulin II respectively.

The large divergence in primary structure is more apparent in species which are not closely related to each other. Rat C-peptide I and II and human C-peptide have thirty-one amino acids each, twenty-one of which are common to all three peptides. The appearance of thirty-one amino acid residues in the C-peptides from three divergent mammalian orders namely, the primate, the rodent and the perissodactyl, and the clear homology of the terminal regions among all the C-peptides strongly suggests that the primate mammalian C-peptide contained at least 31 amino acids.

The large degree of variation in the primary structure of C-peptide between species has necessitated the development of species specific radioimmunoassays. Most naturally occurring peptides and their biosynthetically related peptides are difficult to obtain and therefore synthetic peptides are used increasingly as the starting material for developing radioimmunoassays. Unfortunately the purity of these chemically synthesised compounds is variable. C-peptide can be synthesised using the azide fragment condensation method (Yanaihara et al. 1974, 1978b) or more recently the solid phase method (Igano et al. 1981). The condensation method used to synthesise rat C-peptide I and II involves the stepwise fragment
**Figure 5:1**

**Species variation of amino acid sequence of C-peptides**

<table>
<thead>
<tr>
<th>Human</th>
<th>Rat I</th>
<th>Rat II</th>
<th>Dog</th>
<th>Porcine</th>
<th>Bovine</th>
<th>Monkey</th>
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- amino acids identical in these species with human
condensation from the C terminal 20-31 sequence. The products are then purified by gel filtration or ion exchange chromatography.

Antisera against synthetic rat C-peptide I and II have been produced both in rabbit and guinea pigs (Yanaihara et al. 1978a). The conjugate used for the immunisation was either produced by coupling rat C-peptide II to human serum albumin or adsorbing rat C-peptide I and II onto polyvinyl pyrrolidone which binds non specifically to the peptides. Using these conjugates Yanaihara et al. (1978a) produced three antisera. Each antiserum could detect either rat C-peptide I and II or both depending on the standard and tracer used in the radioimmunoassay. The antiserum produced in the rabbit could detect both C-peptides equally. The C-peptide levels described in this chapter were obtained using this rabbit antiserum. Yanaihara et al. (1978a) stated that the major antigenic site of this antiserum was located in the 20 to 31 amino acid region of the C-peptide which is identical in both rat C-peptides.

The major route for proinsulin conversion to C-peptide and insulin involves trypsin-like and carboxypeptidase B like enzyme activity. (Clark and Steiner, 1969, Kemmler et al. 1971). A minor route in rodents has been described by Tager et al. (1973) resulting in the production of peptide A, an NH$_2$ terminal twenty two residue peptide from rat C-peptide I. This peptide is formed initially by chymotryptic-like cleavage of rat proinsulin I between residues 22 and 23 of the connecting region. This region is then enzymatically cleaved as before, producing two peptides, one with twenty two amino acids and one with seven. (Fig. 5:2) Yanaihara et al. (1980) described a similar fragment cleaved from rat proinsulin II. This fragment has been extracted from rat plasma by gel filtration on Sephadex G-50. The biological importance of either of these peptides after stimulation of the B cell has not yet been studied. However, Yanaihara et al. (1981), using a guinea pig rat C-peptide antiserum, which crossreacted 100% with rat C-peptide AII (obtained from rat proinsulin II) demonstrated that a considerable proportion of the rat C-peptide is released in the form of rat C-peptide IIA in glucose perfused
Figure 5:2

A minor pathway of enzymic cleavage of rat proinsulin I

Proinsulin

connecting region

Proteolytic cleavage

chymotryptic like cleavage

Arg Arg Lys Arg

Proteolytic cleavage

Insulin

Peptide A (22 amino acids)
rat islets. Immunohistochemical staining with antisera raised against rat C-peptide I and II have demonstrated that these peptides are stored in the same B cell although the amino acid sequence of the peptides are different (Yanaihara et al. 1980). There is some evidence for the direct autoregulation of insulin (Iversen and Miles, 1971, Turner and Johnstone, 1973, Loreti et al. 1974) in whole pancreas, pieces of pancreatic tissue and isolated islets using exogenous insulin in a variety of animals including man. However, other workers have failed to demonstrate any autoregulation (Malaisse et al. 1967, Sando et al. 1970) This failure could be attributed to the failure to use species specific insulin. However, since C-peptide is secreted in equimolar amounts with insulin it is possible that the autoregulation of insulin is due to other B cell products such as C-peptide and proinsulin, in addition to insulin. The evidence for a possible physiological role for C-peptide in insulin regulation is also conflicting.

Toyota et al. (1975) investigated the effect of synthetic rat C-peptide I in the perfused isolated rat pancreas on insulin secretion in the presence of 16.6 mM glucose. They demonstrated that C-peptide inhibited glucose induced insulin secretion in the rat pancreas and suggested that C-peptide may have a possible regulatory role in insulin secretion. They postulated that a defective pancreas where C-peptide may accumulate in the B cell may lead to diabetes because the excess C-peptide inhibits insulin release. Wojcikowski et al. (1977) demonstrated using isolated perfused rat pancreas that synthetic human, rat I and rat II C-peptides inhibited glucose stimulated insulin release to varying degrees. The largest degree of inhibition was demonstrated when human C-peptide was present. Both early and second phases of glucose stimulated insulin secretion were inhibited. Stimulation of isolated pancreas with arginine in the presence of human C-peptide attenuated both phases of glucagon secretion. Physiological C-peptide concentrations in the extracellular space of the islet cell are estimated to be approximately $10^{-6}$ to $10^{-5}$ molar at maximum. This calculation is based on insulin
measurement as no rat C-peptide assay was available at the time of the study. In their experiments Wojcikowski et al. (1977) used a C-peptide concentration of $10^{-8}$ M which caused a significant effect. Although the degree of inhibition of glucose stimulated insulin secretion was greatest with human C-peptide, rat C-peptide II gave an appreciable degree of inhibition. Inhibition of insulin secretion was only slight with rat C-peptide I suggesting that the difference in effectiveness is not due to species heterogeneity but possibly due to small variations in the tertiary structure. Wojcikowski et al. (1977) suggested that C-peptide may be added to the list of participants involved in the interplay of islet cell hormones. However, Dunbar et al. (1976) working with isolated hamster islets demonstrated inhibition of glucose stimulated insulin release with proinsulin and insulin but not C-peptide. Inhibition of insulin secretion was almost total with proinsulin. In this study an undescribed C-peptide was used and, therefore, because of the large variation in C-peptide between species the data must be regarded with some scepticism. Further studies in isolated rat islets by Yasuda et al. (1976) did not demonstrate any inhibitory effect on insulin secretion of rat C-peptide II and human C-peptide although rat C-peptide I did have an inhibitory effect. The results produced from these two studies, may have been due to some disturbance of islet function by collagenase treatment. Studies in isolated rat pancreas by Kaneko et al. (1978) did not demonstrate any inhibition of glucose stimulated insulin release by rat C-peptide II. However, the infusion of C-peptide was at a lower concentration than that used in the studies of Wojcikowski et al. (1977).

The close relationship between the gastrointestinal hormones and insulin release has long been known. McIntyre et al. (1964) demonstrated that intrajejunal glucose resulted in a greater insulin response than intravenous glucose. One of the major gut peptides that contributes towards this increased insulin response is GIP. GIP release has been shown to be inhibited by a number of substances released from the pancreas. A number of workers have investigated the response of GIP to oral fat after intravenous injection of insulin or infusion of glucose and all have found that
GIP secretion is inhibited (Brown et al. 1975, Crockett et al. 1976, Ross and Dupré 1978, Ebert et al. 1979). Other islet hormones, namely, glucagon (Ebert et al. 1977), somatostatin (Pederson et al. 1975) and C-peptide (Dryburgh et al. 1980) have all been found to inhibit GIP release. Investigations into whether C-peptide may be involved in the negative feedback control of GIP has been studied in this department by Dryburgh et al. (1980). Using the rat intestinal perfusion model they were able to demonstrate that exogenous rat C-peptide II inhibited fat stimulated GIP release at doses which were only three times those encountered in the normal fed rat. Plasma glucose levels were significantly depressed in the group of animals receiving C-peptide alone. This may have been due to depression of glucagon levels by C-peptide which has already been demonstrated by Wojcikowski et al. (1977).

Care must be taken when using synthetic substances, as a substitute for the naturally occurring compounds. In the process of chemical synthesis small peptide fragments are often produced as a side reaction and if not removed can contaminate the synthetic material. The purity of these compounds is, therefore, extremely important and sufficient care has to be taken to ensure that the synthetic compounds used are homologous. Nevertheless, naturally extracted substances are not necessarily pure and may also be contaminated. Purification is, therefore, a very important process in obtaining compounds for use in vivo and in vitro studies. The development of biosynthetic compounds and the use of high pressure liquid chromatography has made it possible to obtain compounds which can be used in the knowledge that purity is maximal but the purification procedures necessary are tedious, time-consuming and also require a relatively large amount of starting material, which is not always possible.

In this chapter various investigations have been carried out in order to elucidate whether endogenous C-peptide inhibits fat stimulated GIP release in the rat. The stimulation of endogenous C-peptide enables any responses due to contamination of the exogenous C-peptide to be eliminated. In these studies, GIP secretion was
stimulated using either an in situ intestinal perfusion of fat or by dosing the rats orally in the conscious state. C-peptide was stimulated by intravenous injection of tolbutamide in either the jugular or tail vein and the biological effects of insulin were masked by the simultaneous injection of insulin antiserum. Due to the species variation in the primary structure of C-peptide a specific radioimmunoassay was set up and validated.

5:2 The immunoreactive rat C-peptide radioimmunoassay

Immunoreactive rat C-peptide was measured by a modification of a method described by Yanaihara (1979 Personal communication). The antiserum, standard and tyrosylated rat C-peptide I were all kindly donated by Professor N. Yanaihara.

5:2:1 Materials and Methods

(a) Antiserum

The antiserum (batch no. R.901 12.15.76) was raised in a rabbit against rat C-peptide II which was adsorbed on to polyvinylpyrrolidine.

(b) Iodination methods

(i) Lactoperoxidase method. This was a modification of the Thorell and Johansson (1971) method. All reactions were carried out in a plastic autoanalyser cup at ambient temperature. The reactants were mixed with a small magnetic flea. Iodination procedure was as follows:-the reagents being added in the following order.

1. 5 μg tyrosylated rat C-peptide I dissolved in 50 μl of 0.04M sodium acetate buffer pH 5.6.
2. 50 μl 0.4M sodium acetate buffer pH 5.6.
3. 18.5 MBq (0.5mCi) in a 5μl aliquot of Na$^{125}\text{I}$.
4. 10 μl 0.003% solution hydrogen peroxide (100 vols) dissolved in 0.04M sodium acetate buffer pH 5.6.
5. 10 μl containing 500 ng of lactoperoxidase 0.04M sodium acetate buffer pH 5.6

After 15 seconds.

6. 240 μg sodium metabisulphite dissolved in 200 μl of 0.04M sodium acetate pH 5.6.
The reactants were then loaded onto the purification column. The reaction time was increased to 10 minutes in the course of developing the assay to investigate whether any increased incorporation of $^{125}$I into the rat C-peptide occurred.

(ii) Chloramine T method

The tyrosylated rat C-peptide I was iodinated using a modification of the Hunter and Greenwood (1962) chloramine T method. Rat C-peptide I was weighed out and dissolved in a small quantity of dilute acetic acid. The tyrosylated rat C-peptide I was then diluted in 0.05M phosphate buffer pH 7.4 to a concentration of 100 μg/ml. This solution was, then, aliquoted into plastic autoanalyzer cups containing 5 μg of tyrosylated rat C-peptide I and stored at -20°C until required. Iodination was as follows:- the reagents being added in the following order.

1. 5 μg tyrosylated rat C-peptide I dissolved in 50 μl of 0.05M sodium phosphate buffer pH 7.4
2. 50 μl of 0.05 M sodium phosphate buffer pH 7.4
3. 18.5 MBq (0.5mCi) in a 5μl aliquot of Na$^{125}$I.
4. 20 μg chloramine T dissolved in 10μl of 0.05M sodium phosphate buffer pH7.4
5. 25μg sodium metabisulphite dissolved in 200 μl of 0.05M sodium phosphate buffer pH7.4

After 10 seconds

6. 500 μg potassium iodide dissolved in 50 μl of 0.05M sodium phosphate buffer pH7.4

The reactants were mixed throughout this process with a small magnetic flea. They were then loaded on to the purification column. Similar iodinations using different time intervals were carried out in the course of assay development.

(c) Purification of iodinated rat C-peptide I

A 30 x 1.1 cm column of Sephadex G15 was equilibrated with 0.05 M sodium phosphate buffer pH7.4 containing 0.1% BSA. The iodination mixture was applied to the column and the iodinated C-peptide was eluted from the column with 0.05 M
sodium phosphate buffer pH7.4 containing 0.1% HSA. The unreacted iodide peak was eluted after the protein peak. The purified tracer was aliquoted and stored at -20°C.

(d) Standard

(i) Preparation of standard

Synthetic rat C-peptide I was used to produce the standard for the assay. The standard was weighed out on a Kahn microbalance and dissolved in a small quantity of 6 M acetic acid. This solution was made up to the concentration required aliquoted in 20 ng aliquots into freeze drying vials and lyophilized. The vials were stored at -20°C.

(ii) Investigation of standard purity using high pressure liquid chromatography

High pressure liquid chromatography was used to investigate the purity of the rat C-peptide I standard. Chromatography was carried out at constant pressure using a Varian high pressure liquid chromatography machine equipped with a variable wavelength spectrophotometer.

Organic solvents used were HPLC grade. Buffers and salt solutions were made up with Analar or Aristar reagents using double glass distilled water.

Compounds for chromatography were freshly dissolved with 0.05M sodium phosphate buffer pH7.4. The HPLC column used 250 x 5mm I.D. packed with Hypersil-ODS and operated at ambient temperature. Buffer A was 0.1M sodium phosphate buffer pH 2.5/20% acetonitrile and buffer B was 0.1M sodium phosphate buffer pH 2.5/60% acetonitrile. A linear gradient was supplied from 20-50% acetonitrile with a flow rate of 1 ml/min. The variable wavelength detector was set at 230 nm and the chart speed was 60 cm/hr.

(e) Assay procedure

(i) Rat C-peptide assay

The diluent buffer used in the assay was 0.05M sodium phosphate buffer pH7.4 containing 1g BSA per litre.

Standard curves were constructed using rat C-peptide I in the range
0.5-20 μg/Litre diluted in buffer.

To a series of polystyrene LP3 tubes were added either 50 μl of standard or 50 μl of plasma sample plus 100 μl antiserum at a final dilution 1:2400 diluted in assay buffer. 50 μl of rat C-peptide free plasma was also added to the standard curve tubes. Standard and samples were set up in duplicate. A set of tubes were set up without antiserum to measure non-specific binding of the samples and standard curve. The counts in these tubes were subtracted from the total $^{125}$I rat C-peptide bound when calculating the amount of immunologically active rat C-peptide in unknown samples. All tubes were mixed on a Vortex mixer and incubated at 4°C for 24 hours. Tracer was diluted to give approximately 6000 cpm/100 μl (8000 dpm/100 μl). Tubes were then mixed on a Vortex mixer and incubated for a further 24 hours at 4°C. Separation of bound from free $^{125}$I tyr C-peptide was carried out by double antibody precipitation. The antirabbit γ globulin (Batch no. HP/D/66-IC) was used at a final dilution of 1:100. Double antibody precipitation time was reduced by the addition of polyethylene glycol 6000 at a final concentration of 2%, was added to the tubes. They were mixed on a Vortex mixer and incubated at 4°C for 2 hours. The tubes were then spun in a refrigerated centrifuge for 25 minutes at 2000 g and the supernatent was aspirated. The protein precipitate which contained the antibody bound $^{125}$I rat C-peptide was counted in an automatic gamma counter. The amount of immunologically active rat C-peptide in unknowns and standards was determined by subtracting the number of counts in the non-specific tube. The corrected values were then used in the construction of a standard curve and in calculating results. The protocol of the rat C-peptide assay is shown in Table 5:1.

(ii) Preparation of rat C-peptide free plasma

Fasted blood was obtained by cardiac puncture from anaesthetised rats. The plasma was treated with agarose charcoal according to the method of Stockhill (1979).
Table 5:1

Protocol for Rat C-peptide radioimmunoassay

<table>
<thead>
<tr>
<th>Tube</th>
<th>Buffer</th>
<th>A/S</th>
<th>CSS</th>
<th>Std.</th>
<th>Label</th>
<th>NRS</th>
<th>Dab</th>
<th>4% PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>150</td>
<td>-</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Non-specific binding (NSB)</td>
<td>150</td>
<td>-</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Binding (MB)</td>
<td>-</td>
<td>150</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero Binding (Zero)</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top Standard</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

24 h at 4°C  24 h at 4°C  2 h at 4°C
(iii) **Validation of rat C-peptide assay**

(1) **Recoveries**

2.5, 5, 10, 20 ng of rat C-peptide I were each added to 1 ml of plasma obtained from fasted Wistar rats.

(2) **Parallelism of standard curves with circulating endogenous rat C-peptide**

A plasma sample which had a high endogenous rat C-peptide level was serially diluted with rat C-peptide free plasma.

5:2:2 **Results**

(a) **Iodination methods**

The lactoperoxidase iodinations produced tracers which bound a maximum of only 10% of the tracer to the antibody in conditions of antibody excess. The most successful tracer was produced when tyrosylated rat C-peptide I was iodinated for 15 seconds with chloramine T. Forty per cent of this tracer was bound in conditions of antibody excess.

(b) **Purification of tracers**

The elution profiles on G15 columns of the lactoperoxidase iodinated products are shown in Figures 5:3 and 5:4.

The elution profiles on G15 columns of the chloramine T iodination are shown in Figures 5:5 and 5:6. Antiserum dilution curves using these tracers are shown in Figure 5:7. These tracers were aliquoted into small containers and stored at -20°C.

(c) **Standard**

The purity of the rat C-peptide I standard was investigated using high pressure liquid chromatography. This synthetic compound contained four peaks which are eluted from the column fairly close to each other (Figure 5:8).

(d) **Assay conditions**

The effect of increasing the preincubation time and varying the period the tracer was present in the tube is shown in Figure 5:9. A 24 hour preincubation period and 24 hours in contact with the tracer before separation using double antibody technique were chosen for all future work.
Figure 5.3
Lactoperoxidase iodination profile - (15 second iodination)

Peak A  Iodinated Rat C-peptide I
Peak B  Free Iodide

Figure 5.4
Lactoperoxidase iodination profile - (10 second iodination)

Peak A  Iodinated Rat C-peptide I
Peak B  Free Iodide
Chloramine T iodination (15 sec) elution profile

Peak A: Iodinated Rat C-peptide I
Peak B: Free Iodide

Figure 5.6

Chloramine T iodination (30 sec) elution profile

Peak A: Iodinated Rat C-peptide I
Peak B: Free Iodide
Figure 5:7

Antiserum Dilution Curves using tracers produced by Chloramine T iodination

% of \(^{125}\)I C-peptide of Total

-- 15 sec Chloramine T iodination
-- 30 sec Chloramine T iodination

Antiserum dilution

1:100 1:200 1:400 1:800 1:1600
Figure 5:8

High pressure liquid chromatogram of rat C-peptide I
(synthetic)

Buffer A 0.1 M Sodium phosphate buffer pH2.5 / Acetonitrile 20%
Buffer B 0.1 M Sodium phosphate buffer pH2.5 / Acetonitrile 60%
Flow rate 1 ml min\(^{-1}\)
Chart Speed 60 cm hr\(^{-1}\)
Wavelength 230 nm
(i) **Standard curve**
Under the conditions described for the rat C-peptide radioimmunoassay increasing quantities of rat C-peptide I displaced $^{125}$I tyrosylated rat C-peptide I in the range 0.5 to 20 µg/l (Fig. 5:10). The non-specific binding varied with the age of the tracer but was always less than 10% of the total.

(ii) **Sensitivity**
The limit of sensitivity of the assay defined as two standard deviations from the zero standard (Fieldman & Rodbard, 1971) was 65 pg.

(e) **Validation of rat C-peptide radioimmunoassay**

(i) **Precision**
The intra-assay coefficient of variation at a mean plasma level of 2.3 µg/l was 11.5 (n = 6) and at 9.6 µg/l was 10.0% (n = 6).

The interassay coefficient of variation of four serum samples was calculated (Table 5:2).

(ii) **Recoveries**
The mean recovery of exogenous rat C-peptide when added to fasted rat plasma was 93%.

(iii) **Parallelism**
Plasma samples containing a high concentration of endogenous rat C-peptide serially diluted in charcoal extracted plasma were superimposable upon the rat C-peptide standard curve (Figure 5:9).

5:2:3 **Discussion**
The large species variation in the primary sequence of C-peptide has made it impossible to use the one assay for detection of C-peptide levels in most animals. The production of a specific antiserum which can detect both rat C-peptide I and II has been described by Yanaihara et al. (1978a). This antiserum cross reacts equally with both C-peptides. The cross reactivity of this antiserum with rat C-peptide A I and AII is less than 0.001%. Yanaihara et al. (1981) have suggested that the concentration of these products may be higher than originally expected of a minor
Table 5:2

Inter assay precision of plasma samples containing a range of levels of endogenous Rat C-peptide

<table>
<thead>
<tr>
<th>Mean Rat C-peptide value (µg/Litre)</th>
<th>No.of observations</th>
<th>SD</th>
<th>% Coeff of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.05</td>
<td>9</td>
<td>0.125</td>
<td>6.1</td>
</tr>
<tr>
<td>4.05</td>
<td>9</td>
<td>0.28</td>
<td>7</td>
</tr>
<tr>
<td>9.5</td>
<td>9</td>
<td>0.58</td>
<td>6.1</td>
</tr>
</tbody>
</table>
Figure 5:9

Optimisation of assay condition

- 24 h preincubation/24 hr label
- 48 h preincubation/24 hr label
- 24 h preincubation/48 hr label
Superimpossibility of serially diluted plasma samples diluted in rat C-peptide free serum upon a synthetic rat C-peptide standard similarly diluted

- Sample 1
- Sample 2
- Rat C-peptide standard in charcoal-extracted serum
pathway but it is unlikely that C-peptide Al and All interfere in the assay to any significant extent.

The difficulty of extracting naturally occurring rat C-peptide I and II has lead to synthesis of these peptides by the condensation technique. These synthetic peptides have been used to develop antisera, produce standards and tracers for radioimmunoassays. The high pressure liquid chromatogram of rat C-peptide I standard demonstrated its lack of purity, as 4 distinct components. Further studies will have to be completed with rat plasma and assessment of the immunogenicity of the high pressure liquid chromatography fractions using the antiserum to determine which peak is the rat C-peptide I. The levels of rat C-peptide measured in the rat plasma must, therefore, not be regarded as absolute values.

The problems encountered in obtaining a high specific activity tracer may have been due to impurities in the preparation of tyrosylated rat C-peptide I. A high pressure liquid chromatogram was not run and, therefore, the purity of the tyrosylated peptide could not be assessed. In conditions of antibody excess the maximum binding of the tracer varied between 30 -40% of total counts added.

5:3 Endogenous C-peptide induced inhibition of fat-stimulated GIP release.

Study 1.

5:3:1 Materials and Methods

Thirty male Wistar rats weighing between 250 and 290 g fed on normal laboratory diet were used in this investigation. The rats were fasted in metabolic cages for 24 hours prior to the investigation. The animals were anaesthetised with intraperitoneal (I.P.) pentobarbitone (100 mg/kg body weight). The abdomen was opened along the midline and a polyethylene catheter (internal diameter 4mm) inserted into the upper intestine through a small incision in the region of the duodenal bulb. An incision was made in the terminal ileum and the contents of the gut flushed out with saline at 37°C. A second polyethylene catheter was then placed in the lower incision to allow free drainage of gut perfusates. The upper polyethylene catheter was attached to a peristaltic pump allowing perfusion of the gut with either 10%
bath. The gut perfusion was carried out for 30 minutes, 10 minutes after the start of perfusion an injection was given into the tail vein. Blood was collected immediately after the perfusion period from the vena cava. A 100 μl aliquot of whole blood was deproteinised with sodium tungstate and stored at 4°C until assay. The remainder was placed in lithium heparin tubes which were immediately spun and the plasma was aliquotted and stored at -20°C. Animals were sacrificed immediately after blood sampling.

5:3:2 Experimental protocol

(1) Gut perfusion with 10% Intralipid at 37°C at a rate of 2 ml/min for 30 min. 1 ml intravenous (I.V.) saline (0.154 mol/l) was given as a bolus into the tail vein (n = 11).

(2) Gut perfusion with saline (0.154 mol/l) at 37°C at a rate of 2 ml/min for 30 min. 1 ml I.V. saline (0.154 mol/l) was given as a bolus into the tail vein (n = 6).

(3) Gut perfusion with 10% Intralipid at 37°C at a rate of 2 ml/min for 30 min. 1 ml of glucose (0.5 gm/kg - 0.5 ml) and tolbutamide (83 mg/ml -0.5 ml) was given as a bolus into the tail vein (n = 6).

(4) Gut perfusion with 10% Intralipid at 37°C at a rate of 2 ml/min for 30 min. 1 ml of glucose (0.5 gm/kg - 0.25 ml), tolbutamide (83 mg/ml - 0.5 ml) and insulin antibodies (0.25 ml - Batch no. MF/GP/5-IA) (n = 6).

5:3:3 Analyses

(i) Glucose analysis

Blood glucose was determined in duplicate by a glucose oxidase method (Trinder, 1969).

(ii) Immunoreactive GIP (IR - GIP) radioimmunoassay

Immunoreactive GIP was measured by a double antibody radioimmunoassay (Morgan et al. 1978). A porcine standard was used, parallelism was demonstrated between the standard curve and a serially diluted sample of rat plasma containing high levels of endogenous GIP. The assay sensitivity was 110 ng/Litre (2 standard
deviations from zero) and the interassay coefficient of variation was 4.1% at 2677 ng/Litre at 22.5% at 138 ng/Litre.

(iii) Immunoreactive rat C-peptide (IR - RCP) radioimmunoassay

Immunoreactive rat C-peptide was measured according to the method described earlier in this chapter.

5:3:4 Preparation of globulin fraction of insulin antiserum

Insulin antiserum (batch no. MF/GP/5-IA) was placed in glass beakers containing a small magnetic bar. The serum was allowed to mix slowly on magnetic stirrers. Saturated ammonium sulphate at room temperature was placed in a microburette. The ammonium sulphate was then added dropwise until half the original volume of the serum had been added. The mixture was then centrifuged at 2500 rpm for 10 minutes and the supernatant discarded. The precipitate was then resuspended in distilled water and dialysised against distilled water for 48 h. The dialysate was then aliquoted into freeze drying vials and lyophilised. The vials were then stored at -20°C until required. The binding capacity of the insulin antiserum was calculated from a Scatchard plot.

5:3:5 Results

The IR-GIP, IR-RCP and blood glucose levels are shown in Table 5:3 and Figure 5:11.

Immunoreactive GIP (IR-GIP) levels were significantly increased (p < 0.02) in the group perfused with Intralipid compared with the control group (saline perfusion). No significant difference was observed in the IR-GIP levels between any of the groups perfused with 10% Intralipid (Groups 1, 3 and 4). However, when data from groups 3 and 4 were combined (those animals which had high circulating C-peptide levels) a significant inhibition (p < 0.05) of IR-GIP levels was observed in these animals compared with rats only Intralipid perfused (Group 1).

The immunoreactive rat C-peptide (IR-RCP) levels were significantly increased in group 4, one of the groups of rats which had had B cells stimulated, compared with saline injected rats (Group 1) (p < 0.05). The addition of insulin antiserum to the
Table 5:3

Endogenous C-peptide induced inhibition of IR-GIP release: Study 1

\( \bar{x} \pm \text{SEM} \)

<table>
<thead>
<tr>
<th>Groups</th>
<th>IR-GIP (ng/Litre)</th>
<th>IR-RCP (( \mu \text{g}/\text{Litre} ))</th>
<th>Glucose (mmol/Litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gut perfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intralipid/IV</td>
<td>356 ± 44</td>
<td>15.1 ± 2.02</td>
<td>4.8 ± 0.27</td>
</tr>
<tr>
<td>saline ( n = 11 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gut perfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline/ IV</td>
<td>179 ± 22</td>
<td>16.65 ± 2.2</td>
<td>4.4 ± 0.53</td>
</tr>
<tr>
<td>saline ( n = 6 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gut perfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intralipid/IV</td>
<td>263 ± 50</td>
<td>28.8 ± 7.1</td>
<td>4.9 ± 0.29</td>
</tr>
<tr>
<td>glucose + tolbutamide ( n = 6 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gut perfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intralipid IV</td>
<td>253 ± 28</td>
<td>55.2 ± 15.1</td>
<td>5.3 ± 0.82</td>
</tr>
<tr>
<td>glucose + tolbutamide + insulin antibodies ( n = 6 )</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.11
Plasma IR-GIP, IR-RCP and blood glucose levels in response to IV infusions during gut perfusion with Intralipid 10% or saline (0.154 mol/Litre) in anaesthetized rats - Study 1

Mean ± SEM
** = p < 0.02
* = p < 0.05

1. IV saline
2. IV saline
3. IV Glucose and tolbutamide
4. IV Glucose, tolbutamide, insulin antibodies
cocktail of glucose and tolbutamide resulted in an increase in the circulating C-peptide levels compared with rats receiving only the cocktail. Mean C-peptide levels in group 3, the group receiving only tolbutamide and glucose, were elevated compared to their saline control, but the elevation failed to achieve statistical significance. Glucose levels in all groups showed no statistical difference.

The binding capacity of the insulin antiserum injected into the rats was calculated from a Scatchard plot (Fig 5:12). The antiserum had a binding capacity $15.9 \times 10^{-12}$ mol/Litre and equilibrium constant $21.43 \times 10^{10}$ L/mol. The serum from each rat was screened for the presence of antibodies in the group given insulin antibodies. All rat sera showed insulin antibodies present at the end of the study and the residual insulin binding was calculated to be 3.6 ng insulin per rat.

Discussion

The plasma GIP levels reported in this study were only significantly depressed in the presence of high circulating C-peptide levels when data from both B cell stimulated groups were combined. Increasing the number of animals in each group may have been sufficient to obtain significance in each group.

The low circulating levels of antibodies at the end of the study and the similar glucose levels in all groups of rats indicated that only a small proportion of the antibodies injected were passing into the circulation. A probably explanation is that the high concentration of antibodies used caused the collapse of the vein and the antibodies remained in the interstitial tissues.

The level of C-peptide present after stimulation with glucose and tolbutamide without insulin antibodies was not significantly elevated. The doses of glucose and tolbutamide may have been insufficient but more likely the route of administration prevented the entire dose reaching the circulation. Since the tail vein as an intravenous route was unsuitable for the purpose of the study an alternative route the jugular vein was investigated.
Scatchard plots of insulin antiserum. (Batch no. MF (GP/S-IA))

\[ K = 21.42 \times 10^{10} L/mol \]
\[ n = 15.9 \times 10^{-12} mol/L \]
5:4 Endogenous C-peptide induced inhibition of rat stimulated GIP release.

Study 2

5:4:1 Materials and Methods

Forty male Wistar rats weighing between 250 and 290 g fed on normal laboratory diet were used in this investigation. The rats were fasted in metabolic cages for 24 hours prior to the investigation. The procedure followed was exactly the same as Study I except that the substances were injected into the animals via the jugular vein. A small polyethylene cannula attached to a 26 gauge needle was placed into the external jugular and kept patent with saline (0.154 mol/Litre). The I.V. cannula was connected to a syringe and injected 10 minutes after the start of the gut perfusion.

5:4:2 Experimental Protocol

1. Gut perfusion with saline (0.154 mol/Litre) at a rate of 2 ml/min for 30 minutes. 1 ml. I.V. saline (0.75 ml) and normal guinea pig serum (0.25 ml).
2. Gut perfusion with 10% Intralipid at 37°C at a rate of 2 ml/min for 30 minutes. 1 ml I.V. saline (0.154 mol/Litre - 0.75 ml) and normal guinea pig serum (0.25 ml).
3. Gut perfusion with 10% Intralipid at 37°C at a rate of 2 ml/min for 30 minutes. 1 ml I.V. glucose (0.5 gm/kg - 0.25 ml), tolbutamide (8.3 mg/kg - 0.5 ml) and normal guinea pig serum (0.25 ml).
4. Gut perfusion with 10% Intralipid at 37°C at a rate of 2 ml/min for 30 minutes. 1 ml I.V. glucose (0.5 gm/kg - 0.25 ml), tolbutamide (83 mg/kg -0.5 ml) and insulin antibodies (0.25 ml Batch no. MF/GP/6-VA).

5:4:3 Analyses

See Study 1.

5:4:4 Preparation of globulin fraction of insulin antiserum and normal guinea pig serum

The procedure followed was exactly the same as in Study 1 except that another batch of insulin antiserum was used (Batch no. MF/GP/6-VA).
Results

The IR-GIP, IR-RCP and glucose levels are shown in Table 5:4 and Figure 5:13. Immunoreactive GIP (IR-GIP) levels were significantly increased in the group perfused with Intralipid (Group 2) compared with the saline perfused group (Group 1) (p < 0.005). There was a significant inhibition (p < 0.005) of IR-GIP levels in groups 3 and 4 given glucose and tolbutamide with or without insulin antibodies compared with control rats (Group 2). No significant difference was noticed between the rats receiving normal guinea pig IgG and insulin antibodies.

No significant difference was observed in immunoreactive rat C-peptide (IR-RCP) levels in Intralipid and saline gut perfused group (Group 1 and 2). A significant increase in IR rat C-peptide levels was noticed in the two groups (Groups 3 and 4) given glucose and tolbutamide with and without insulin antibodies compared with the control group (Group 2) (p<0.0025 p< 0.05) respectively. Immunoreactive rat C-peptide levels were not statistically different between Group 3 and 4.

Blood glucose levels were similar in Group 1 and 2. A significant difference (p < 0.005) was observed in the group of rats given the insulin antibodies (Group 4) compared with rats given normal guinea pig serum (Group 3).

The binding capacity of the insulin antiserum injected into the rats was calculated from a Scatchard plot (Figure 5:14). The antiserum had a binding capacity 1.82 x 10^{-11} moles/Litre and an equilibrium constant of 7.8 x 10^{10} L/mol. The antiserum was made up in half its original volume on resuspending the lyophilised material, therefore, each animal received 500 µl of the original antiserum. All rat sera in Group 4 showed insulin antibodies present at the end of the study and the residual binding capacity was calculated to be 0.6 µg insulin per rat.

Discussion

In this study fasting plasma GIP levels were elevated compared with the previous investigation. A significant depression of plasma GIP levels was observed in those rats with high circulating C-peptide levels either with or without insulin antibodies compared with the rats given intravenous saline. Immunoreactive C-peptide levels
### Endogenous C-peptide induced inhibition of GIP release: Study 2

\[ \bar{x} \pm \text{SEM} \]

<table>
<thead>
<tr>
<th>Groups</th>
<th>IR-GIP (ng/Litre)</th>
<th>IR-RCR(μg/Litre)</th>
<th>Glucose(mmol/Litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Gut perfusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline IV</td>
<td>305 ± 28</td>
<td>5.98 ± 1.29</td>
<td>7.66 ± 0.37</td>
</tr>
<tr>
<td>saline + NGPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>Gut perfusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>intralipid IV</td>
<td>680 ± 18.8</td>
<td>5.6 ± 1.1</td>
<td>8.6 ± 0.43</td>
</tr>
<tr>
<td>saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>Gut perfusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>intralipid IV</td>
<td>glucose, tolbutamide and NGPS</td>
<td>458 ± 15.9</td>
<td>12.3 ± 1.2</td>
</tr>
<tr>
<td>Group 4</td>
<td>Gut perfusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>intralipid IV</td>
<td>glucose, tolbutamide and insulin antibodies</td>
<td>490 ± 32.8</td>
<td>10.4 ± 2.0</td>
</tr>
</tbody>
</table>
Figure 5:13

Plasma IR-GIP, IR-RCP and glucose responses to IV infusions during gut perfusion with intralipid 10% or saline (0.154 mol/Litre) in anaesthetized rats - Study 2

mean ± SEM
* p < 0.0005
** p < 0.0025
a p < 0.05

N.G.P.S. = Normal Guinea
Serum
1 Saline/Normal guinea pig
2 IV saline/NGPS
3 IV Glucose, Tolbutamide
4 IV Glucose, Tolbutamide, Insulin antibodies

□ Gut perfusion 10% intralipid
☒ Gut perfusion saline
Scatchard plot of Insulin Antiserum - batch no. MF/GP/6-VA

\[ k = \text{equilibrium constant} \cdot 7.8 \times 10^{10} \text{ L/ml} \]
\[ n = \text{binding capacity} \cdot 1.8 \times 10^{-11} \text{ mol/Litre} \]
were elevated compared with the control group in both groups receiving the B cell stimuli. Blood glucose levels were significantly elevated in the group given insulin antibodies indicating that circulating insulin was being removed by the antibodies thus reducing the rate of removal of glucose.

The gut perfusion technique used in the two studies was first described by Doluisio et al. (1969). English et al. (1975) used this technique to study the effect of three polyene macrolides on cholesterol absorption from the gut. This method of gut perfusion has been used by several workers to investigate the release of GIP in response to various nutrients in the rat. Watt et al. (1978) perfused the intestines of rats with glucose, a highly significant response was produced in GIP levels in portal blood after 15 minutes which plateaued by 30-45 minutes. The perfusion with a fat emulsion, 10% Intralipid, produced a slower rise in portal GIP levels which were significantly higher after thirty minutes but had increased further by 45 minutes (Dryburgh et al. 1980).

The experiments employing the gut perfusion technique used a perfusion rate of 120 ml/min, as this was the flow rate used in previous experiments. This is far in excess of the physiological flow rate and it is quite probable that the intestinal absorption rate of fat is diminished. Studies have shown that GIP release is dependent on the absorption of nutrients. Investigations using non-dietary fibres and selective carbohydrates inducing GIP release have demonstrated not only the importance of nutrients specific molecular structure but also the rate of nutrient absorption. In certain diseases which are characterised by malabsorption a diminished GIP response is observed, lending further weight to the suggestion that absorption of nutrients is required for GIP release (Creutzfeldt et al. 1976, Besterman et al. 1979.)

This study provides evidence that endogenous C-peptide does inhibit fat stimulated GIP release. The circulating GIP levels after perfusion with Intralipid were significantly raised but were only 2-3 fold higher than basal levels, in contrast to a 4-5 fold elevation that is expected after an oral fat load in humans. The gut
perfusion technique is clearly unphysiological and therefore a change in experimental protocol is desirable. Oral fat dosing as an alternative to gut perfusion was chosen as being closest to physiological conditions. In order to maximise the GIP response to fat the rats were pretreated on a high fat diet as it had been reported (Deschodt-Lanckman et al. 1971) that a high fat diet increases pancreatic lipase activity in rats, increasing the rate of absorption of a fat load and hence should increase the GIP response.

5:5 Endogenous C-peptide induced inhibition of fat stimulated GIP release in fat pretreated rats.

5:5:1 Materials and Methods

Thirty male Wistar rats weighing between 250 and 290 g were fasted for 24 hours prior to the study. They were given 3 ml of triolein for 4 days prior to the study as well as being allowed free access to standard laboratory food. The animals were anaesthetised two hours after oral dosing. A small polyethylene cannula attached to a 26 gauge needle was then placed into the external jugular and kept patent with saline (0.154 mol/l). Blood was collected by cardiac puncture then put into lithium heparin tubes and immediately spun in a refrigerated centrifuge. Plasma for hormone and glucose estimation was aliquoted and stored at -20°C. The animals were sacrificed immediately after blood sampling.

5:5:2 Experimental Protocol

(1) Control group given 1 ml saline orally.

(2) 1 ml triolein orally 2 h prior to 1 ml I.V. saline (0.154 mol/Litre - 0.75 ml) and normal guinea pig serum (0.25 ml).

(3) 1 ml triolein orally 2 h prior to 1 ml I.V. glucose (0.5 gm/kg - 0.25 ml), tobutamide (83 mg/kg - 0.5 ml) and normal guinea pig serum (0.25 ml).

(4) 1 ml triolein orally 2 h prior to 1 ml I.V. glucose (0.5 gm/kg - 0.25 ml), tobutamide (83 mg/kg - 0.5 ml) and insulin antibodies (0.25 ml - batch no. MF/GP/6-VA).
Analyses see Study 1 except for glucose analysis

(a) Glucose analysis

Plasma glucose was measured by glucose oxidase method (Beckman glucose analyser) using the enzyme hexokinase.

Preparation of globulin fraction of insulin antiserum and normal guinea pig serum

The procedure followed was exactly the same as in Study 1 except that another batch of insulin antiserum was used (Batch no. MF/GP/6-VA)

Results

The IR-GIP, IR-RCP and plasma glucose levels are shown in Table 5:5 and Fig. 5:15. The plasma immunoreactive GIP following oral triolein was significantly increased in all groups compared with the control rats (Group 1). However, no significant difference was observed in IR-GIP levels between any of the groups which had received oral triolein.

Immunoreactive plasma rat C-peptide (IR-RCP) levels were significantly higher (p < 0.0005) in the group given oral triolein (Group 2) than in the saline dosed group (Group 1). Significantly raised IR-RCP levels were observed in those groups receiving the B cell stimuli with or without insulin antibodies compared with the control group (Group 2) (p < 0.0005).

Plasma glucose levels were significantly raised in the group given insulin antibodies compared with those receiving normal guinea pig IgG (p < 0.0025). Both these groups had significantly elevated plasma glucose levels compared with controls (Group 2) (p < 0.0005).

The binding capacity of the antiserum injected into the rats was the same as in Study 2, as the same antiserum was used. The sera from the rats (Group 4) were screened for the presence of insulin antibodies. All rat sera showed insulin antibodies present at the end of the study and the residual insulin binding capacity was calculated to be 9.45 ng insulin per rat.
Table 5:5

Endogenous C-peptide induced inhibition of GIP release in fat pretreated rats

mean ± SEM

<table>
<thead>
<tr>
<th>Groups</th>
<th>IR-GIP (ng/Litre)</th>
<th>IR-RCP (μg/Litre)</th>
<th>Glucose (mmol/Litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No triolein</td>
<td>329 ± 21</td>
<td>1.87 ± 0.24</td>
<td>3.4 ± 0.10</td>
</tr>
<tr>
<td>IV saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triolein</td>
<td>687 ± 60</td>
<td>10.1 ± 1.56</td>
<td>3.2 ± 0.20</td>
</tr>
<tr>
<td>IV saline, NGPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triolein</td>
<td>683 ± 84</td>
<td>26 ± 0.57</td>
<td>8.8 ± 0.86</td>
</tr>
<tr>
<td>IV Glucose, tolbutamide, NGPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triolein</td>
<td>665 ± 59</td>
<td>23.7 ± 1.2</td>
<td>13.3 ± 0.45</td>
</tr>
<tr>
<td>IV Glucose, tolbutamide, and antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.15

Endogenous C-peptide induced inhibition of GIP release in fat pretreated rats

Mean ± SEM
* p < 0.0005  IR-GIP (ng/Litre)
a p < 0.0025

IR-RCP (μg/Litre)  Plasma Glucose (mmol/Litre)

KEY
☐ Oral triolein
☑ Oral saline
1 IV saline
2 IV saline and NGPS
3 IV Glucose, Tolbut
4 IV Glucose, Tolbut and Insulin antibody
5:5:6 Discussion

Rats were maintained on a short term high fat diet in order to produce an increased GIP response to fat. Fat pretreatment did not result in a significant increase in GIP above those reported in Study 2 but the experimental design was a more physiological one.

No statistical difference was observed in GIP levels between any of the groups of animals receiving triolein. The previous studies had demonstrated inhibition of fat stimulated GIP release by both endogenous insulin and C-peptide. This suggests that the short term fat pretreatment may affect the control of GIP release. A defective feedback mechanism of insulin on fat stimulated GIP release in obese subjects has been demonstrated by Creutzfeldt et al. (1978). This defect can be explained by decreased number of receptors (Roth et al. 1975) on the GIP cell. The presence of C-peptide receptors has not been reported. It is possible to speculate that fat pretreatment causes a defect in the feedback mechanism on the GIP cell. The measurement of plasma C-peptide levels in each of the studies, reported in this chapter, showed a large variation in the basal values depending on the techniques used in the investigations. Comparison of C-peptide values in the two gut perfusion studies showed a two-fold difference in the basal values. In these studies the route of administration of intravenous substances was either via the tail or jugular vein. It seems unlikely that changing the route of administration of the intravenously substances would alter the basal C-peptide levels. The gut perfusion technique necessitates a number of procedures involving handling of the gut which may affect basal C-peptide values. Measurement of rat C-peptide levels after oral triolein showed very low basal values indicating that the gut perfusion technique may have affected the C-peptide levels.

Previous studies in this department by Dryburgh et al. (1980) demonstrated that fat stimulated GIP release was inhibited by exogenous C-peptide. In this chapter investigations have been reported that indicate that endogenous C-peptide may indeed play a role in the control of GIP release. Several pathological conditions
which have exaggerated GIP responses with low circulating C-peptide levels due to
defective secretion of the B cell have been reported, in untreated juvenile onset
diabetics (Ebert and Creutzfeldt, 1978), mild chronic pancreatitis (Ebert et al.
1976', Botha et al. 1978) and after duodenopancreatectomy (Creutzfeldt et al.
1976'.) These exaggerated GIP responses could, therefore, be explained by the low
C-peptide levels being unable to regulate GIP secretion. Pretreatment of rats with
oral fat appears to affect the inhibition of GIP release by both insulin and
C-peptide. Further studies investigating whether short term high fat diets affect
the inhibition of GIP release using exogenous insulin were carried out in both rats
and man and are described in the following Chapter. The inhibition of fat
stimulated GIP release by endogenous C-peptide should be studied in orally dosed
rats maintained on normal laboratory food. Unfortunately, due to the short supply
of insulin antiserum this could not, at present, be investigated.
CHAPTER VI

THE EFFECT OF HIGH AND LOW FAT DIETARY REGIMENS
ON THE ENTEROINSULAR AXIS RESPONSES TO FAT AND GLUCOSE
IN RAT AND MAN.
6:1 Introduction

In the previous chapter inhibition of fat stimulated GIP release by endogenous insulin or C-peptide could not be demonstrated in fat pretreated rats. A more detailed investigation of the phenomenon was, therefore, carried out using exogenous insulin whose inhibitory properties on fat stimulated GIP have previously been documented.

As long ago as 1927 (Sweeney, 1927) dietary composition was shown to influence glucose tolerance in healthy individuals. Himsworth (1933, 1934) confirmed and extended this information to conclude that a high fat diet decreased glucose tolerance and that this decrease was due to changes in susceptibility to insulin. A number of workers (Zaragoza-Hernandez & Felder, 1970, Susini & Lavau, 1978, Lavau et al. 1979) have demonstrated that short-term feeding of rats on a high fat diet in vivo and in vitro will induce an insulin resistant state.

Insulin resistance may occur due to an alteration at any one or all of three levels (1) prior to the interaction of insulin with the receptors (2) at the level of the receptor or (3) at steps distant to the insulin receptor interaction. Causes of insulin resistance at the prereceptor level include factors that reduce free insulin concentration, such as increased insulin degradation or insulin binding to proteins other than the receptor. At the level of the receptor, alteration in receptor affinity or concentration also result in a decrease in biological response to a given concentration of free hormone. Finally, any changes in the 'coupling function' between the concentration of hormone-receptor complexes and the final biological response would also result in tissue resistance to insulin action. Insulin resistance may be defined as a state where normal concentrations of hormone produce a less than normal biological response.

The initial step in the action of insulin on a tissue is to bind with a specific receptor on the membrane of the cell. Decreased cellular insulin receptors have been observed in a variety of tissues from obese animals (Kahn et al. 1973, Olefsky, 1976a) and man (Bar et al. 1976, Olefsky, 1976). A potential causal
relationship between decreased insulin receptors and insulin resistance is obvious. However, the quantitative relationship between decreased insulin receptors and the biological effect of insulin is not straightforward because of the presence of "spare" receptors on insulin target tissues (Kono & Barham, 1971). The functional consequences of a moderate decrease in insulin receptors is a shift in the insulin biological function dose response curve, resulting in a decreased response to submaximal insulin concentrations but no change in the maximal response to the hormone (Kono & Barham, 1971). Receptors are continually being synthesised and degraded (Kahn, 1976). A variety of factors regulate receptor concentrations. Plasma insulin levels appear to be a major factor and the effect is dependent on the duration of exposure to the hormone. Grundleger and Thenen (1982) demonstrated a decreased receptor number in soleus muscle of rat fed a high fat diet for a short time (10 days) but no change in receptor affinity. Other workers (Ip et al. 1976, Sun et al. 1977) observed a greater than 40 per cent decrease in insulin binding and receptor number, but no change in the affinity constant of either class of binding site in isolated fat cells or liver plasma membranes obtained from rats fed on a high fat, carbohydrate free diet. Other workers (Olefksy, 1976, Lavau et al. 1979, Grundleger and Thenen, 1982) have suggested that the decreased binding of insulin was not fully accounted for by decreased receptor number therefore additional defects distal to the insulin receptor complexes may exist. Glucose transport has been investigated in fat fed rats. Rats maintained on this diet transport glucose into muscles and adipocytes more slowly than rats maintained on a high carbohydrate diet (Lavau et al. 1979, Grundleger and Thenen, 1982). Grundleger and Thenen (1982) showed that this impairment process of glucose metabolism was of far greater importance than the decrease in glucose transport. The glucose transport process, although depressed, may not be the rate limiting step. Studies investigating the effect of a high fat diet on the intracellular metabolism of glucose in adipocytes and muscle cells have shown an impairment in insulin
responsiveness. Increased fatty acid uptake and decreased conversion of glucose to fatty acids occurs in animals fed a high fat diet. The main function of adipose tissue is the synthesis and storage of lipids and mobilisation of this fuel storage for the provision of energy. In the adipocytes, obtained from fat fed rats, fatty acid synthesis is strongly inhibited and lactic acid production is increased together with glycerogenesis and glycogenesis (Susini & Lavau, 1975). In muscle tissue, a high fat diet, inhibits glycolysis whilst glycogen storage is only slightly depressed in both the basal and insulin stimulated states (Grundleger and Thenen, 1982). Studies on enzymes such as hexokinase II (Marshall et al. 1976), pyruvate dehydrogenase (Griglio et al. 1969) and glucose 6 phosphate dehydrogenase (Lavau et al. 1979) have all shown decreased activity in tissues obtained from rats maintained on high fat diets. This would suggest that the unresponsiveness of the glucose metabolic pathway to insulin originates in the reduction in intracellular capacity to handle glucose entering the cell in response to the hormone. A modulation of insulin action via alterations of intracellular metabolism suggests a fine adjustment that allows a much more sensitive response than would be accomplished by the mere modification of the insulin receptor.

Reports in the literature show either no change (Lavau et al. 1979) a moderate decrease (Zarazoya-Hermans and Felder, 1970) or a rise (Grundleger and Thenen, 1982) in basal plasma insulin levels in rats maintained on a high fat diet. The conflicting findings could, however, be due to variations in the age, the strain of rat, type of diet and time maintained on diet.

The relationship between the gastrointestinal hormone GIP and insulin release in response to oral glucose is well known. Several substances have been shown to inhibit GIP release namely insulin (Brown et al. 1975), somatostatin (Pederson et al. 1975), glucagon (Ebert et al. 1977) and C-peptide (Dryburgh et al. 1980). A number of factors are responsible for affecting GIP release. Absorption of
nutrients is necessary for GIP secretion, as has been demonstrated by studies using disaccharidase inhibitors (Ebert and Creutzfeldt, 1978) and non-nutrient dietary fibres (Morgan et al., 1979; Jenkins et al., 1980). Changes in diet have been shown to effect GIP secretion. Reiser et al. (1980) placed volunteers on diets providing 30% of total energy as either sucrose or wheat starch. They demonstrated that after oral sucrose a greater GIP response was observed in those subjects maintained on the sucrose diet, and concluded that induction of the intestinal enzyme sucrase on the high sucrose diet resulted in facilitation of GIP release because of the increased rate of absorption of sucrose.

Obesity is characterised by hyperinsulinaemia both in fasted and stimulated states and the resistance to both endogenous and exogenous insulin in obesity has been shown to be a progressive condition that comprises two main phases - an early phase without insulin resistance and a later phase in which insulin resistance becomes more apparent. The latter condition is associated with a decreased number of insulin receptors in all target tissues (Kahn et al., 1973). The decrease in receptors is related to the degree of hyperinsulinaemia and returns towards or to normal when the hyperinsulinaemia is corrected (Assimacopoulos-Jeannet and Jeanrenaud, 1976).

Hyperinsulinaemia in obesity may be caused, at least in part, by an overactive enteroinsular axis. An exaggerated GIP response after ingestion of a mixed meal has been observed in obese subjects (Ebert et al., 1976) in whom exogenous insulin fails to inhibit fat stimulated GIP release (Creutzfeldt et al., 1978). After 21 days of starvation their GIP levels after a glucose load tend towards normal (Willms et al., 1978). Similar results were obtained on diets providing only 800 calories per day consumed for three weeks, thus the GIP response may be more dependent upon previous diet than on body weight. Ebert et al. (1976) have shown that a diminished GIP response was observed in obese individuals after only seven days on a reduced energy intake. The mechanism causing hypersecretion of GIP release in obesity may, therefore, be due to adaptation of the GIP cells to the incoming nutrient load and the ability of insulin to regulate GIP release.
The ability of exogenous insulin to inhibit fat stimulated GIP secretion in both rats and humans maintained on low and high fat diets was investigated. In the human studies the experimental design was such that the high and low fat diets were not isocaloric and therefore body weight was carefully monitored. Investigations into GIP responses to oral fat and glucose were also carried out.

6:2 Materials and Methods

6:2:1 Investigations of the responses of the enteroinsular axis of rats pretreated on a high fat diet

Male Wistar rats weighing between 240-290 g were used in these investigations. The 180 rats were divided into two groups. The first group (fat-pretreated group) were given 3 ml triolein/day orally for 4 days prior to the experiments as well as being allowed free access to standard laboratory food. This provided 20% of total energy as fat. The second group (untreated low fat group) was fed on standard laboratory food which contained 3.5% energy as fat. Rats were weighed each day and fasted for 24 h prior to each experiment. Three experimental protocols were performed on fat-pretreated and untreated rats during which they were anaesthetised with intraperitoneal (IP) pentobarbitone (100 mg/kg body weight). Blood was collected by cardiac puncture into lithium heparin tubes and the samples were immediately centrifuged. Plasma for hormone and glucose estimation were divided and stored at -20°C.

6:2:2 Experimental Protocol

(a) Time course of responses to oral fat

Fat pretreated and untreated groups of rats were each divided into five sets (six rats/set). Set A from each group received no triolein. Animals in the other four sets (B - E) of each group received 1 ml triolein (8 kcal) orally. Blood was collected by cardiac puncture at 0 min (Set A) and at 30, 60, 120 and 180 min. after oral dosing (Sets B - E respectively).
(b) Effect of fat pretreatment on responses to oral fat with and without intraperitoneal insulin

Groups of fat pretreated and untreated rats were each divided into three sets (10 rats/set) and given: Set A - 1 ml triolein orally 2 h before IP insulin (1 U/kg body weight); Set B - 1 ml triolein orally 2 h before IP saline (154 mmol/l); Set C - 1 ml saline (154 mmol/l) orally 2 h before IP saline. Blood was collected by cardiac puncture 20 min after the IP injection in all cases.

(c) Effect of fat pretreatment on responses to oral glucose with and without intraperitoneal insulin

Groups of fat pretreated and untreated rats were each divided into three sets (10 rats/set). Set A was given 3 ml 2.78 M glucose (5.8 kcal) orally 40 mins before IP saline (154 mmol/l); Set B: 3 ml saline (154 mmol/l) orally 40 min before IP saline (154 mmol/l) Set C: 3 ml 2.78M glucose (5.8 kcal) orally 40 min before IP insulin (1 U/kg body weight). Blood samples were collected by cardiac puncture 20 min after IP injection in all cases.

6:2:3 Investigation of the response of the enteroinsular axis in man on a high fat and low fat diet

9 volunteers (6 male 3 female) aged between 23-31 years (28 ± 1.87 ± SEM) within 20% of ideal body weight (Metropolitan life assurance tables) were investigated. Informed consent was obtained from all volunteers. There was considerable variation in the normal dietary fat intake of the volunteers ranging from 12-157 g fat per day (99 ± 20.1 g). The percentage of energy derived from fat in their diets varied from 20-50% per day (38 ± 2.5 g). The study was initially carried out in 5 subjects. A further 4 subjects were investigated because one of the initial group had to be excluded because of his excess fat intake. The first group (n = 5 3 males 2 females) ate a low fat diet providing less than 30 g of fat per day (low fat) for nine days. After a further fifteen day period on their usual dietary regimen the volunteers ate a high fat diet for nine days. This was achieved by supplementation of their low fat diets with 250 ml of double cream and butter per day providing 140 g fat. They were, therefore, consuming in excess of 140 g fat per day. Their
energy intake during this time was similar to their normal intake.
The second group of volunteers were maintained on a reversed dietary regimen i.e. high fat before low fat diet. This second group was carefully assessed beforehand to ensure only those whose fat intake was usually less than 130 g of fat per day took part. All volunteers were weighed throughout the studies. Three test meals were given to each volunteer. Blood was collected by means of an indwelling catheter inserted into the antecubital vein and kept patent with 0.12M sodium citrate. Blood samples were collected into lithium heparin and fluoride oxalate tubes. The samples were immediately centrifuged and aliquoted. Plasma was stored at -20°C until assayed.

6.2.4 Experimental Protocol
(a) Effect of a high and low fat diet on responses to oral fat
200 ml of double cream (96 g fat) was given to the second group of volunteers (4) on day nine of the low and high fat dietary regimens after an overnight fast. Two volunteers from the first group were also given a fat load after being maintained on a high fat diet for a second time. Two basal blood samples were taken prior to ingestion of the cream. Blood samples were then taken after 30, 60, 90, 120, 150, 180 and 210 minutes.

(b) Effect of a high and low fat diet on responses to oral fat with exogenous insulin
Following an overnight fast on day seven of both the low and high fat diets all the volunteers were given oral fat followed by intravenous insulin. Two basal blood samples were taken prior to ingestion of the cream (96 g fat) and blood samples were then taken 30, 50 and 60 min then insulin (0.2 U/kg body weight) was given intravenously to each volunteer. The first group had insulin injected via the blood sampling catheter while the second group had the insulin injected into the other arm. Blood samples were taken 90, 120, 150, 180 and 210 min after the start of the test.
(c) Effect of a high and low fat diet on responses to oral glucose

On day nine of the low and high fat diets the first group of volunteers (5) were given 75 g of liquid glucose orally (Hycal). After an overnight fast two basal blood samples were taken and then the Hycal made up to 250 ml with water was given to each volunteer. Blood samples were taken at 30, 60, 90, 120, 150, 180 and 210 min after the glucose load.

6:2:5 Chemical analyses

Plasma immunoreactive rat insulin was measured by a double antibody radioimmunoassay employing an antiserum raised against porcine insulin (batch no. MF/GP/10-VA), iodinated bovine insulin and a rat insulin standard (lot no. RC 791009). The sensitivity of the assay was 0.5 μg/Litre and the interassay coefficient of variation was 15.6% at 0.5 μg/Litre and 6.7% at 6.7 μg/Litre.

Plasma immunoreactive human insulin was measured by a double antibody radioimmunoassay employing an antiserum raised against porcine insulin (HP/GP/3-VA), iodinated bovine insulin and a human insulin standard (NISBC 66/3004). The sensitivity of the assay was 2.5 mU/Litre and the interassay coefficient of variation was 15% at 6.5 mU/Litre and 7.4% at 46 mU/Litre.

Plasma immunoreactive human C-peptide was measured by the method described in Chapter II. The assay has a detection limit of 10 pg/tube (10 ng/Litre). The interassay coefficient of variation was 24.5% at 0.44 μg/Litre and 9% at 3.7 μg/Litre.

Immunoreactive GIP was measured by a double antibody radioimmunoassay (Morgan et al. 1978). A porcine GIP standard was used but parallelism was demonstrated between the standard curve and serially diluted samples of both rat and human plasma containing high levels of endogenous GIP. The assay sensitivity was 110 ng/Litre and the interassay coefficient of variation was 4.1% at 2677 ng/Litre and 22.5% at 138 ng/Litre.

Rat plasma glucose was measured using a glucose oxidase method. Glucose estimation on human samples utilised the enzyme hexokinase in an automatic glucose analyser. Plasma triglyceride were measured in rat plasma by a fully enzymatic U.V. kit and in human samples by an enzymatic colorimetric kit.
6:2:6 Statistical analyses

Results were compared using Student's t test for unpaired, paired data and analysis of variance.

6:3 Results

6:3:1 Investigation of the responses of the enteroinsular axis of rats pretreated on a high fat diet

The untreated control rats were slightly, but significantly heavier than the fat-pretreated rats, throughout the study. There was, however, no significant weight gain in either group of rats on completion of the fat-pretreatment (control rats 297.0 ± 4.14 before pretreatment versus 297.5 ± 4.13 g after pretreatment p NS; fat pretreatment rats 280.2 ± 4.96 g before pretreatment versus 279.0 ± 10 g after pretreatment p NS; x ± SD n = 60). There were no significant differences in fasting plasma glucose, insulin or GIP levels between the two groups on completion of the fat-pretreatment.

(a) Time course of response to oral fat

Mean GIP levels were consistently higher in animals in the fat-pretreated sets, but the differences did not reach statistical significance at any single time point (Figure 6:1). However, when the data was subjected to analysis of variance (two factor with replication) the two groups were found to be significantly different at the 5% level.

Plasma triglyceride concentrations (Figure 6:2) were significantly higher in the fat pretreated rats than in the controls at 120 and 180 min (p < 0.01) and were associated with marked lipaemia in the former but not the latter.

(b) Effect of fat pretreatment on response to oral fat with or without exogenous insulin

In the untreated control group of rats administration of IP insulin caused a significant inhibition of triolein stimulated GIP release (Plasma GIP levels in animals given oral triolein + IP saline 853 ± 93 ng/Litre in those given triolein + IP insulin 663 ± 49 ng/Litre p < 0.025) not observed in the fat-pretreated rats which...
Figure 6:1
Plasma immunoreactive GIP levels following oral triolein (1 ml) in fat pretreated and untreated control rats.
Figure 6:2
Plasma triglyceride levels following oral triolein (1 ml) in fat pretreated and untreated control rats

- Pretreated rats
- Untreated rats

\[ \pm \text{SEM} \]
* \( p < 0.01 \)
showed similar triolein-stimulated GIP levels whether they were given IP insulin or saline (1008 ± 95 versus 1116 ± 100 ng/Litre pNS). Plasma glucose levels in rats given IP insulin after oral fat fell significantly less in the pretreated than in the untreated controls (2.6 ± 0.48 versus 1.57 ± 0.15 mmol/Litre p < 0.05) although plasma insulin levels 20 min after insulin injection were similar in the two groups (4.1 ± 1.7 versus 4.7 ± 1.22 µg/Litre pNS, Fig 6.3).

(c) Effect of fat pretreatment on the response to oral glucose with and without intraperitoneal insulin

Following oral glucose, fat-pretreated rats had significantly higher levels of plasma glucose (10.2 ± 0.39 versus 8.9 ± 0.41 mmol/Litre p < 0.025) and insulin (6.2 ± 1.2 versus 2.5 ± 0.59 µg/Litre p < 0.01) one hour after dosing than untreated controls (Figure 6:4). Plasma GIP levels showed a tendency, significant at the 10% level, to be higher in the fat-pretreated group compared with their untreated controls.

No significant difference was observed in plasma immunoreactive insulin and plasma glucose levels in pretreated rats given oral glucose intraperitoneal insulin compared with the untreated control group.

6:3:2 Investigation of the responses of the enteroinsular axis in man on high and low fat diets

No significant change was observed in the weight of the volunteers during the entire study (Table 6:1). Basal triglyceride, insulin, C-peptide and GIP levels were not significantly different on the two dietary regimens. Basal LSI levels were elevated on the high fat compared with the low fat diet (29 ± 2.9 versus 19 ± 1.44 LSI ± SEM p < 0.025). Dietary energy intake records were maintained throughout the study periods (Table 6:2).

(a) Effect of high and low fat diets on responses to oral fat

Figure 6:5 shows the plasma immunoreactive GIP concentrations which were not significantly different after fat loads on high and low fat diets. Basal plasma glucose levels were similar throughout the fat loads on both diets. Plasma C-peptide and insulin levels showed no significant rise after oral fat throughout the study. No significant difference was observed between insulin and C-peptide levels on the two diets.
Figure 6:3
Plasma glucose IM- insulin and IM- GIP levels following oral triolein (1 ml) in fat pretreated and untreated control rats.

- Untreated rats
- Pretreated rats

± SEM

GLUCOSE (mmol/l)

INSULIN (ug/l)

GIP (ng/l)
Figure 6:4

Plasma glucose, IM-insulin and IM-GIP levels following oral glucose (5 mg/kg body weight) in fat pretreated and untreated control rats

- Untreated rats
- Pretreated rats

SEM
GLUCOSE (mmol/l)

INSULIN (µg/l)

GIP (ng/l)

* P<0.05
** P<0.025
### Table 6:1

**Effect of high and low fat diets on bodyweight in man**

Mean ± SD  \( n = 9 \)

<table>
<thead>
<tr>
<th>Diet Type</th>
<th>Start (Day 1)</th>
<th>End (Day 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low fat diet</td>
<td>66.4 ± 9.6 kg</td>
<td>66.1 ± 10.1 kg</td>
</tr>
<tr>
<td>High fat diet</td>
<td>66.4 ± 9.3 kg</td>
<td>66.4 ± 10.1 kg</td>
</tr>
</tbody>
</table>
### Table 6:2

**DAILY ENERGY AND FAT INTAKE**

Mean ± SD

<table>
<thead>
<tr>
<th></th>
<th>Mean Energy Intake/day</th>
<th>Mean Fat Intake/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kJ (Kcal)</td>
<td>g</td>
</tr>
<tr>
<td><strong>GROUPS 1 &amp; 1.1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((n = 8))</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>USUAL DIET</strong></td>
<td>523 ± 228 (2188 ± 952)</td>
<td>80 ± 52</td>
</tr>
<tr>
<td><strong>S.L.F.</strong></td>
<td>299 ± 89 (1253 ± 393)</td>
<td>21 ± 10</td>
</tr>
<tr>
<td><strong>S.H.F.</strong></td>
<td>556 ± 79 (2325 ± 331)</td>
<td>152 ± 11</td>
</tr>
</tbody>
</table>

**S. L. F.** Short term low fat diet.
**S.H. F.** Short term high fat diet
Figure 6.5 Plasma immunoreactive GIP responses to oral fat on low and high fat diets
(b) Effect of high and low fat diets on responses to oral fat with exogenous insulin

All volunteers experienced a number of side effects approximately twenty minutes after insulin infusion. These lasted for varying lengths of time and included sleepiness, sweating, deterioration in vision and finally hunger. All volunteers stated that after the second insulin stress test these effects were less dramatic and persistent.

One volunteer from the initial group was excluded from the study since his normal dietary fat intake (157 g of fat per day) was very high.

After oral fat, plasma GIP secretion was inhibited by exogenous insulin on the low fat diet but not on the high fat diet in the remaining eight volunteers (Fig. 6:6) (AUC plasma GIP: 3979 ± 547 pgml⁻¹.hr (x ± SEM) after fat + insulin versus 5490 ± 599 pgml⁻¹.hr after fat alone on LFD, p < 0.01 5652 ± 962 pgml⁻¹.hr versus 4503 ± 654 pgml⁻¹.hr on HFD, p NS). The volunteer with the normal high fat intake showed no suppression of GIP release with IV insulin on the low fat diet.

Plasma glucose levels (Figure 6:7) were significantly lower on the low fat diet after the insulin compared with the high fat diet at all timepoints. Plasma glucose levels were plotted as a percentage fall of glucose levels from basal values since basal levels were elevated on the high fat diet (Fig. 6:8) Normoglycaemia was achieved faster whilst on the high fat diet than on the low fat diet.

Plasma insulin levels were measured in four volunteers. No significant difference was observed in basal levels or in the disappearance rate of exogenous insulin.

Figure 6:9 shows the mean plasma C-peptide levels which were not significantly different on either diet.

Plasma triglyceride levels were significantly raised 180 min after oral fat on the high fat compared with the low fat diet (1.6 ± 0.2 versus 1.14 ± 0.12 mmol/Litre p< 0.025) calculation of the area under the curves showed a significant increase in plasma triglyceride levels on the high fat compared with low fat diet (AUC triglycerides 1.27 ± 0.09 mmol/Litre .hr (x ± SEM) on HFD than on LFD 6.38 ± 0.12 mmol/Litre .hr. p < 0.005) (Fig. 6:10)
Figure 6:6 Plasma immunoreactive GIP levels after oral fat followed by IV insulin on low and high fat diets

GIP (ng/l)

- High fat diet
- Low fat diet
I ± SEM
N=8
* p<0.05
** p<0.025
*** p<0.0125
**** p<0.005

Insulin 0.2 U/kg b.w.

oral fat 100g

0 60 120 180 Time (min)

3000
2000
1000
Figure 6.7  Plasma glucose levels after oral fat followed by IV insulin on low and high fat diets.

<table>
<thead>
<tr>
<th>GLUCOSE (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High fat diet</td>
</tr>
<tr>
<td>Low fat diet</td>
</tr>
<tr>
<td>I ± SEM</td>
</tr>
<tr>
<td>N = 8</td>
</tr>
</tbody>
</table>

oral fat
100g

Insulin 0.2U/kg b.w.

Time (min)
Figure 6:8 Percentage change in plasma glucose levels after intravenous insulin on low and high fat diets.

**GLUCOSE - % Fall (basal level)**

- High fat diet
- Low fat diet

** ± SEM**

N=8

**INSULIN 0.2 U/kg b.w.**

- * p<0.05
- ** p<0.0125
Figure 6:9  Plasma immunoreactive C-peptide levels after oral fat followed by intravenous insulin on low and high fat diets.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Peptide (ug/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N=8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

- High fat diet
- Low fat diet
Figure 6:10 Incremental plasma triglyceride levels on both low and high fat diets.

- Low fat diet
- High fat diet

* * p < 0.05
** * p < 0.005

Oral fat alone + IV Insulin
(c) **Effect of high and low fat diets on the response to oral glucose**

Plasma insulin levels were not significantly different after oral glucose on the two diets (Figure 6:11). Plasma C-peptide levels were significantly elevated 150 min after oral glucose on the low fat compared with the high fat diet (3.3 ± 0.7 versus 1.8 ± 0.3 μg/Litre p < 0.025) (Fig. 6:12). Correlation of insulin and C-peptide levels between 60-210 min after oral glucose was carried out. A significant correlation was obtained on the low fat diet (coeff. of corr. = 0.47 p < 0.05) but not the high fat diet (coeff of corr = 0.28 pNS).

The plasma glucose levels were statistically similar on both dietary regimens following oral glucose. (Fig. 6:13).

6:4 **Discussion**

The dietary intake of rats can be controlled within very narrow limits. Normal fat intake of rats maintained in laboratory conditions is approximately 3.5% of their diet, which is very low when compared with levels usually found in Western man's diet. Oral fat dosing of rats increases their fat intake to 21%, which, although abnormal for the rat is closer in composition to a typical Western diet.

Humans have a large degree of variation in their daily fat intakes. In most prosperous countries fat usually contributes 35% to 45% of the total energy intake while in poorer countries this figure is about 15% or even lower. In order to maintain human subjects on diets of varying fat content the majority of dietary fat can be withdrawn and replaced by one substance so that the fat intake can be carefully controlled. The amount of fat in the diet prior to the study appears to effect the GIP responses to an oral fat load in rats. However, human subjects showed no change in GIP response which may be explained by the large variation in their normal dietary fat intake.

Maintaining both rats and humans on either short-term high or low fat dietary regimens results in no change in weight. Grundleger and Thenen (1982) maintained rats on both high fat and high carbohydrate diets for ten weeks and observed no increase in body weight in either case. Lavau et al (1979) fed rats low or high fat
Figure 6:11 Plasma immunoreactive insulin levels after oral glucose (75 g) on low and high fat diets.

- High fat diet
- Low fat diet

\[ \text{INSULIN (mU/l)} \]

\[ I \pm \text{SEM} \]

\[ N = 5 \]
Figure 6:12
Plasma immunoreactive C-peptide levels after oral glucose (75 g) on low and high fat diets.

C-Peptide (ug/l)
- High fat diet
- Low fat diet

± SEM
* p<0.025
Figure 6.13

Plasma glucose levels after oral glucose (75 g) on low and high fat diets.

GLUCOSE (mmol/l)

- High fat diet
- Low fat diet

±SEM

N=8

0 60 120 180
Time (min)
diets and observed no difference in their weights after four weeks, although epididymal adipose tissue and fat cell size increased during the high fat diet. On the high fat dietary regimen both rats and humans showed increased triglyceride levels following oral fat compared with a low fat diet. This increase was more marked in the rats than in the human subjects. Adaptation of the enzyme pancreatic lipase to dietary lipids has been reported in the rat (Deschodt-Lanckman et al. 1971). Unsaturated fatty acids stimulate lipase significantly more than saturated fatty acids. This increase in activity of pancreatic lipase has been reported in other animals (Behrman and Kare, 1969, Wills and Hinners, 1968). Dietary enzyme adaptation has been reported to take only 2 to 3 days, the enzyme levels being stabilised after 5 to 7 days. However, it has been suggested that changes, such as increased absorption of lipids, depends also on the adjustment of other digestive processes such as gastric emptying or intestinal motility.

GIP levels were elevated following oral fat in rats pretreated on a high fat diet compared with their unpretreated controls but no such increase occurred in the human subjects. A possible explanation for this difference is that the habitual fat intake of the human subjects was much greater than that of the rats and therefore the proportional increase in fat intake was not as great in the human subjects when placed on the high fat diet. The fat intake of the human volunteers was extremely variable ranging from 12 to 157 g per day considerable more variation than observed in the rats. At the end of the human study one volunteer was excluded who had a normal dietary fat intake of approximately 157 g per day while the other participants had an intake of less than 127 g of fat per day. These volunteers when on the high fat diet had a minimum fat intake of 135 g per day, resulting in a large increase in fat content compared with the low fat regimen and an increase of greater than 34% above their normal dietary intake.

There is some evidence that the rate of absorption is a factor in determining the magnitude of the GIP response. A reduction of nutrient absorption rate in certain diseases, such as untreated coeliac disease (Creutzfeldt et al. 1976) and marked
malabsorption (Besterman et al. 1979) and the addition of the unabsorbable carbohydrate guar gum to the meal (Morgan et al. 1979; Jenkins. 1980) have all been shown to depress GIP response to oral nutrients.

Exaggerated GIP responses to a mixed meal have been reported in obese subjects (Creutzfeldt et al. 1978). These could, however, be reduced by dietary restriction (Willms et al. 1978). Reiser et al. (1980) showed that maintenance on a high sucrose diet resulted in a greater GIP response to sucrose compared with subjects on high starch diets. Diet composition, therefore, appears to be more relevant than body weight in determining the magnitude of GIP response to oral nutrients. The data from the rat study support this concept but because human subjects habitually consume more fat than rats longer term dietary modification may be necessary in order to show any modification of GIP secretion in response to oral fat in man.

A number of workers (Brown et al. 1975; Crockett et al. 1976) have shown that intravenous infusions of insulin or glucose reduced the rise in fat stimulated GIP levels and, therefore, a negative feedback control of insulin on GIP release has been suggested. In the rat study the untreated group showed inhibition of fat stimulated GIP release by exogenous insulin but this was not observed in the fat pretreated group. In the human studies, the eight volunteers, who had a fat intake of less than 130 g per day, showed inhibition of GIP release to insulin on the low fat diet but no effect was apparent on the high fat regimen. One subject in the initial study had a habitual high fat intake and appeared insensitive to insulin on both dietary regimens. Creutzfeldt et al. (1978) have observed in obese subjects that there is also a defective insulin feedback mechanism. The experimental design of the human studies, was such that low and high fat diets were randomised. It was therefore, possible to show that the sensitivity of the GIP cells to insulin could be reversed after a short-term dietary change.

A degree of insulin insensitivity was observed in both rats and human on short term high fat diets. This was more marked in the rodent study. Other workers
(Lavau et al. 1979, Grundleger and Thenen 1982) have investigated the effect of high fat diets in order to establish what causes the change in insulin sensitivity. Investigation into insulin receptor number in human monocytes (Pederson et al. 1980) and rat soleus muscles (Grundleger and Thenen, 1982) have showed that they are altered by dietary changes. Grundleger and Thenen (1982) have demonstrated that although receptor number decreased on a high fat diet, post receptor changes were far more important in contributing to insulin sensitivity.

In obesity insulin resistance also occurs at the level of the target tissues. The B cells of obese animals oversecrete insulin and the hepatic clearance of insulin is decreased. The concentration of insulin in the portal vein is raised so that more insulin reaches peripheral tissues (Karakashi et al. 1976). Insulin and C-peptide levels showed a positive correlation at the 5% level on the low fat diet after oral glucuose. No such relationship could be reported on the high fat diet. More data is required from a large number of subjects before any definite conclusions can be drawn.

Exogenous insulin (0.2 U/kg bodyweight) in normal subjects generally results in mild to moderate neuroglycopenic symptoms commencing twenty to thirty minutes after the insulin injection and lasting ten to thirty minutes. In the human studies all subjects experienced such symptoms on both dietary regimens. The initial insulin stress test appeared to produce the most severe symptoms irrespective of the regimen and the after effects were prolonged for a considerable period. The plasma glucose levels although significantly elevated on the high fat diet after the insulin stress test had little effect on the actual recovery time and severity of the symptoms experienced by the subjects. It would, therefore, appear that the reduced severity of the symptoms experienced after the second insulin stress test was due to the mental and physical awareness of the effects of insulin.

In conclusion, increased intake of a particular nutrient i.e. fat resulted in a state of mild insulin insensitivity in both man and rats and hypersecretion by GIP cells in rats. Pathological conditions characterised by hyperinsulinism such as obesity
(Creutzfeldt et al. 1978) and maturity onset diabetes (Brown et al. 1975) may be partly due to an overactive enteroinsular axis caused by previous dietary intake.
CHAPTER VII

FINAL DISCUSSION
7:1 Human C-peptide radioimmunoassay

An assay for the measurement of human C-peptide has been developed suitable for routine hospital laboratory use. Although there have been previous reports of C-peptide assay described in the literature, there have been problems concerning the standardisation of the assay which were still unresolved when this assay was developed.

A prerequisite for developing a radioimmunoassay is an adequate supply of C-peptide for use as immunogen and standard. Human C-peptide is required since there is a large species variation in the primary structure of this peptide. The rarity of natural human C-peptide has made it essential to synthesise the peptide in order to obtain sufficient quantities for radioimmunoassay development. Recently a biosynthetic human C-peptide has been produced which will hopefully be available in sufficient quantity to overcome this problem. Natural human C-peptide contains thirty-one amino acids. However, some "so called" synthetic C-peptides contain extra amino acids and functional groups. Kaneko et al. (1974) reported the development of a human C-peptide assay using antisera raised against a 35 amino acid peptide, whose crossreactivity with natural human C-peptide was claimed to be similar to the synthetic peptide. Work described in Chapter II has demonstrated that many C-peptide standards are not pure or do not always run parallel to endogenous C-peptide depending on assay conditions. The lack of an international C-peptide standard has lead to the development of many different 'in house' standards. It will not be until biosynthetic human C-peptide is available in sufficient quantity and purity, that the problem of standardisation will be finally resolved.

Normal mean overnight fasting C-peptide values vary widely between laboratories ranging between 1.1-2.45 µg/Litre (Block et al. 1972, Kaneko et al. 1974, Heding and Rasmussen. 1975, Beischer et al. 1976). This may be due, in part, to the lack of an international standard. However, there have been reports that circulating C-peptide is heterogenous and this may contribute to the large variation in fasting
values. Various closely related peptides with different immunogenic properties have been identified in serum (Kuzuya et al. 1977, Kakita et al. 1980). A number of workers have reported that different C-peptide antisera gave different values with the same plasma sample (Heding, 1975, Kuzuya et al. 1978). Serum samples have been shown to contain small and large molecular weight substances whose chemical identity is unknown and which crossreact in the C-peptide assay (Kuzuya et al. 1977, 1978, Kakita et al. 1980).

The production of a human C-peptide antiserum raised against the peptide coupled to ovalbumin by gluteraldehyde at the N terminal glutamic acid residue has been described in Chapter II. This is the first report of an antiserum raised against C-peptide using this conjugation method, and has theoretical advantages over the carbodiimide method of conjugation used by other workers (Melani et al. 1970, Beischer et al. 1976) which has been discussed previously. The C-peptide antiserum was raised in a primitive breed of sheep (Soay) since attempts in rabbits proved unsuccessful. The Soay was chosen since these animals have previously proved successful in producing antiserum to non immunogenic compounds and a better immune response should occur theoretically in primitive breeds of animals. Human C-peptide antiserum has been raised by other workers in goats, guinea-pigs and rabbits (Melani et al. 1970, Kaneko et al. 1974, Heding, 1975, Beischer et al. 1976). Due to the low immunogenicity of C-peptide high titre antiserum was not obtained from the sheep. Sufficient antiserum was, however, obtained due to the large volume of blood which could be obtained from the animal.

C-peptide and insulin are released in equimolar amounts from the pancreas. However, the measurement of C-peptide, as well as insulin, can provide additional information on B cell function and in certain situations, outlined below, it has advantages over insulin measurements.

The difference in the half lives between insulin and C-peptide makes them suitable for investigating different aspects of B cell function. The half-life of C-peptide has been reported to be longer than that of insulin. Published data concerning the
half-life of C-peptide have reported values anywhere between 11-33 minutes (Horwitz et al. 1973. Munemura et al. 1974. Kutuza and Maksuda 1976, Faber et al. 1976, Krause 1977). This large disagreement in the calculated half-life of C-peptide is due, in part, to experimental design and degree of crossreactivity of proinsulin in the C-peptide assay. The slower metabolism of C-peptide compared with insulin makes it impractical to measure it when short, rapid, changes in B cell secretion are being studied. Insulin is, therefore, a better indicator of rapid changes in B cell secretion because of its shorter half-life. However, when looking at an overall effect it is possible to measure C-peptide levels at less frequent intervals than insulin and still obtain a reliable estimation of the integrated B cell response.

Another method of obtaining an estimation of integrated B cell function is to measure urinary C-peptide levels. The C-peptide radioimmunoassay can be easily modified to measure levels in urine. Several workers have reported C-peptide levels in urine (Kuzuya et al. 1976, Horwitz et al. 1977, Meistas et al. 1981). The urinary clearance of C-peptide is approximately 5% compared to 0.2% for insulin. This makes it possible to measure urine C-peptide levels in clinical situations where insufficient or no serum can be obtained, for example, in infants.

Differences in the basal circulating concentrations of C-peptide and insulin have meant that there are advantages in measuring C-peptide rather than insulin in basal conditions and when the B cell is suppressed. Mean fasting plasma C-peptide level in normal subjects, measured in the assay described in Chapter II, was $1.38 \pm 0.61 \mu g/Litre$. The assay was developed to be extremely sensitive having a limit of detection calculated to be 10 pg (Feldman and Rodbard, 1971) corresponding to a concentration in unextracted plasma of 0.1 pg/Litre which is well below normal fasting levels. However, fasting plasma insulin levels in normal subjects are approximately 10-fold lower between 0.12 - 0.2 pg/Litre (3-5 mU/Litre) which is near, and sometimes, below the detection limit of most insulin assays. The measurement of fasting C-peptide levels would, therefore, be expected to give more reliable results.
The C-peptide primary sequence is contained within the proinsulin molecule and therefore antisera prepared against human C-peptide crossreact with human proinsulin. The C-peptide antiserum described in Chapter II has a crossreactivity of 10 per cent with proinsulin. This is lower than the proinsulin crossreactivities previously reported for other human C-peptide assays which are generally between 15-66 % (Melani et al. 1970, Kaneko et al. 1974, Cerrigill et al. 1980). It is important to know the degree of crossreactivity in the assay but whether a high or low crossreactivity is advantageous depends on the clinical situation. Most insulin antisera, however, generally crossreact to a very much larger degree with human proinsulin than does the C-peptide antiserum described in Chapter II. In addition, assessment of some insulin antisera has shown that the crossreactivity with proinsulin is variable, dependant upon the relative concentration of proinsulin and insulin.

The addition of a tyrosine residue to the N terminal end of the C-peptide molecule made it possible to produce an iodinated peptide. Difficulties were, however, encountered in producing a tracer of sufficiently high specific activity for use in an assay without causing damage to the peptide. Eventually a lactoperoxidase method was used in which the reaction was stopped after only fifteen seconds. Other workers have reported no such difficulties in producing an iodinated C-peptide either using lactoperoxidase or chloramine T methods (Kaneko et al. 1974, Beischer et al. 1976) which, once again, suggests that the nature of the synthetic peptide must be important.

7:2 B cell function in normal human subjects: the relevance of immunoreactive C-peptide measurements.

The large variation in reported fasting C-peptide values makes it essential to establish our own reference plasma C-peptide levels using the assay described in Chapter II, in response to a variety of stimuli. The measurement of C-peptide levels in normal subjects has led to several observations.

The correlation between fasting plasma C-peptide levels and body weight was
calculated in normal non-obese volunteers since Krause (1977) had reported the dependence of basal plasma C-peptide levels on body weight. This might have been expected since insulin and body weight have previously been observed to be correlated (Bagdade et al. 1967).

Blood samples were obtained hourly from ten normal non-obese subjects for 24 hours after a test meal. Calculation of mean fasting plasma C-peptide levels from 10 hours after the meal gave a mean value of $1.7 \pm 0.2 \, \mu g/Litre$. Previously reported mean fasting plasma C-peptide levels were $2.53 \pm 3.8 \, \mu g/Litre$ over a 24 hour period but this data was obtained from six normal pregnant women allowed three meals during the study (Lewis et al. 1976).

Ingestion of a mixed meal at 1000 h produced an increase in C-peptide levels approximately 4-fold above basal values. Ingestion of the same meal at 0200 h produced a significantly greater integrated C-peptide and insulin response. However, as the sampling time points in this study were hourly it is difficult to draw any firm conclusions and the six meal study was, therefore, carried out.

The "six meal" study clearly showed that peak plasma insulin levels varied depending on the hour at which the meal was eaten. Meals were eaten on six separate occasions at different times in a 24 hour period. Variation in the time of the meal also affected the time at which peak post prandial plasma insulin and C-peptide levels were obtained. Plasma insulin levels reached a maximum approximately thirty minutes after the test meals consumed at 1000 h and 0200 h. However, maximum levels occurred 90-120 minutes after the other meals. This variation was also reflected in the C-peptide data. Insulin secretion is dependent not only upon the circulating glucose levels but also upon insulinotropic gut hormones which are secreted when carbohydrates are taken orally. This component of insulin secretion has been termed by Unger and Eisentraut (1969) "the enteroinsular axis". A potential major component of the enteroinsular axis is the gastrointestinal hormone, GIP. In order to ascertain to what extent the changes in B cell response were due to changes in the enteroinsular axis when meals were
eaten at different times of the day volunteers were given i.v. glucose loads at 
1000 h and 0200 h on different occasions. Differences in B cell response to 
intravenous glucose at different times were very small. The two intravenous 
glucose loads gave similar peak and integrated plasma insulin responses. However, 
the integrated C-peptide response was slightly but significantly greater in response 
to the test carried out at 0200 h. These results demonstrate that the longer half-
life of C-peptide has made it possible to detect small difference in the B cell 
response to glucose loads which is not possible by monitoring insulin levels and 
sampling at the same time points.

Since differences in maximum insulin levels were observed when nutrients were 
given orally at different times of the day it is suggested that changes in the 
enteroinsular axis might be partly responsible. However, the height of peak plasma 
GIP levels following a meal did not depend on the time at which the meal was 
eaten. The nature of the component of the enteroinsular axis which might be 
causing these differences in insulin secretion observed, therefore, remains 
unknown.

Integrated C-peptide and insulin responses in the first six hours after each meal 
were compared. It was unexpected to find that they did not show any significant 
correlation. A similar lack of correlation was found in response to intravenous 
glucose.

Insulin and C-peptide are mainly metabolised by different organs in the body. A 
large proportion of secreted insulin is extracted during its initial transit through 
the liver (Samols and Ryder, 1961) while C-peptide is not extracted to any 
significant degree (Stoll et al. 1970), the kidney being the major organ responsible 
for its degradation and removal.

It was possible to correlate peripheral insulin and C-peptide in seventeen non-obese 
volunteers in the fasting state. However, no such correlation was observed after 
intravenous glucose or a test meal. The extent of correlation of insulin and 
C-peptide is related to hepatic insulin clearance on the one hand and the removal
of C-peptide by the kidneys on the other. In chronic renal failure patients both insulin and C-peptide levels have been reported to be elevated. Jaspan et al. (1977) reported that such patients had no correlation between plasma insulin and C-peptide levels. A reduction in hepatic insulin clearance has been observed in subjects with mild glucose intolerance. Malmquist et al. (1981) studied normal and mildly glucose intolerant males and demonstrated that insulin C-peptide ratios were elevated in the latter. The volunteers in the present study were neither obese or glucose intolerant and had normal hepatic and renal function. The reason for the failure to correlate stimulated insulin and C-peptide levels is not clear, although this might be possible if the number of volunteers in the study and the frequency of sampling were to be increased.

7.3 The diagnostic use of C-peptide estimations.

Human C-peptide plasma levels have been estimated in a wide variety of clinical conditions (Horwitz et al. 1978, Molar et al. 1978). The C-peptide assay described in Chapter II has been used to assess plasma C-peptide levels in certain specific clinical conditions. Its main use has been in the diagnosis of patients with insulin producing tumours. The major clinical manifestation in these patients is hypoglycaemia due to inappropriate secretion of insulin. The definitive diagnosis of an insulinoma takes place in three stages

(a) The suspicion and demonstration of recurrent hypoglycaemic attack followed by

(b) biochemical analyses to determine the cause of the hypoglycaemia and finally, if hyperinsulinism (or more correctly inappropriate insulin secretion) is demonstrated.

(c) localisation of the tumour (Marks, 1981)

A wide variety of tests have been used in the assessment of insulinoma patients. The most common is the prolonged or overnight fast with the measurement of plasma glucose, insulin and C-peptide levels. The demonstration of inappropriately high C-peptide and insulin with low glucose values distinguishes insulinoma patients.
from all other patients with hypoglycaemia. However, the prolonged fast requires hospitalisation and close observation and so may be impractical both for the hospital and the patient. Insulin stress tests have therefore been advocated if hypoglycaemia cannot readily be induced or if prolonged fasting is not possible. The development of the C-peptide radioimmunoassay enables B cell secretory function to be assessed during insulin induced hypoglycaemia. Impairment of B cell suppression during this test has been clearly demonstrated in two patients described in Chapter IV compared to normal non-obese subjects. The major advantage of this test is that it is suppressive rather than a provocative test, it is of short duration and it can be carried out on outpatients. However, when performing an insulin stress test, care must be taken to ensure that hypoglycaemia is induced in order to evaluate the C-peptide results and that the patient has adequate renal function. Failure to meet these criteria would produce results which would be meaningless.

Insulinoma patients have similar or slightly raised fasting insulin C-peptide ratios compared with normal subjects. One patient in our series with a disseminated malignant insulinoma had an elevated ratio suggesting that chronic insulin overproduction or possibly the presence of liver metastases had affected hepatic insulin clearance.

Monitoring fasting C-peptide and plasma glucose levels in normal subjects and insulinoma patients clearly demonstrated the difference between the two group as shown in Chapter IV. However, these results represent only a small series of patients and the possibility cannot be excluded that a larger series of subjects will contain some whose fasting C-peptide and glucose values fall in an area where they are difficult to assess.

A number of other mainly provocative tests have been used, in the past, to investigate patients suspected of having insulinomas. Nowadays their use is confined only to a few cases where diagnosis is inconclusive, in which they may provide additional useful information.
The value of C-peptide levels in the detection of factitious hypoglycaemia due to exogenous insulin has been clearly demonstrated when undetectable or low C-peptide levels are found in conjunction with high circulating insulin values. Although the frequency of these cases is uncommon C-peptide estimation is valuable in demonstrating unequivocally the administration of insulin.

**7:4 The physiological role of C-peptide.**

Circulating C-peptide was considered to be biologically inert. Its physiological function was assumed to be confined to aiding the biosynthesis of insulin by holding the amino acid sequence of the insulin precursor in the correct three dimensional structure to facilitate alignment of the A and B chains of insulin. However, studies using synthetic C-peptide have suggested that it may have several other biological roles.

C-peptide may be involved in islet cell hormone release (Toyota et al. 1976, Wojcikowski et al. 1977). However, as discussed in Chapter V the evidence is conflicting, with other workers demonstrating that this peptide has no effect in the isolated islet or pancreas (Yasuda et al. 1976, Kaneko et al. 1978). The concentration of C-peptide used and isolation procedures varied and these factors, were probably responsible for the different results obtained.

The close relationship between the gastrointestinal hormones, GIP and insulin release has long been known. GIP has been shown to be inhibited by a number of substances released from the pancreas including insulin (Brown et al. 1975) and somatostatin (Pederson et al. 1975). Previous work in this department has suggested a possible role for C-peptide as an inhibitor of fat stimulated GIP release. Dryburgh et al. (1980) demonstrated that exogenous rat C-peptide inhibited fat-stimulated GIP release in the rat. However, the purity of the exogenous C-peptide (synthetic rat C-peptide II) was not assessed and the concentration of this peptide in the rats was approximately three times higher than circulating levels in normal fed rats.
Studies were, therefore, carried out to ascertain whether endogenous C-peptide produced the same effect. In the course of the work it became clear that the experimental design was very important. GIP was initially stimulated in the rats by perfusion of the intestines with a fat emulsion. Fat stimulated GIP release was inhibited in the presence of high circulating endogenous C-peptide levels when insulin bioactivity was blocked by means of insulin antiserum. However, the gut perfusion technique resulted in an unphysiologically fast transit time for the emulsion through the gut with the result that fat absorption was probably impaired. GIP release is dependent upon absorption of nutrients and using the gut perfusion technique fat stimulated plasma GIP levels were low, only approximately twice basal levels. In addition, problems were encountered in the route of intravenous administration of tolbutamide and insulin antiserum to ensure reproducible high circulating C-peptide levels. Care also needed to be taken to avoid unnecessary handling of the gut and pancreas to prevent falsely elevated C-peptide levels.

Further studies were, therefore, carried out orally dosing rats with fat to eliminate the problems of the gut perfusion technique. In order to attain a maximum GIP response to an oral fat load rats were pretreated with oral fat for four days prior to the experiment as this had previously been shown to increase the GIP response (Kwasowski, 1983). It was an unexpected finding that in these fat-pretreated rats fat-stimulated GIP levels were not reduced in the presence of high circulating endogenous C-peptide. Obese subjects with abnormal glucose tolerance have been reported to have a defective feedback mechanism of insulin on GIP release (Creutzfeldt et al. 1978). It is, therefore, possible to suggest that the inhibition of C-peptide on GIP release was effected by fat pretreatment in the rats. At this point, however, the supply of insulin antibodies necessary in these experiments was exhausted. In view of the results obtained from the fat pretreated rats, it was decided to investigate this aspect further. Studies were, therefore, carried out to investigate the effect of insulin, a known inhibitor of GIP release, in fat pretreated rats.
The work carried out so far indicates that endogenous C-peptide is, in fact, an inhibitor of fat stimulated GIP release, further investigation is required in rats maintained on normal laboratory diets, subject to adequate supplies of insulin antiserum being available. The recent availability of biosynthetic human C-peptide will enable investigation in man to be carried out and should resolve the question of the relative importance of C-peptide and insulin in the feedback inhibition of GIP.

The effect of high fat diets on the enteroinsular axis in man and rats.

The lack of inhibition of fat stimulated GIP secretion in the presence of high circulating C-peptide levels in fat pretreated rats could be explained by a defective feedback inhibition mechanism. Studies were, therefore, carried out in similarly treated rats given insulin, a known inhibitor of fat stimulated GIP release (Brown et al. 1975). No inhibition of GIP release by exogenous insulin was observed in rats maintained on short term high fat diets. However, insulin induced inhibition was demonstrated in rats maintained on a low fat dietary regimen. This study could, therefore, explain the lack of C-peptide inhibition of GIP release in fat pretreated rats described previously.

A mild state of insulin insensitivity was also observed in the fat pretreated rats after exogenous insulin. Other workers (Lavau et al. 1979, Grundleger and Thenen, 1980;) have observed similar changes in insulin sensitivity in rats maintained on short-term high fat diets.

Further studies were carried out to investigate whether this lack of GIP inhibition by exogenous insulin observed in rats could be induced on a high fat dietary regimen in man. Previous workers (Brown et al. 1975) have shown that exogenous insulin inhibits fat stimulated GIP release in volunteers consuming a 'normal' diet although the fat intake of the subjects was not stated. Studies were initially carried out in five volunteers maintained on nine day low fat and high fat diets. Volunteers were maintained on their 'normal' diets for 15 days between the low and high fat dietary regimens. One volunteer had to be excluded since inhibition of GIP release by exogenous insulin could not be demonstrated on either dietary
regimen. This volunteer had a high 'normal' fat intake of 157 g of fat per day. To increase the numbers in the study, a further group of four volunteers was, therefore, chosen, whose 'normal' fat intake was below 130 g. This second group of volunteers were maintained on a reverse dietary regimen compared with the first group i.e. high fat followed by low fat diet.

Inhibition of fat stimulated GIP release by exogenous insulin was demonstrated in these subjects maintained on a low fat dietary regimen. However, as in rats, volunteers showed no such inhibition when on the high fat diet. Due to the experimental design of the human study it was possible to demonstrate the reversibility of insulin insensitivity on the GIP cells. When maintained on the high fat diet subjects showed no inhibition of GIP release by exogenous insulin but nine days on a low fat diet was sufficient to re-establish inhibition by insulin. Creutzfeldt et al. (1978) observed a defect in the insulin feedback mechanism on GIP release in obese subjects. Our findings suggest that these results might be explained by the previous dietary history of the subjects rather than their body weight. Insulin inhibition of GIP release was not, however, investigated in a subsequent study by Creutzfeldt's group (Willms et al. 1978) on the effect of dietary restriction and starvation on GIP release in obese subjects. Creutzfeldt et al. (1978) also reported an exaggerated GIP response in obese subjects given a mixed high energy meal. However, starvation or dietary restriction with minimal weight loss reduced the GIP response (Willms et al. 1978). In view of the results described in Chapter VI a possible explanation of this data could lie in changes in the feedback control of GIP by insulin and C-peptide which are secreted in response to a mixed meal. In addition short term high fat feeding in rats resulted in significantly elevated GIP release in response to fat calculated from time course data. This effect was not observed in the human experiments although exaggerated GIP secretion in obese subjects is well documented. The dietary fat intake of a rat is normally very low unlike a typical western human diet and this may explain the differences observed between the two species. Both rat and human studies suggest
that previous diet is more relevant than body weight in determining the magnitude of the GIP response to oral nutrients.

Dietary adaptation to a high fat diet resulted in increased absorption of triglycerides and a degree of insulin insensitivity in both rats and man. GIP release in response to fat was elevated in the rat. The elevated GIP release in rats may be dependent on both increased absorption of fat and insulin insensitivity of GIP cells. However, in man, insensitivity of the GIP cells to exogenous insulin was present without an elevated GIP response to fat on the high fat diet. It would be interesting to ascertain whether a longer period of exposure to a high fat diet in man would lead to hypersecretion of GIP in response to oral fat alone.

The implication of these studies indicate that previous diet is important in determining the GIP release to a particular substance. An exaggerated GIP release to nutrients results in an overactive enteroinsular axis causing hyperinsulinaemia.

7:6 Future work.

The measurement of human C-peptide levels has proved clinically useful in certain conditions for example insulinomas. However, proinsulin levels are often elevated in such patients. Immunoreactive insulin and C-peptide data are difficult to interpret due to varying degrees of crossreactivity of the antisera with proinsulin. The estimation of proinsulin levels would, therefore, be desirable to enable insulin and C-peptide results to be more accurately interpreted. Biosynthetic human proinsulin and C-peptide will shortly become available in larger quantities as a result of change in commercial processes for manufacturing human insulin for diabetics. It will, therefore, be possible to develop a direct radioimmunoassay for proinsulin suitable for routine hospital laboratories.

The availability of biosynthetic human C-peptide suitable for infusion into human volunteers will be very useful in the elucidation of the physiological role of C-peptide. Investigations into both the endocrine role of C-peptide and its possible paracrine role within the islets should prove rewarding.

Lack of time prevented the follow up in the human dietary studies of the single
volunteer with a habitually high fat consumption who demonstrated a defective feedback inhibition of GIP. It would be interesting to ascertain whether giving a long-term high fat diet to human volunteers has a more permanent effect upon the feedback inhibition of insulin upon GIP. These studies would extend our understanding of the role of diet in the activity of the enteroinsular axis and the secretion of insulin and may ultimately prove relevant in the investigation of various disease states, for example, coronary artery disease, where hyperinsulinism appears to be implicated.


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Publications:


