GROWTH HORMONE AND ITS RELATIONSHIP TO THE MICROVASCULAR COMPLICATIONS OF DIABETES MELLITUS.

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Physicians of the utmost fame
Were called at once; but when they came
They answered, as they took their fees,
´There is no cure for this disease´.

from ´Henry King´
Hilaire Belloc
´Cautionary Verses´

TABLE OF CONTENTS

Chapter 5

5.1 The change in serum GH induced by lowering of the plasma glucose within the physiological range in 3 normal subjects

5.2 Change in plasma glucose, serum insulin and glucose infusion rate during glucose clamp experiments in diabetics at diagnosis and after 6 months insulin therapy

5.3 Change in serum GH during the steady state periods of the glucose clamp experiments at diagnosis and after 6 months insulin therapy

Chapter 6

6.1 Scatterplot of unidirectional [^{11}C]3-O-methyl-D-glucose extraction in normal subjects and diabetics studied at euglycaemia and hyperglycaemia

Chapter 7

7.1 Profile of serum GH from a diabetic subject with proliferative retinopathy separated on Sephadex

7.2 Composite native PAGE profiles of serum GH from patients with proliferative retinopathy, synthetic 22k GH and pituitary extracted GH

7.3 HPLC profiles of serum GH from a diabetic subject with proliferative retinopathy, a normal subject, synthetic 22k GH and pituitary extracted GH

Chapter 8

8.1 24 hour profiles of serum GH and IGF-I from 4 diabetic subjects with proliferative retinopathy

8.2 Scatterplot of serum IGF-I values from normal subjects and diabetic subjects separated according to retinopathy grade

Chapter 9

9.1 24 hour GH profiles of 4 diabetic subjects with proliferative retinopathy before and after maximum dosage of SMS 201-995

Chapter 10

10.1 Proposed sites of 2 main abnormalities accounting for GH hypersecretion in diabetes.

LIST OF TABLES

All studies and experiments contained in this work were designed and carried out by the author with the advice and guidance of the author's supervisor, Dr Eva Kohner. All were approved by the Ethical Committee of the Royal Postgraduate Medical School and Hammersmith Hospital.

There have been 4 main areas of collaboration :-

In the follow-up study of patients with proliferative retinopathy treated by ^{90}Y pituitary ablation, data were collected with help from Dr T.J.Fallon. The author was responsible for the analysis of the medical details, and Dr Fallon for the ophthalmological data, although there was discussion and consultation on both sides throughout.

The experiments examining the effect of insulin and glucose on pituitary cells in culture were performed at the suggestion of the author. The work was carried out in collaboration with Dr E.Adams, and resulting hormonal assays carried out by the author.

Experiments examining glucose transport across the blood brain barrier were again carried out at the instigation of the author. The work was performed in collaboration with the staff of the Medical Research Council Cyclotron Unit at The Royal Postgraduate Medical School, and in particular with Dr D.J.Brooks who developed the methodology. The author's contribution was to design the study, and to maintain conditions of constant plasma glucose and insulin during scans.

The section involving high pressure liquid chromatography was performed in the laboratory of Professor S.R.Bloom. All work was performed by the author, under the watchful eye of Mr Ying Li.

Finally, all assays for growth hormone and insulin-like growth factor-I were carried out by the author.

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SUMMARY

The aim of this work was to discover possible causes of growth hormone (GH) hypersecretion in diabetic subjects, and to examine the possibility that the GH so produced is relevant to the development of diabetic microvascular disease.

To reassess the hypothesis that GH plays a role in the genesis of diabetic microvascular complications, patients who had undergone pituitary ablation for treatment of retinopathy were followed up. Between 1960 and 1976, 117 patients had an ^{90}Y pituitary implant. The 5 year mortality of these patients was 17.6% and the 10 year 51%, figures lower than generally reported for patients with proliferative retinopathy. Between 7 and 13% developed renal failure post-ablation, again lower than generally reported for unablated diabetics. The 100 patients operated on between 1965 and 1976 were included in the ophthalmological follow-up. Of these patients, the visual acuity in the better eye at the time of operation was 6/12 or more in 84%, this percentage remaining similar at the time of 5 and 10 year follow-up. By 5 years, grading of new vessels on the disc had improved from an initial grading of 2.7 ± 1.6 to 0.8 ± 1.2 (mean$\pm$SD, $p<0.001$), and by 10 years there was no disc neovascularisation in any eye. There was similar improvement in peripheral new vessels, hard exudates, microaneurysms and haemorrhages. It is concluded that pituitary ablation had a beneficial effect on the course of diabetic microvascular complications.

After brief confirmation of the fact that GH hypersecretion can be demonstrated in patients with retinopathy using the 'in house' GH radioimmunoassay, an investigation of aspects of the GH

regulatory pathways in normal and diabetic subjects was carried out to suggest potential causes of GH hypersecretion in diabetes.

Using growth hormone releasing factor (1-44) (GRF), it is demonstrated that hyperglycaemia leads to a suppression of GRF stimulated GH secretion in normal subjects. Only 3 of 6 diabetic subjects studied in a similar fashion demonstrated suppression. In pituitary cells in culture, neither glucose nor insulin produced any effect on GRF stimulated GH secretion. It is concluded that hyperglycaemia produces suppression of GH by its action at hypothalamic level, and it can be inferred that there is a defect in GH regulation at this level in diabetes, perhaps mediated by lack of, or resistance to, somatostatin.

Regulation of GH secretion in response to changes in plasma glucosewas further examined by assessing the change in serum GH in response to a fall in plasma glucose from 12 to 6 mmol/l in 3 normal subjects. It was found that such a fall in plasma glucose is capable of stimulating a rise in serum GH, which mechanism may contribute to GH hypersecretion in treated diabetic subjects. In further studies, serum GH was measured in 13 diabetic subjects during the steady state periods of glucose clamp experiments at plasma glucose 12mmol/l at the time of diagnosis of diabetes, and after 6 months insulin therapy. It is shown that at diagnosis, diabetics are still capable of producing GH while hyperglycaemic, but insulin therapy abolishes this phenomenon. No correlation was found between measurements of insulin sensitivity and serum GH levels suggesting that GH hypersecretion in diabetes is the result of poor glycaemic control and not the cause, as has been suggested.

Still examining the central regulation of GH secretion in diabetes, glucose transport across the blood-brain barrier (BBB) was examined using [^{11}C]3-O-methyl-D-glucose and positron emission tomography (PET) to exclude the possibility that central neuroglycopaenia leads to over-production of GH in diabetes. Five diabetic subjects, including 3 with severe microvascular disease, took part in 8 PET studies at euglycaemia and hyperglycaemia, with and without insulin infusion. Four normal subjects acted as controls. Glucose extraction fractions were similar in euglycaemic diabetics and normal subjects. At hyperglycaemia, extraction fractions were reduced, but in absolute terms, the quantity of glucose crossing the BBB was unchanged. The presence or absence of insulin made no difference to glucose transport across the BBB. It is concluded that there is no abnormality of glucose transport across the BBB in diabetic subjects, including those with microvascular disease, and there is no cause for GH hypersecretion in diabetes at this level. Further, the BBB does not appear to be an insulin sensitive organ.

At circulatory level, a possible cause for GH hypersecretion might arise from altered proportions of GH forms with altered biological activity. Examination of GH forms extracted from diabetic serum and analysed using high pressure liquid chromatography and native polyacrylamide gel electrophoresis revealed no difference from normal.

Measurement of serum insulin-like growth factor-I (IGF-I) was carried out to assess the action of GH at tissue level. Patients with active proliferative retinopathy were found to have significantly higher serum IGF-I levels than both normal subjects and patients with inactive retinopathy. A case history is

presented demonstrating a simultaneous rise in serum IGF-I and deterioration in retinopathy, but the case is complicated by other factors, and definite conclusions cannot be drawn. Finally, IGF-I was measured in the vitreous of 7 patients with proliferative retinopathy and 3 control subjects, but no significant difference was detected. These results are discussed.

Amongst the serum IGF-I levels measured in the diabetic subjects, a significant negative correlation was found between IGF-I and HbA1 ($r= -0.3$, $p<0.01$). This suggests that poor glycaemic control in diabetes leads to failure of generation of IGF-I in response to GH, and provides another possible cause for GH hypersecretion.

Finally, to assess the contribution of GH to the development of diabetic microvascular complications, a trial of long acting somatostatin analogue was undertaken in 4 patients with proliferative retinopathy. After treatment with large doses of SMS 201-995 (500ug thrice daily), only 2 demonstrated a modest decrease in 24 hour GH levels. It is concluded that an element of somatostatin resistance exists in diabetes, and that somatostatin analogue will not be an effective method of treatment for proliferative diabetic retinopathy.

In conclusion, regarding the mechanism of GH hypersecretion in diabetes, 3 possible mechanisms are identified - somatostatin resistance induced by chronic hyperglycaemia, GH release induced by fluctuating plasma glucose, and failure of IGF-I generation secondary to poor glycaemic control. Regarding the role of GH and IGF-I in the genesis of diabetic microvascular complications, it is concluded that there is presently insufficient evidence to assign a specific role for either GH or IGF-I.

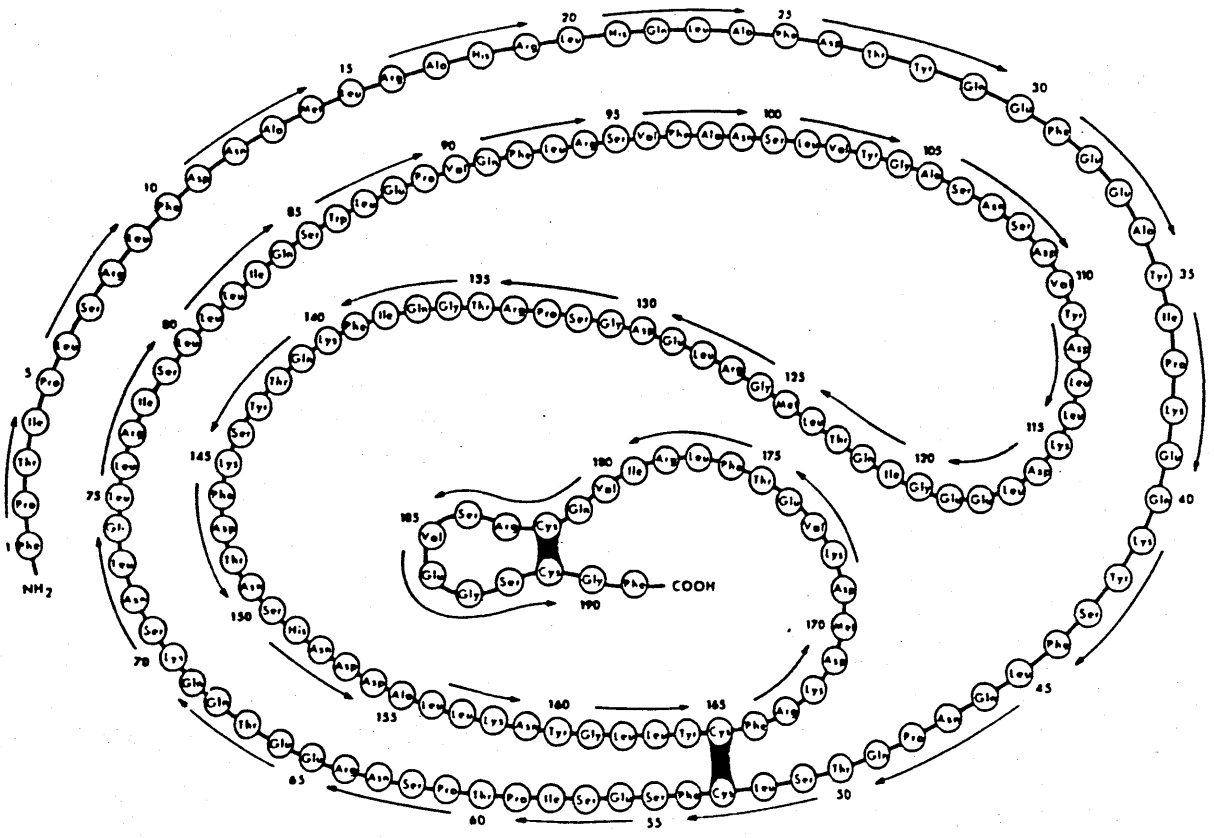
Interest in growth hormone (GH) in relation to diabetes mellitus essentially springs from the observation that GH levels are elevated in diabetes. At the same time, the improvement in diabetic microvascular complications following hypophysectomy or pituitary ablation has been ascribed to removal of the GH stimulus following these procedures. In the 1960s and '70s, Lundbaek (Lundbaek et al., 1970, Lundbaek, 1976) was the leading proponent of the view that GH was a 'permissive' factor in the genesis of diabetic microvascular complications. Since that time, although the idea has descended into the folklore of diabetes, little constructive research has been carried out into the concept. Most work on the subject now only highlights further instances or groups of patients providing more evidence of disturbed GH regulation in diabetes. The following work is an attempt to define possible causes of GH hypersecretion in diabetes and discover whether elevated GH levels contribute to the development of diabetic microvascular complications.

The biochemistry, physiology and pathophysiology of growth hormone

1.1 The chemistry of growth hormone.

The ability of pituitary extracts to stimulate growth was recognised in the 1920s (Evans and Long, 1921), and attempts to purify the factor responsible soon followed. By the middle 1940s, a protein hormone was isolated from beef anterior pituitary gland which was designated growth hormone or somatotropin (Li and Evans, 1944). Early work on the chemistry of GH led to proposals for a 2 chain molecule. More recent results have shown that the major form of the hormone in man has a molecular weight of 22,000 and consists of 191 amino acid residues in a single chain (figure 1.1). The first complete amino acid sequence of GH was described in 1966 (Li et al., 1966), but several errors were subsequently discovered, and the definitive sequence must be considered to be that derived from the corresponding nucleic acid sequence (Martial et al., 1979).

The primary structure contains 2 disulphide bridges, one of which links distant parts of the molecule while the other forms a small loop near the C-terminus. The detailed 3-dimensional structure of GH is unclear, but it is known that the molecule is spherical and contains a good deal of helix, some 50-60% of the polypeptide chain having this conformation (Bewlay and Li, 1975). GH has an isoelectric pH of 4.9, and the tertiary structure is robust since on heating to 100^{o}C or treatment with acid, there is little conformational change and much of the biological activity is retained. Further, disruption of the disulphide bridges, by



The amino acid sequence of human growth hormone.

Figure 1.1

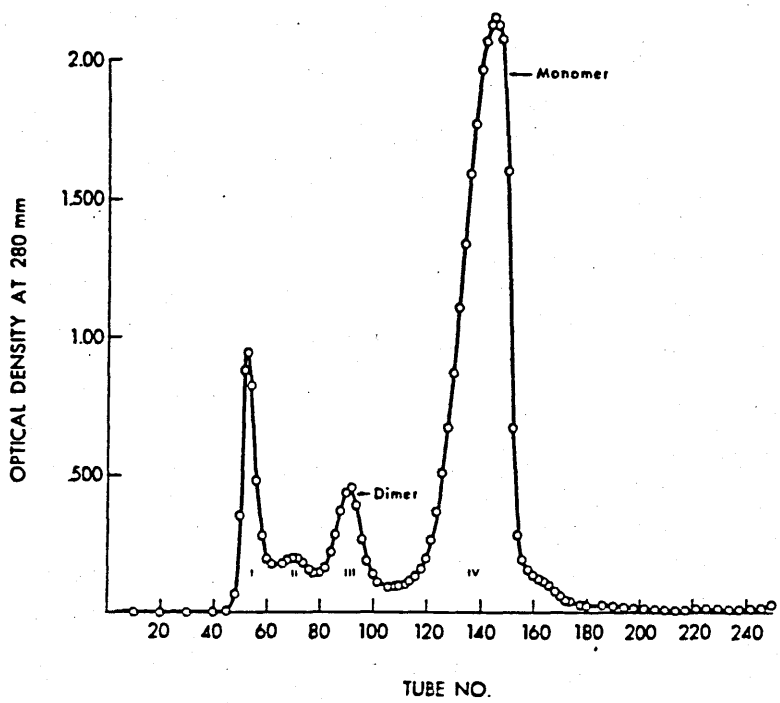
oxidation or reduction, yields a derivative with similar 3-dimensional structure and almost full growth promoting activity (Wallis et al., 1985). These considerations are of importance when considering preparation of samples for chromatographic separation.

1.2 Circulating forms of growth hormone.

There is a tendency for GH to aggregate to form polymers, some 17% of pituitary and 30% of circulating GH existing in this form (Frohman et al., 1972, Wright et al., 1974). Thus, using gel chromatography, both pituitary and plasma GH can be separated into monomeric 'little' GH, dimers ('big' GH) and larger aggregates ('big big' GH) (figure 1.2). The larger forms probably represent post-translational aggregation of GH during storage in the pituitary gland. Some 60-80% of the polymeric GH can be dissociated by treatment with 8M urea, and this fraction represents non-covalently bonded aggregates of GH. The residual large forms are the 'true' dimers of GH which are partly linked covalently by disulphide bridges. Such forms are potentially important, but their significance remains to be elucidated. It seems unlikely, however, that they represent precursors of the hormone.

It has also been proposed that an 80k form of GH exists which is not an aggregated form (Ellis et al., 1978), although further work is required to confirm that this is a true variant.

Chromatographic methods with greater resolving power than simple gel chromatography, especially polyacrylamide gel electrophoresis (PAGE) and isoelectric focussing, have revealed that monomeric GH contains a number of other variants of the basic 22k molecule. Firstly, there is a group which differ in charge only (Chawla et



Profile of plasma GH run on Sephadex G-100.
(reproduced from Bewlay and Li, 1975).

Figure 1.2

al., 1983), but since these are found only in the pituitary gland and not in the circulation they will not be considered further here. Secondly, there are a number of mass variants. The 22k form makes up 80-90% of circulating GH. A 20k form, making up 5-15% of circulating GH has also been identified and characterised, and is identical to the 22k form excepting that the amino acid residues 32-46 are deleted (Lewis et al., 1980a, Lewis, 1984). It is of interest that the beginning of the deleted sequence corresponds exactly to the position of an intervening sequence in the human GH gene. This suggests that the 20k form of GH is not a post-translational form of 22k GH, but is specifically coded for in a gene with a long intervening sequence (Wallis, 1980). There is also a 24k form of GH (Singh et al., 1974). This material is identical to the 22k form of GH except that it is nicked between residues 139 and 140 without any amino acid deletion. This variant makes up only 1% of circulating GH but is present in greater quantities in the pituitary gland. It is thought to arise as a posttranslational modification of the 22k form.

1.3 Growth hormone as a prohormone.

There is much evidence that peptides which represent only part of the amino acid sequence of GH may retain significant biological activity (Wilhelmi, 1982). For example, a peptide corresponding to residues 96-134 of bovine GH has been shown to retain 10% of the growth promoting activity of the parent hormone. Of considerable interest is the work on partial digestion of GH with the enzyme plasmin. Under controlled conditions, this enzyme cleaves human GH on the C-terminal side of residues 134 and140, releasing a hexapeptide, and leaving 2 large peptides joined by a disulphide bridge. This derivative retains almost full

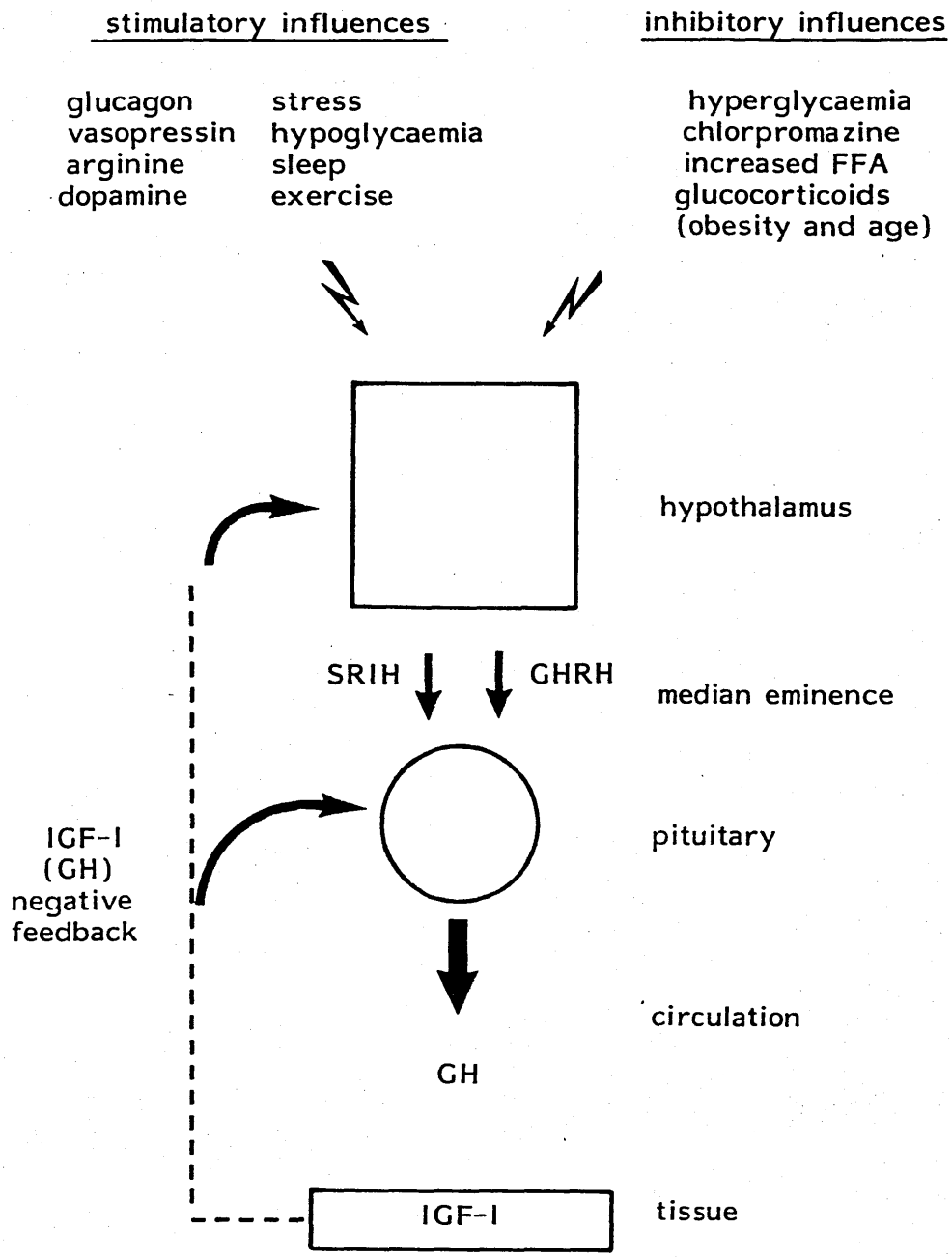
biological activity. The 2 component peptides can be separated after cleavage of the disulphide bridge, and the larger of the 2 retains reduced but significant bioactivity in several bioassays. Digestion with several other enzymes has confirmed the concept that not all of the GH molecule is required for retention of at least some biological activity (Lewis et al., 1980b). This is surprising since GH is a large protein with a clear-cut 3-dimensional structure. Little, if any, of the tertiary structure can be retained in these enzymic digests, suggesting that the tertiary structure may not be the crucial element in the biological actions of GH. This fact has led to the view that monomeric GH may be a prohormone, and specific fragments are cleaved from the molecule at tissue level to effect the diverse functions of the hormone (Lewis, 1984).

1.4 The growth hormone receptor

Consistent with the above hypothesis is the fact that the GH receptor appears to be a complex structure, several different forms being present. Receptors from a number of species have been characterised, and they appear to have a molecular weight of about 300,000, probably with a subunit molecular weight of 40-70,000 (Haeuptle et al, 1983).

1.5 The regulation of growth hormone secretion.

A schematic representation of the regulation of GH secretion is shown in figure 1.3. The precise mechanisms remain to be elucidated, but evidence to date suggests a complex interplay of anatomical sites and neurotransmitters. Input from neural pathways from the higher centres of the brain are mediated in part by the sympathetic and parasympathetic nervous systems. The bulk of evidence suggests that alpha-adrenergic receptors



Schematic representation of growth hormone regulation.

Figure 1.3

stimulate and beta adrenergic receptors inhibit GH secretion caused by stress (Blackard and Heidingsfelder, 1968). Parasympathetic stimuli contribute to the GH bursts seen during sleep (Mendelson et al., 1978) and exercise (Few and Davies, 1980). Sleep induced GH secretion can be abolished by the use of atropine (Taylor et al., 1985). Specific anatomical location of the areas of the brain which are important in the regulation of GH indicate that the ventromedial hypothalamus (VMH), arcuate nucleus and limbic system are involved (Merimee, 1979). The VMH, with noradrenaline as the neurotransmitter, is thought to contain the glucose sensitive cells which modulate GH secretion in response to changes in plasma glucose (Frohman and Bernardis, 1968), although the lateral hypothalamus is also thought to be important in this respect (Himsworth et al., 1972). The arcuate nucleus, with dopamine as the neurotransmitter is thought to be responsible for the increase in GH seen in response to l-Dopa infusion (Boyd et al., 1970), since l-Dopa is capable of crossing the blood brain barrier where it is converted to dopamine. The limbic system, with serotonin as neurotransmitter, is thought to be responsible for GH secretion during deep sleep.

The role of the various neural pathways and transmitters is currently an area of active research. Particular interest is focussed on the role of atropine and other cholinergic receptor blockers since they are capable of suppressing GH secretion in a number of situations, and could thus be of therapeutic benefit if side effects were minimised. However, they also provide an example of the difficulty in assigning a site and mechanism of action for any given agent. Muscarinic receptors are found in the median eminence and the pituitary gland itself (Burt and Taylor, 1980, Casanueva et al., 1983). Thus, atropine could block

GH secretion either by affecting secretion of inhibitory and stimulatory agents from reaching the pituitary gland, or by blocking GH secretion from the pituitary gland itself. Both views have been suggested (Young et al, 1979, Casanueva et al., 1986) and the question remains to be settled.

The hypothalamus controls GH synthesis and release by means of the stimulatory and inhibitory factors, growth hormone releasing hormone or factor (GRF) and somatostatin (variously known as growth hormone release-inhibitory hormone or somatotropin release inhibitory factor, SRIF) (Reichlin, 1974). GRF is found in its largest form as a 44 amino acid peptide, (Guillemin et al. 1982), although a 40 amino acid form may also occur naturally (Rivier et al., 1982). Administered exogenously, the residues 1-29 have been shown to retain almost full GH stimulatory activity (Grossman et al., 1984). GRF is found in highest concentrations in the infundibular nucleus of the hypothalamus in man (Werner et al., 1986).

Somatostatin was first isolated and characterised in 1973 (Brazeau et al. 1973). It is a peptide chain of 14 amino acid residues, although a 28 amino acid form is also found in the circulation having full biological activity and probably physiological significance. Somatostatin is widely distributed throughout the body, including the brain, hypothalamus, D-cells of the islets of Langerhans, and the gut. In the hypothalamus, it is found principally in the VMH and perimammilary area (Hokfelt et al., 1975).

In response to the various stimuli, GRF and somatostatin are produced in the hypothalamus and reach the anterior pituitary gland via the portal system of capillaries in the median eminence. Here they act upon the acidophilic GH producing cells

stimulate or inhibit GH production. GRF appears to act by stimulating cyclic AMP leading to activation of protein kinases. Somatostatin does not appear to alter levels of cyclic AMP, probably acting at a later stage in the secretory pathway altering the flux of calcium into and out of the somatotroph (Wallis et al., 1985).

As a consequence of the above influences, GH is released into the general circulation where it in turn stimulates the production of insulin-like growth factor-I (IGF-I). There is presently some controversy regarding subsequent events in the feed back mechanism controlling GH secretion. Certainly the IGF-I produced as a result of GH stimulation is capable of inhibiting further GH production by direct action at pituitary and hypothalamic level (Berelowitz et al., 1981). It is less clear whether GH itself, independently of IGF-I, participates in the autoregulatory process as was originally proposed (Sakuma and Knobil, 1974).

1.6 The actions of growth hormone.

The actions of GH present something of a puzzle in that the hormone is clearly involved in growth but also plays a role in the metabolic changes associated with fasting. The requirements of each are obviously opposite, one being an anabolic and the other a catabolic state. A list of the functions of GH is given in table 1.1. These anabolic effects are measured by the weight gain test in which the increase in body weight in a hypophysectomised rat is measured during 10 daily injections of GH, and the tibia assay in which the growth of the proximal epiphysis of the tibia of a hypophysectomised rat is measured after 4 daily injections of GH. Other assays measure the metabolic action of the hormone on muscle, adipose tissue and

... the latter target organ measures the lactogenic properties of the hormone which in excess can cause galactorrhoea. The diabetogenic action of GH is measured by the presence of hyperinsulinaemia and hyperglycaemia 10 hours after the injection of the GH preparation in a rat.

Table 1.1

The Effects of Growth Hormone.

Metabolic	- stimulates amino acid transport
	- stimulates protein transport
	- stimulates DNA/RNA synthesis
	- stimulates polyamine synthesis
	- stimulates lipolysis
	- inhibits insulinactiononglucose metabolism
Physiological	- increases renal blood flow, glomerular filtration rate and tubular reabsorption of phosphate
	- increases basal metabolic rate
	- stimulates new bone formation
	- stimulates erythropoeisis
	- expands extracellular fluid space
Anatomical	- accelerates linear growth
	- reduces adipose mass and enlarges lean body mass (muscle, liver, kidney, heart, GI tract, pancreas, skeleton, connective tissue).

It is clear that the major function of GH is to promote proportionate growth of both soft and skeletal tissue (Cheek and Hill, 1974). These actions are mediated at least in part by IGF-I

(Phillips and Vasiliopoulou, 1980). However, since the time course of some of the metabolic effects of GH are rapid, it is probable that the hormone has some independent intrinsic activity. When GH is administered to an animal in vivo, it causes general nitrogen retention with a fall in levels of urinary nitrogen and blood urea (Kostyo and Nutting, 1974). These effects are largely due to an increase in protein synthesis, and occur within 30 minutes. Similar effects have been demonstrated in vitro in the rat diaphragm. In the perfused liver, GH stimulates amino acid uptake and the synthesis of all forms of RNA, which accounts for some of the effects on protein synthesis, (Jefferson et al., 1972) but this is not the sole mechanism since inhibition of RNA synthesis does not totally block this action, and GH may affect the efficiency of the ribosomes by selective enzyme stimulation (Korner, 1969).

GH also has an effect on lipid metabolism (Goodman and Schwartz, 1974) causing a drop in the level of non-esterified fatty acids (NEFA), followed by a prolonged increase. This appears to be due to increased utilisation of lipids, increased uptake of NEFA by muscle preceding increased output by adipose tissue. The overall effect is that GH appears to divert energy metabolism from carbohydrate to lipid metabolism, thereby counteracting the action of insulin. The actions of GH on lipid metabolism are particularly marked in man, where GH levels are elevated on fasting, and in this context the hormone presumably plays a role in stimulating increased lipid utilisation in the fasting state. In the rat, on the other hand, GH levels fall on fasting (Tannenbaum et al., 1979), leading to the suspicion that the actions of GH on lipid metabolism are not of prime importance in the physiological adjustments to fasting.

GH has also been shown to be involved in the differentation of several cell types. Studied in considerable detail has been the conversion of fibroblasts of the 3T3 line to adipocytes (Morikawa et al., 1982), which requires the presence of GH. IGF-I has no effect on this transformation.

1.7 The effects of GH on carbohydrate metabolism and glucose counterregulation.

In vivo, GH has both insulin-like and diabetogenic effects (Altszuler, 1979). Infusion studies in man demonstrate a transient hypoglycaemia, lasting 15-30 minutes, followed by prolonged hyperglycaemia. The latter appears to be due to increased glucose output by the liver, reduced glucose uptake by the peripheral tissues, and possibly by direct inhibition of insulin secretion by the pancreas (Rizza et al., 1982, Bratusch-Marrain et al., 1982). The overall effect is to antagonise the actions of insulin, causing a rise in plasma glucose. Since hypoglycaemia is a potent stimulus for the secretion of GH (Roth et al., 1963), it would seem logical that GH plays a part in glucose counterregulation, contributing to the spontaneous rise in plasma glucose following hypoglycaemia. However, a number of factors suggest that GH does not play a significant part in this process (Cryer and Gerich, 1985). The fact that the increase in plasma glucose following GH infusion is delayed for some hours would make GH secretion a remarkably inefficient defence mechanism against hypoglycaemia. Experimental evidence, studying recovery from hypoglycaemia during SRIF infusion, suggests that glucagon and adrenaline are the important factors since replacement with GH did not affect the speed of recovery (Cryer, 1981). Further, the fact that hypoglycaemia causes a fall in

plasma GH in the rat (Panison and Tannenbaum, 1985) again suggests that GH is not of prime importance in this respect in mammalian physiology.

1.8 The actions of the GH mass variants.

From the above account, it is apparent that the actions of GH are diverse and sometimes conflicting. In view of the multiple forms of GH which have been isolated in man (see section 1.2), it has been suggested that each action of GH is produced by a particular GH form or fragment. This view has received most publicity from the observation that the 20k form of GH lacks the early insulin-like action of the 22k form (Frigeri et al., 1979). Most of the other variants of GH have also been reported to possess differing bio-activity as measured by the various bioassays. Thus, compared to the 22k form, the covalently bonded dimers of GH have been reported to be 10% as active in the weight gain assay and 50 % as active in the lactogenic assay (Lewis et al., 1977). The 80k variant has been reported to be 50-200 times as active as the 22k form in the tibia assay (Ellis et al., 1978), and the 24k variant to be inactive in the tibia assay but more potent on the lactogenic assay (Lewis et al., 1980b).

There have been reports suggestive of the existence of bio-inactive GH (Kowarski et al., 1978, Hayek et al., 1978, Fraser et al., 1980, Rudman et al., 1981) based on case reports of children with short stature, normal plasma GH levels measured by radioimmunoassay but reduced activity on radioreceptor assay. Characteristically, administration of exogenous GH to these patients reverses the growth retardation. It is not known whether the GH produced by these individuals represents one of the forms catalogued above with reduced activity, or is an undescribed

form. The gene family coding for GH is found on the long arm of chromosome 17, and a number of mechanisms which might produce an abberrant form of GH have been proposed (Chawla et al., 1983).

1.9 Growth hormone and diabetes.

Historically, the relationship between GH and diabetes is often related to the observation that insulin requirements in diabetic dogs were much reduced following hypophysectomy (Houssay and Biassoti, 1931). Retrospectively it is apparent that abolition of GH secretion was partly responsible for this phenomenon. This might have passed merely as a point of interest were it not for the observation in 1953 that the hormones of the anterior pituitary may play some part in the pathogenesis of diabetic retinopathy. This interest arose from a case history (Poulsen, 1953) reporting on a woman of 30 years with poorly controlled diabetes who had had frequent admissions to hospital with ketoacidosis. She was documented as having diabetic retinopathy, but following a post-partum uterine haemmorhage resulting in pituitary necrosis, it was observed that her retinopathy progressively improved and eventually disappeared. Since there was no alternative treatment for proliferative diabetic retinopathy at that time, this observation prompted trials of hypophysectomy and pituitary ablation for treatment of diabetic retinopathy resulting in the opinion that this was an effective form of treatment (Luft et al., 1955, Joplin et al., 1965, Bradley et al., 1965, Oakley et al., 1969, Kohner et al., 1970, Panisset et al., 1971, Kohner et al., 1972, Kohner et al., 1976). The mechanism of such improvement remains obscure. Certainly a limited trial of adrenalectomy for patients with proliferative retinopathy was without benefit, thereby excluding an effect of

[illegible] secretion (Martins, 1962, Graef and Maier, 1962). It was further observed that the efficacy of pituitary ablation for treatment of diabetic retinopathy depended upon the completeness of ablation (Joplin et al., 1967) and a good correlation with the degree of reduction in GH secretion was found (Wright et al., 1969). Post ablation, all patients were put on replacement therapy with prednisone, thyroxine and sex steroids with no detrimental effect on the retinopathy. This again suggested GH was the active agent in this context since it was the major anterior pituitary hormone which was not replaced. With the development of the radioimmunoassay for GH, it was soon found that 24 hour serum levels of GH are elevated in patients with insulin-dependent diabetes (Hansen and Johansen, 1970), and that levels are inappropriately high for the level of glycaemia in non-insulin dependent diabetes (Hansen, 1973, Kjeldsen et al., 1975). In 1970, GH was proposed as a permissive factor in the pathogenesis of diabetic microvascular disease (Lundbaek et al., 1970), citing as evidence the fact that GH administration can cause capillary basement membrane thickening in experimental diabetes. One of the most often quoted pieces of work which seemed to confirm that GH was related to the development of diabetic microangiopathy was the fact that GH deficient dwarfs did not develop retinopathy despite the presence of diabetes, and the fact that these dwarfs had thinner capillary basement membranes than control diabetic subjects (Merimee et al., 1970). Experimental evidence that GH might be a contributory factor in the development of microvascular disease, perhaps in combination with hyperglycaemia, was derived from experiments in mice (Ostreby et al., 1978). In these experiments, GH was infused into diabetic and non-diabetic animals, and it was found that the

diabetic mice demonstrated greater thickening of the capillary basement membrane compared to non-diabetic animals similarly treated. However, this does not answer the most often quoted criticism of the GH hypothesis, this being the fact that patients with acromegaly and impaired glucose tolerance do not develop retinopathy any more often than diabetics without acromegaly. There has been no satisfactory response to this point.

Regarding the observation that patients with diabetes display GH hypersecretion, with an elevated baseline and frequent GH spikes, this has been developed since the time of first description with the further observation that this is aggravated by poor glycaemic control (Johansen and Hansen, 1971, Hansen, 1971, Vigneri, 1976). It was also discovered that diabetic subjects have an increased or abnormal GH response to various stimuli, including TRH (Dasmahapatra et al., 1981, Blickle et al., 1982, Chiodera et al., 1984) arginine (Burday et al., 1968, Waldhausl, 1972), hypoglycaemia (Powell et al., 1966, Beaumont et al., 1971, Sonksen et al., 1972), exercise (Hansen, 1973, Passa et al., 1974), dopamine (Lorenzi et al., 1980), insulin in the absence of hypoglycaemia (Sharp et al., 1984a) and recently GRF (Kaneko et al., 1985). In most of these studies, patients with retinopathy are said to have a greater response than patients without.

The mechanism of GH hypersecretion in diabetes has received little attention, but a number of isolated observations have been made. Firstly, it was found that the increased GH response to arginine is not suppressed under conditions of hyperglycaemia, as in normal subjects (Burday et al., 1968). However, early work with somatostatin suggested that GH secretion in diabetic subjects can be inhibited with this peptide (Hansen et al., 1973,

Mortimer et al., 1974). In an attempt to explain the high GH levels seen in diabetes, it was proposed that plasma GH is cleared at a slower rate than normal in diabetic subjects (Sperling et al., 1973). However, accepting this to be the case, it does not explain the more frequent GH peaks seen in diabetes and is not considered to be a major contributory factor in the causation of GH hypersecretion in diabetes. A further interesting hypothesis was that the blood brain barrier in diabetic subjects is abnormally permeable to large molecules which are then capable of acting upon the GH regulatory centres. The evidence for this is based upon infusion studies of dopamine in normal and diabetic subjects (Lorenzi et al., 1980). Dopamine caused a rise in plasma GH in the diabetic, but not the normal subjects. Since dopamine does not normally cross the blood brain barrier, it was proposed that the permeable blood brain barrier in diabetic subjects allowed access of dopamine to the hypothalamic centres concerned with GH regulation and caused an increase in GH acting through the arcuate nucleus. There have been no follow-up studies to examine this possibility.

A final point of interest is that in patients with secondary diabetes, due to pancreatic destruction or removal, GH levels are also elevated (Lundbaek and Hansen, 1980). This suggests that the elevated GH levels seen in diabetes are secondary to lack of or resistance to insulin and its metabolic consequences, rather than to any other feature of insulin-dependent or non-insulin-dependent diabetes.

Attempts have been made to produce a unifying hypothesis, incorporating diverse theories on the pathogenesis of diabetic microvascular disease, including the GH hypothesis. For instance, it is known that patients with diabetic retinopathy have

increased platelet aggregation and increased levels of von Willebrand factor (Bensoussan et al., 1975, Porta et al., 1981). It was proposed that GH causes the release of von Willebrand factor from vascular endothelial cells, thus providing a direct link between GH and abnormal platelet function in diabetes (Sarji et al., 1977). However, this theory is presently out of favour (Lamberton et al., 1984).

Accepting that GH levels in diabetes are elevated, a number of studies have attempted to assess the metabolic consequences of such elevation. For example, at one extreme it has been suggested that the high GH levels cause poor glycaemic control in insulin-dependent diabetes, and not the other way round as is generally thought (Press et al, 1984). There have also been attempts to relate high nocturnal GH levels in subjects with insulin-dependent diabetes to the 'dawn phenomenon', where the blood sugar rises in the early hours of the morning (Skor et al., 1985).

1.10 Insulin-like growth factor-I.

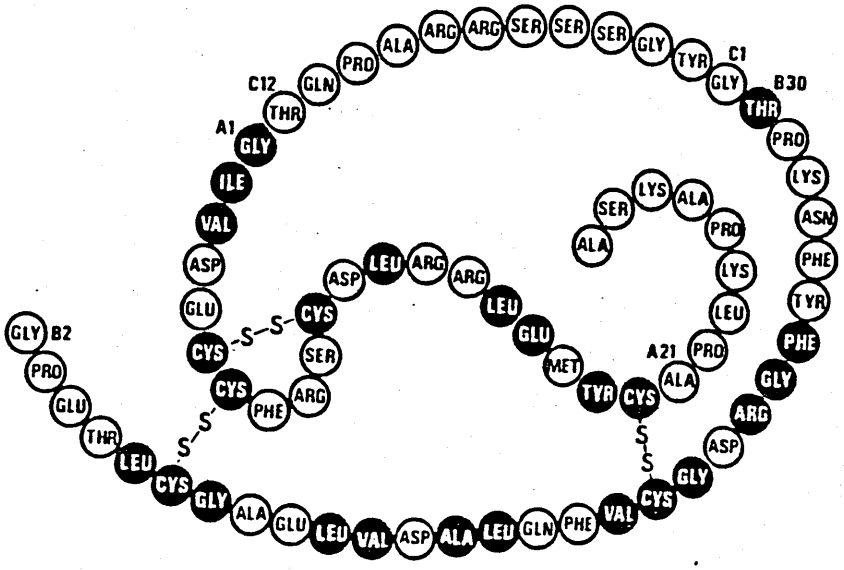
Many of the actions of GH are mediated through the action of insulin-like growth factor-I (IGF-I).With the isolation of IGF-I and subsequent development of sensitive assays, there has been considerable interest in this peptide in relation to diabetes in the hope that further information on the link between GH and the microvascular complications of diabetes might be gained.

1.11 The chemistry of insulin-like growth factor-I.

The chemical properties and biological actions of IGF-I have been extensively reviewed recently in view of the rapidly expanding body of knowledge on the subject (Humbel, 1984, Zapf et al., 1984).

Early confusion concerning the nature of insulin-like growth factors arose from the fact that a number of different groups were working independently along the same lines. Studying the effects of GH, Salmon and Daughaday (Salmon and Daughaday, 1957) observed that serum from GH treated hypophysectomised rats stimulated the incorporation of sulphate into cartilage in vitro, but added GH was without effect. They postulated that the effects of GH are mediated through a 'sulfation factor' which was later renamed somatomedin (Daughaday, 1972). Other workers found that only a small fraction of the insulin-like effect of serum could be neutralised by anti-insulin serum, leading them to the isolation of non-suppressable insulin-like activity (NSILA) (Froesch et al., 1963). Attempts to purify NSILA lead to further confusion since a number of peptides, somatomedin (Sm) A,B and C, were isolated. Sm-B was later found to be a contaminant, and Sm-A is likely to prove to be identical with IGF-II. Sm-C has now been proved to be identical to IGF-I (Klapper et al., 1983). However, the legacy of this confusion is that the terms somatomedin and insulin-like growth factor are used synonymously. In the present work, the term insulin-like growth factor will be used. Since the function of IGF-II is not known, and there have been no reports of any relation to diabetes, it will not be considered further here.

The amino acid sequence of IGF-I is shown in figure 1.4. There is marked sequence homology with proinsulin which is even more marked with respect to 3-dimensional structure. This is important in the context of the actions of IGF-I (Nissley and Rechler, 1984) since the similarity also extends to the receptors for IGF-I and insulin. An IGF receptor, designated type I, has been isolated with a M_r of 130,000 which binds preferentially to IGF-



The amino acid sequence of human IGF-I.

Identical positions as in human insulin are indicated in black.

Figure 1.4

I, but also recognises insulin. This is undoubtedly the IGF-I receptor. Further, many similarities have been observed between this type-I IGF receptor and the insulin receptor (Czech, 1982). Thus, the actions of IGF-I and insulin are qualitatively but not quantitatively similar, and indeed the two peptides may be able to act through the receptors of each. For instance, in adipose tissue, IGF-I stimulates glucose transport and lipid synthesis from glucose, and in muscle, IGF-I is also capable of stimulating glucose transport. Equally, in sufficiently high concentrations, insulin is capable of acting as a growth factor. This is an important consideration in tissue culture work where insulin is often added to the medium in quantities such that it is undoubtedly acting through the IGF-I receptor, and erroneous conclusions can be drawn if this is not considered.

The actions of IGF-I on cell growth and division have been recently reviewed (Van Wyk, 1984). Based on work on the c3T3 mouse fibroblast, it has been proposed that platelet derived growth factor or fibroblast growth factor first render the cell 'competent' by passing it through the G_0 phase of the cell cycle. IGF-I is termed a' progression factor' since it is one of the factors necessary to pass the cell through the G_1 phase of the cycle leading to mitosis.

In the circulation, IGF-I is bound to two binding proteins of molecular weight 150,000 and 40,000 (Hintz, 1984). The precise role of the binding protein is an area of active research, but it is proposed that the binding of IGF-I to its binding protein is a form of storage since the half-life of the unbound peptide is minutes only. Further, the relationship between the 150,000 and 40,000 molecular weight proteins remains to be elucidated, but it is thought that GH can influence the half-life of IGF-I by

increasing levels of the 150,000 molecular weight protein which is GH dependent. The presence of a binding protein is of considerable importance in designing an assay for IGF-I as most antibodies will not bind to the peptide unless it is first stripped from the binding protein.

Circulating levels of IGF-I in adult life are in the region of 200ng/ml, almost all of which is bound to binding protein. Levels increase during childhood to reach a peak at the time of adolescence, and thereafter decline (Hall and Sara, 1984). A clear demonstration of the way in which serum levels of IGF-I are GH dependent was provided by experiments in which GH, administered intramuscularly to normal volounteers, was followed 18-36 hours later by a rise in IGF-I (Copeland et al., 1980). The fact that there is a considerable lag period between a rise in GH and change in IGF-I, and the fact that that the half life of protein bound IGF-I is in the order of 3 hours, contribute to the lack of variation in IGF-I levels compared to the wide fluctuations in GH.

It was initially thought that the main site of production of IGF-I was the liver. However, as the number of cell types and tissues documented as being capable of producing IGF-I increases, a paracrine or autocrine model has been proposed (Underwood et al., 1980). Thus it is now thought that each cell type produces its own IGF-I, and levels in serum may be the result of production from a number of sites.

1.12 Insulin-like growth factor-I and diabetes.

Measurement of plasma levels of IGF-I in diabetes has produced conflicting results, presumably resulting from differing assay techniques since both bioassays and radioimmunoassays have been

employed. Further, many of the early studies were carried out on very small patient numbers. Thus, levels in diabetes have been reported as high (Cohen et al., 1977), normal (Zapf et al., 1980, Blethen et al., 1981, Tamborlane et al., 1981, Horner et al., 1981, Lamberton et al., 1983, Merimee et al., 1983) or low (Winter et al., 1979, Amiel et al., 1984). From the above review, however, it can be seen that the majority of workers have found plasma levels of IGF-I to be normal in diabetics as a whole.

A number of the above studies noted a negative correlation between plasma levels of IGF-I and glycaemic control as measured by glycosylated haemoglobin (HbA1) (Winter et al., 1979, Blethen et al., 1981). This suggests that poor glycaemic control leads to a failure of generation of IGF-I in response to GH, a finding akin to that reported in malnutrition (Clemmons et al., 1981). Further evidence that this may be the case is provided by studies demonstrating that improvement in glycaemic control in subjects with insulin-dependent diabetes leads to a rise in IGF-I despite a fall in GH (Tamborlane et al., 1981, Rudolf et al., 1982), Amiel et al., 1984). However, not all studies have found this to be the case (Merimee et al., 1984). Nevertheless, two other studies provide evidence that poor diabetic control does lead to impairment of generation of IGF-I. Firstly, it has been shown that during periods of severe metabolic decompensation in subjects with insulin-dependent diabetes, levels of IGF-I are low despite high circulating GH (Rieu and Binoux, 1985). Secondly, children with poorly controlled diabetes have been demonstrated to produce lower levels of IGF-I in response to a standard dose of GH than children with better glycaemic control (Lanes et al., 1985).

If glycaemic control were the only factor involved, it would be

expected that diabetics would have low levels of IGF-I. However, studies of patients with retinopathy suggest that another factor is involved. Two groups have reported that patients with proliferative retinopathy have higher levels of IGF-I than patients without retinopathy (Ashton et al., 1983, Merimee et al., 1983). Some doubt must remain on this point since Merimee et al. found high IGF-I levels only in patients with 'rapidly progressive' retinopathy, which group constituted only 7 out of a total study population of 80 patients. Unfortunately, the age of these 7 patients was not given, and since this type of retinopathy is found most commonly in patients in their early twenties (the mean age of the study patients was 46 years) and there is a negative correlation between age and IGF-I, it may be that these patients with high IGF-I were simply younger. A further study was unable to confirm that patients with retinopathy had high IGF-I levels (Lamberton et al., 1984). However, the finding that levels of IGF-I are elevated in the vitreous from the eyes of patients with proliferative diabetic retinopathy (Grant et al., 1986) has kept the controversy alive.

A further complicating factor concerning levels of IGF-I in diabetes exists. It has been pointed out (Phillips, 1986) that IGF-I levels in diabetes are higher when measured by radioimmunoassay than when measured by bioassay. Further, the negative correlation between glycaemic control and IGF-I levels is said to be more marked in studies using bioassay (Winter et al., 1979, Amiel et al., 1984). Accordingly, it has been proposed that 'somatomedin inhibitors' exist which block the biological actions of IGF-I in various conditions, including diabetes. Chromatographic studies suggest that these inhibitors are peptides of molecular weight of 20-30,000 (Goldstein et al.,

1983), but they remain to be fully characterised. However, the fact that some radioimmunoassay studies have found IGF-I levels to be low in absolute terms in diabetes, and have also demonstrated the negative correlation between IGF-I and measures of glycaemic control casts some doubt on the question of these inhibitors until further evidence is available.

1.13 Concluding remarks

From the above review, it is apparent that GH remains one of the most enigmatic of hormones despite its early isolation and characterisation, and despite the volume of literature on the subject. In the context of diabetes, there have been many observations on disordered GH regulation, but no attempt to explain this. Further, despite the fact that GH has long been thought a permissive factor in the genesis of diabetic complications, the evidence is circumstantial and awaits confirmation. Studies of IGF-I, a more distal point in the chain of events, have failed to resolve the questions. The following chapters, while not claiming to solve the problems, are an account of clinical and biochemical studies carried out on the patients attending the Diabetic Retinopathy Clinic at the Hammersmith Hospital. These observations will be placed within the framework outlined above to see if any further light can be shed on the cause of GH hypersecretion in diabetes, and whether GH is related to the development of diabetic complications.

CHAPTER 2

Long term follow-up of patients with diabetic retinopathy who underwent pituitary ablation with yttrium-90.

INTRODUCTION.

As a first step in assessing the role of GH in the pathogenesis of diabetic complications, follow-up of all patients who had undergone pituitary ablation for treatment of diabetic retinopathy was carried out. Clearly, if such long-term assessment does not confirm the beneficial effect of pituitary ablation on diabetic microvascular complications, then the hypothesis that GH plays a permissive role in the genesis of such complications (Lundbaek et al., 1970, Lundbaek, 1976) is without foundation.

2.1 ^{90}Y pituitary implantation in diabetes.

The first results of the effect of hypophysectomy on diabetic retinopathy were reported in 1955 (Luft and Olivecrona, 1955), but the operation was not without its problems. An alternative method, destruction of the pituitary gland by implantation with ^{90}Y, was used successfully at the Hammersmith Hospital in the late 1950s in patients with breast carcinoma (Fraser et al., 1959). This method was subsequently used in patients with diabetic retinopathy, and in 1962, the first tentative but encouraging results of destruction of the pituitary gland by ^{90}Y implantation on the course of diabetic retinopathy in 10 patients were reported (Joplin et al., 1962), the series later being enlarged (Joplin et al., 1965).

Between 1960 and 1964, ^{90}Y pituitary implantation as a treatment for diabetic retinopathy remained a largely experimental

[illegible] whether the optimum radiation dosage for the retinal features likely to respond were known. For example patients with plaques of hard exudate at the macula and irreversible fibrous retinitis proliferans and retinal detachment were initially treated by this means. Further, the radiation dose at the gland periphery was varied from 50,000 rads to 300,000 rads in order to ascertain the optimum level. By 1965 it was established that 150,000 rads achieved complete ablation in the majority of patients and side effects such as diabetes insipidus and CSF rhinorrhoea were of acceptably low frequency.

Pituitary implantation with ^{90}Y for proliferative retinopathy was carried out in a large series of patients at the Hammersmith Hospital, between 1960 and 1976, use of the procedure for this indication being discontinued with the advent of photocoagulation. The short-term results on retinal features and visual acuity have been reported in various groups of these patients (Joplin et al., 1962, Joplin et al., 1965, Joplin et al., 1967, Oakley et al., 1969, Kohner et al., 1970, Panisset et al., 1971, Kohner et al., 1972, Kohner et al., 1976). The first section of this chapter reports on the medical aspects of long-term follow-up of all patients operated on between 1960 and 1976. The second section describes the ophthalmological outcome in a group of these patients, describing only those operated on after 1965 when the retinal indications for pituitary implantation were known.

2.2 MEDICAL ASPECTS

2.2.1 PATIENTS AND METHODS.

The medical selection criteria for consideration of pituitary

ablation for diabetic retinopathy at the Hammersmith Hospital were :

1. good general health
2. blood urea below 12.5 mmol/l
3. ability to manage diabetes under difficult circumstances

One hundred and seventeen patients who underwent pituitary implantation with ^{90}Y for diabetic retinopathy at the Hammersmith Hospital between 1960 and 1976 have been followed. This number does not include the one patient who died immediately post-operatively since it is not proposed to examine the mortality associated with the procedure. The mean age of the patients was 34.7±10.8 years (mean ±SD) with a range of 16-62 years. The mean duration of diabetes prior to implantation was 18.6±10.0 years. All but one had proliferative retinopathy. Before operation, 13 patients were being treated with diet alone or diet plus oral hypoglycaemic agents and the remainder were on insulin therapy. After implantation, all but one were treated with insulin. The irradiation dose was 300 krad or more in 53.8% of subjects, 150 krad in 40.2% and 50 krads in 6%. Of the total, 22.1% had more than one implant in an attempt to achieve complete ablation.

Patients attended the Hammersmith Hospital for review at least once a year. If this was not possible due to difficulties with travelling, a medical report was obtained from the patient's local diabetic clinic. Where neither of these possibilities was available, the patient was considered to be lost to follow up. Cause of death was taken from the death certificate. Where possible, clinical details were also obtained from the attending physician at the time of death. Results of post-mortem examination were taken into consideration when

available.

Assessment of pituitary function in the early patients consisted of an insulin tolerance test with measurement of GH, metyrapone test with measurement of 17-oxogenic steroids, 48 hour ^{131}I neck uptake and steroid withdrawal with assessement of symptoms. These tests have been changed and superseded over the years to the present methods of pituitary function testing outlined below. For this report, assessment of pituitary function at any time depends on the tests then available.

Patients for re-assessment of pituitary function underwent an insulin tolerance test where there were no contraindications, with simultaneous administration of luteinizing hormone/follicle stimulating hormone releasing hormone (LH/FSH-RH) and thyrotrophin releasing hormone (TRH) (Harsoulis et al., 1973). In view of the fact that the patients were diabetic and hypopituitary, the dose of insulin given was 0.15 units/kg body weight (Actrapid, Novo laboratories). If there was no significant fall in the blood sugar within 30 minutes, a further dose was given as considered appropriate. In all subjects, the plasma glucose fell below 2.5mmol/l, and symptomatic hypoglycaemia was obtained. Blood samples were taken at 30 minute intervals for measurement of GH, cortisol and prolactin and these measurements continued for at least 60 minutes after symptomatic hypoglycaemia was achieved. Patients in whom insulin tolerance testing was contraindicated were given, in addition to LH/FSH-RH and TRH as above, an intravenous bolus of 100ug growth hormone releasing factor (GRF, Peninsula Laboratories, California) with measurement of serum GH at 15 minute intervals for 90 minutes. The maximum GH

output was taken as a measure of the patient's somatotroph function.

All data are expressed as mean ± S.D.

2.2.2 RESULTS

Of the 117 patients, 108 (92.3%) have been successfully followed and 9 (7.7%) lost to follow up (8 of whom were resident abroad). All patients were followed for at least 6 months post-implant, and the longest has now been followed for 21 years. The mean follow up period is 10.0±4.9 years (ie time from implant to death or to the present time if still alive).

Pituitary Function

Of the total number of patients, the mean insulin dose pre-implant was 62.7±24 units, falling 6 months post-implant to 30.4±14.9 units. On evidence available at the time, 90 patients (77%) were thought to have complete pituitary ablation, 22 (18.8%) to be partially ablated (which number includes all 7 patients who had the 50 krad radiation dosage, 2 of whom are still surviving (table 2.1)) leaving 5 (4.2%) in whom insufficient evidence was available. Of the currently surviving 38 patients, 23 were admitted to hospital for reassessment of their pituitary function as above. This number included 7 of the 8 surviving patients who had been thought to have some residual pituitary function. Results from these 7 patients are shown in table 1, where it is seen that one had no detectable pituitary function and the remainder had reduced reserve secretion. The other 16 patients who were tested and had been thought to be completely ablated were all confirmed to be so with no response to any of the stimulatory tests performed.

Table 2.1

Pituitary function tests in 7 patients thought to have partial ablation on original assessment. (Maximum values during combined pituitary function testing)

PATIENT	SEX	IRRADIATION DOSAGE (KRAD)	PLASMA GH (mU/l)	PLASMA CORTISOL (nmol/l)	TSH (mU/l)	LH (U/l)	FSH (U/l)
J. M-S.	F	50	11.0	660	13.5	19.4	8.0
T.H.	M	150	4.2	305	<1	1.8	<1
P.S.	F	50	2.6	750	9.3	17.0	16.6
A.J.	F	300	1.6	176	<1	14.3	8.6
S.B.	F	300	<1	526	2.9	7.5	4.5
R.C.	M	300	<1	<55	<1	1.2	<1
D.B.	F	300	1.7	<55	<1	<1	<1
Normal :			>20	>550	>4.5	>16	>2

Replacement therapy.

Of the 108 patients followed, 96% were taking replacement therapy with prednisone or hydrocortisone and thyroxine. The males were all taking intramuscular injections of Primoteston Depot at 3 to 6 weekly intervals. Amongst the females, it is difficult to persuade them of the need to take oestrogen replacement, and at present, only one of the surviving women is doing so. No subject required any form of therapy for diabetes insipidus beyond 6 months post-implant.

Glycaemic control.

Amongst the survivors, glycaemic control is difficult. The mean glycosylated HbA1 is 10.5±1.5% (normal range 5-8%). The principal problem is with hypoglycaemia since in the majority of patients this is asymptomatic until it results in loss of consciousness. Many patients complain that addition of 1 unit of insulin to their daily dosage leads to troublesome hypoglycaemia, and all are advised not to take long-acting preparations of insulin in the evening to avoid nocturnal hypoglycaemia.

Morbidity

Apart from the general problems associated with hypopituitarism, there have been a number of problems peculiar to these patients with diabetes. In one patient, postural hypotension has been so severe that, despite treatment with mineralocorticoids, she has been confined to a wheelchair. Osteoporosis with bone fractures has been a problem in a number of cases, and in one patient non-union of a fractured shaft of femur required pinning and 2 years to allow return to full mobility. Avascular necrosis of the femoral head on a dose of 5mg of prednisone has been reported in

one case. Problems with hypopituitarism and diabetes are illustrated in the following 2 case histories which are not atypical.

Patient G.D., female with diabetes since the age of 14 years, was found to have 'florid' proliferative retinopathy in both eyes at the age of 31 years. Her visual acuity at the time was 6/9 in the right eye, with repeated vitreous haemorrhages, and 6/60 in the left eye due to retinal detachment. She was treated with a 300 krad ^{90}Y pituitary implant, and at 6 month assessment was thought to be completely ablated. Her daily insulin dosage had fallen from 40 to 24 units daily. She was on replacement therapy with prednisone, thyroxine and oestrogens, although she discontinued the latter since she said they upset her diabetic control and made her feel unwell. Glycaemic control proved difficult, and she suffered from repeated hypoglycaemia. She was commenced on thrice daily Actrapid insulin, to a total of 20 units daily, and began monitoring her home blood glucose 4 times daily. Nevertheless, she is frequently found unconscious due to hypoglycaemia at home. She lives alone and is unmarried. Friends have frequently had to administer intramuscular glucagon, and she has had frequent hospital admissions with hypoglycaemia. Her renal function is normal, with a serum creatinine of 62umol/l, and she has no proteinuria. She maintains 6/6 vision in her right eye.

Patient M.P., female with diabetes since the age of 2 years, was found to have bilateral proliferative retinopathy at the age of 27 years, and treated with a 150 krad ^{90}Y pituitary implant. On assessment at 6 months, she was found to be

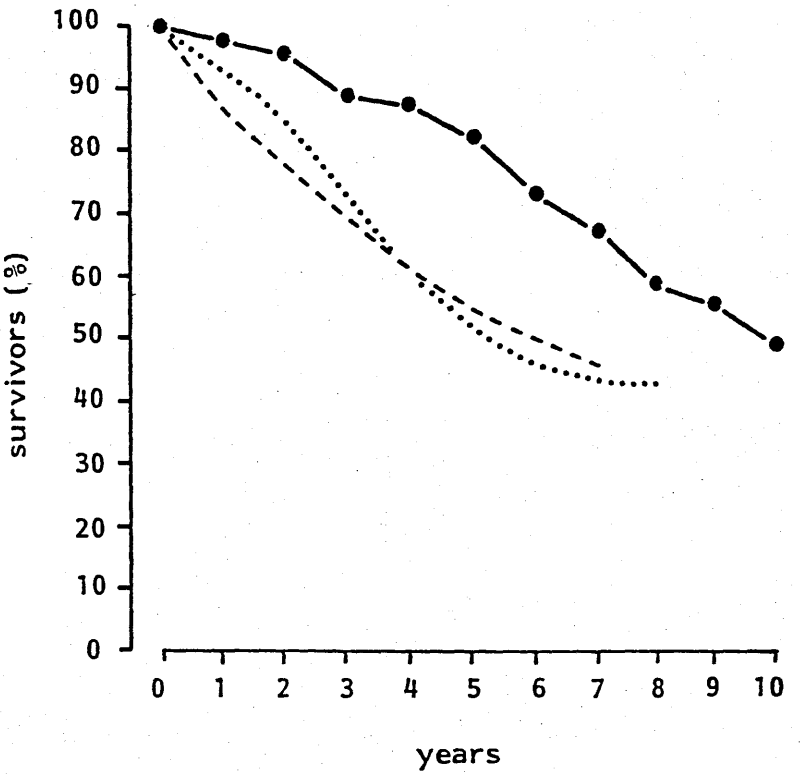
completely ablated and her daily insulin dosage had fallen from 60 to 30 units daily. She was on replacement therapy with prednisone and thyroxine. She developed pulmonary tuberculosis 3 years post-implant and was kept on prophylactic isoniazid in view of her steriod therapy. She had photocoagulation to the left eye in South Africa, and subsequently lost vision in that eye due to retinal detachment. When seen 12 years post-implant, age 39 years, her daily insulin dosage had dropped to 18 units daily, in spite of which she continued to have frequent hypoglycaemic attacks. Despite treatment with oestrogens and calciferol, she complained of back ache, and had sustained several fractures of ribs and fingers. On X-ray examination, she was found to have osteoporosis with collapse of T12 and wedge fractures of L3-4 in addition to 2 fractured ribs. When seen 16 years post-implant, her general health was poor. She was 5 kg less than her pre-implant weight. Her daily insulin dosage was down to 16 units daily, but she continued to suffer from repeated debilitating hypoglycaemic attacks, resulting in a fractured ankle on one occasion. She had never married and had no children. Her plasma creatinine was normal at 86umol/l, and she had no proteinuria. She maintained 6/6 vision in the right eye with no active retinopathy.

Mortality rate.

Of the patients followed, 38 (35.2%) are still alive at the time of writing and 70 (64.8%) dead. The 5 year mortality was 17.6% and the 10 year 51% (figure 2.1).

Causes of death.

Of the 70 patients who have died, 15 (21%) died of the



Percentage survival per year for the 108 patients followed. Survival curves from the studies of Caird et al. (1969) (••••) and Davis et al. (1979) (---) are included for comparison.

Figure 2.1

consequences of hypoglycaemia, infection or adrenal steroid deficiency. The mean survival time in this group was 6.2±3.1 years. These patients are detailed in table 2.2. Subjects B.D. and T.D. both suffered from epilepsy and had grand mal fits secondary to hypoglycaemia which reslted in their deaths. Eight patients (12%) died of unequivocal renal failure (see below). The mean survival time of this group was 6.5±3.4 years. Thirty three patients (47%) died of ischaemic heart disease or cerebral vascular disease, mean survival in this group being 8.2±3.5 years. Fourteen patients (20%) died of miscellaneous causes, including cancer, gastric haemorrhage, and pancreatitis. This group also includes those in whom the death certificate states only bronchopneumonia and the exact cause of death is unknown. Of all patients, both living and dead, there have been no episodes of ketoacidosis.

Renal function.

Assessment of renal function is difficult since the only marker of renal function available consistently in all patients is plasma urea. The presence or absence of proteinuria is not known in all patients.Table 2.3 gives details of all 12 patients who died with an elevated plasma urea (>13mmol/l). One surviving patient has developed rapidly progressive renal failure 14 years post implant, and another has early renal failure, with a plasma creatinine of 154umol/l and poorly controlled hypertension. Thus 13% of patients followed apparently developed some degree of renal failure. This figure is likely to be an overestimate since on examination of the details in table 2.3, some patients had medical conditions which could have contributed to an elevated plasma urea. Subjects M.D., W.F.

Table 2.2

Details of patients who died as a result of hypopituitarism.

PATIENT	AGE AT TIME OF DEATH	YEARS POST-IMPLANT	CAUSE OF DEATH
B.B	33	7	hypoglycaemia
J.B	31	3	hypoglycaemia
A.B	43	0.5	meningitis, adrenocortical insufficiency
D.C.	33	7	hypoglycaemia
R.C.	47	2	meningitis, adrenocortical insufficiency
B.D.	32	8	asphyxia secondary to hypoglycaemia
T.D.	38	8	status epilepticus secondary to hypoglycaemia
E.G.	47	8	meningitis
D.H.	49	4	pneumonia, adrenocortical insufficiency
G.K.	34	10	pneumonia, hypoglycaemia
R.N.	29	6	hypoglycaemia
J.P.	35	11	hypoglycaemia
W.R.	61	3	meningitis, hydrocephalus
W.R.	46	6	pneumonia, adrenocortical insufficiency
M.W.	45	9	influenza

Table 2.3

Patients who died with an elevated plasma urea.

PATIENT	AGE AT IMPLANT (years)	AGE AT DEATH (years)	UREA AT IMPLANT (mmol/l)	UREA AT DEATH (mmol/l)	PROTEINURIA (Y/N)	CAUSE OF DEATH
D.B.	34	40	10.8	34.6	Y	myocarditis
M.D.	48	60	5.8	13.0	N	cardiac failure, IHD
D.F.	30	38	5.5	not known	not known	renal failure
W.F.	48	54	6.0	17.0	Y	cardiac failure, IHD
E.H.	27	39	7.8	41.5	Y	renal failure
M.H.	30	33	8.8	30.6	Y	renal failure
C.J.	23	28	7.6	31.3	Y	MI, renal failure
A.P.	42	52	10.7	44.7	Y	renal failure
S.P.	20	23	6.5	not known	Y	renal failure
F.S.	36	39	9.0	not known	Y	renal failure
R.T.	28	35	7.2	45.0	Y	renal failure
A.V.	55	64	7.1	17.2	N	cardiac failure, IHD

MI myocardial infarction
IHD ischaemic heart disease

and A.V. had cardiac failure and were on diuretic therapy. Subject C.V. had a myocardial infarct following which her plasma urea was elevated. In addition, two patients had evidence of renal failure prior to implant with plasma urea greater than 10mmol/l and proteinuria. Thus, only 8 patients (7.4%) could truly be said to have developed renal failure post pituitary ablation.

A plot of plasma urea against time (figure 2.2) shows a brief peak within 6 months of operation followed by a swift return to normal and subsequent slow progressive rise. This is apparent even when the 12 patients who died with an elevated plasma urea are excluded.

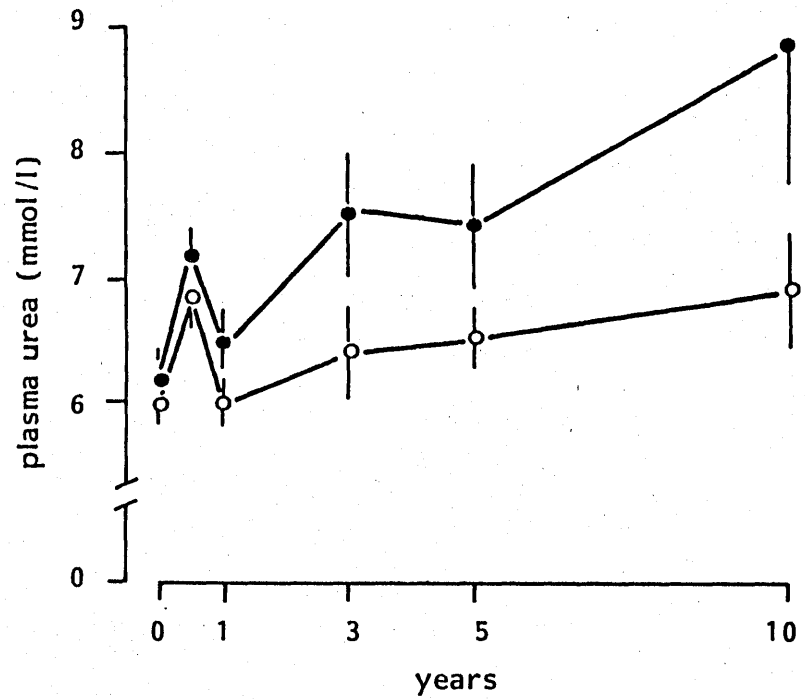
2.3 OPHTHALMOLOGICAL ASPECTS

2.3.1 PATIENTS AND METHODS

One hundred patients who underwent ^{90}Y pituitary implantation for proliferative diabetic retinopathy between 1965 and 1976 were included in the ophthalmological follow-up. Patients were carefully assessed prior to being offered pituitary ablation. They were considered eligible for this treatment if, in addition to the medical criteria quoted in the previous section, they had at least one 'treatable' eye defined as visual acuity of 6/24 or better, the macula not threatened by fibrous retinitis proliferans (RP) and/or detachment, and RP on the disc did not exceed grade 3 out of 5 on the Hammersmith grading system (Oakley et al., 1967).

If complete ablation was not achieved with one operation, and an eye remained treatable, a second operation was offered. Twenty-three patients had two pituitary implants.

Patients admitted to the study were assessed at regular



Plasma urea versus time in all patients (●) and excluding those with eventual renal failure (○).

Figure 2.2

intervals, and once a year underwent a full ophthalmic examination including measurement of best corrected visual acuity and intraocular pressure. Anterior segment examination and retinal evaluation including colour photography of the fundus and fluorescein angiography were also performed. Colour photographs were assessed using the Hammersmith system which separately grades microaneurysms and haemorrhages (MA), new vessels elsewhere (NVE), retinitis proliferans (RP) and hard exudates (HE) on a numerical system between 0 (absent) and 5 (most severe), based on comparison with standard photographs (Oakley et al., 1967). New vessels arising from the disc (NVD) were graded according to the method of Kohner et al. (1971), assessing the number of quadrants of the optic disc crossed by the new vessels, and the distance the vessels extend beyond the disc margin, again assigning a value of 0 to 5. Photocoagulation had been carried out on fifteen patients before they entered the study, and pituitary ablation was performed in these cases because of failure of response.

Ophthalmic records of each patient were recently reviewed. Retinopathy grading and visual acuity immediately prior to implant ('initial') and at one, three, five , ten and fifteen years post-implant, had been recorded and were available for consideration. Original photographs were examined in fifteen per cent to verify the original gradings which were done by different observers over the years. In the sample tested, no changes in grading had to be made. For the purposes of this study 'blindness' is defined as visual acuity of 6/60 or worse. For the purposes of data analysis, in the 23 patients who had a second implant, time of entry into the study was immediately prior to the first operation. Visual acuity

results were converted from Snellen-chart values to numerical scoring to facilitate statistical analysis (Table 2.4).

Data are quoted as mean ± SD throughout. The significance of differences between groups was assessed by Student's t-test.

Table 2.4

Numerical Scoring of Visual Acuity

6/6 = 1	6/60 = 7	
6/9 = 2	3/60 = 8	
6/12 = 3	CF = 9	(CF=Counting Fingers)
6/18 = 4	HM = 10	(HM=Hand Movements)
6/24 = 5	PL = 11	(PL=Perception of Light)
6/36 = 6	NPL = 12	(NPL=No Perception of Light)

2.3.2 RESULTS

Sixty-seven male and 33 female patients underwent pituitary ablation between 1965 and 1976. Mean age at entry was 35±10.5 years (range 16-62 years) and mean duration of diabetes at entry was 17.2±8.7 years (range 1-40 years).

Visual Acuity

At the initial assessment there was a significant difference in the mean visual acuities between right and left eyes: R. 3.2±2.8, L. 4.1±3.4 $p<0.05$ (Table 2.5). Thirty-eight patients were blind in one eye, 14 in the right and 24 in the left. The cause of blindness was usually vitreous haemorrhage or RP causing traction detachment.

Sixty-eight patients were assessed at the five year follow-up. Mean visual acuity had deteriorated, to 4.7±4.2 in the right eye ($p<0.001$) and 4.6±4.2 in the left eye ($p<0.02$). At this stage eight patients were blind in both eyes, secondary to RP/detachment, vitreous haemorrhage or rubeotic glaucoma.

forty-nine patients were analysed at the ten year follow-up. Compared with the five year result, mean visual acuity was almost unchanged at 4.6±4.4 in the right eye ($p=0.057$ on comparison with the 5 year value) and 4.7±4.4 in the left eye ($p<0.05$). Six patients were blind in both eyes, secondary to RP/detachment or rubeotic glaucoma.

Analysing the data by best eye only, 84% of patients had vision of 6/12 or better at the time of initial assessment, declining after 5 years to 81% and by 10 years to 80% (Table 2.6).

Table 2.5

Mean Visual Acuity of Right Eyes

Year	Mean Grading	SD	No of Patients	
Initial	3.2	2.8	100	
One	3.6	3.4	97	*
Three	4.3	3.8	83	**
Five	4.7	4.2	68	**
Ten	4.6	4.4	49	**
Fifteen	4.8	4.5	19	*

Mean Visual Acuity of Left Eyes

Year	Mean Grading	SD	No of Patients	
Initial	4.1	3.4	100	
One	4.5	3.6	97	*
Three	4.8	4.0	83	**
Five	4.6	4.2	68	*
Ten	4.7	4.4	49	**
Fifteen	5.1	4.7	19	

* $p<0.05$
** $p<0.01$ comparing the same eye at initial and subsequent visits

Table 2.6

Grading of better eye from initial to the fifteen year visit

	6/6	6/9	6/12	6/18	6/24	6/36	6/60	3/60	CF	HM	PL	NPL	TOTAL
Initial	55	20	9	10	5	1	0	0	0	0	0	0	100
One	54	21	8	5	2	4	1	0	1	1	0	0	97
Three	44	16	11	0	0	3	3	1	2	2	1	0	83
Five	38	14	3	3	1	1	2	0	1	2	1	2	68
Ten	33	3	3	1	1	2	0	0	1	1	3	1	49
Fifteen	12	2	0	1	0	0	2	0	0	0	0	2	19

CF = counting fingers
HM = hand movements
PL = perception of light
NPL = no perception of light

Disc and Peripheral New Vessels

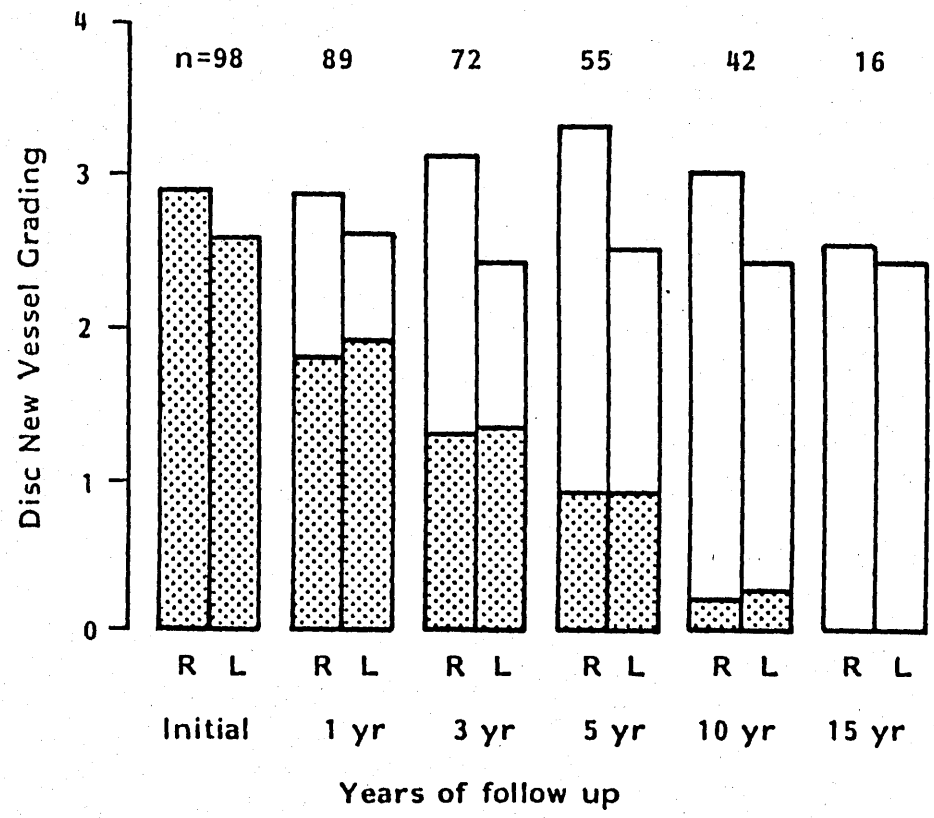
There was a significant improvement in both disc and peripheral neovascularisation. After five years, disc new vessels in both eyes had improved from a mean of 2.7±1.6 to 0.8±1.2 $p<0.001$ (Table 2.7). By ten years there was almost no evidence of neovascularisation in any eye (Fig 2.3). The nineteen patients who have reached the fifteen year follow-up have no evidence of disc neovascularisation, though 4 still have evidence of peripheral new vessels (Table 2.8). The improvement in neovascularisation was matched with a reduction in the incidence of vitreous haemorrhage. At one year 14 (14.4%) patients had one eye that was unassessable because of haemorrhage (2 being unassessable in either eye), while by ten years only 4 (8.5%) patients had this complication.

Retinitis Proliferans

The mean grading for RP increased from 1.0 ±1.3 to 1.8 ±1.3 at ten years (Table 2.9). The increase in RP does not mean that new vessels invariably progressed to RP as some disc vessels resolved without residual fibrous tissue. In general the more severe the neovascularisation at the initial assessment the more likely RP was to develop. RP was the other major cause of eyes becoming unassessable.

Hard Exudates, Microaneurysms and Haemorrhages

There was a significant reduction in the level of exudates from initial to ten years ($p<0.001$, Table 2.10). Those who underwent photocoagulation after implant reduced their level of exudates more rapidly than those who underwent implantation alone. Thus at five years, grading in the right eye was 0.4 ±0.6 in



Disc new vessel grading (all patients) from initial assessment to 15 years. Shaded areas represent mean grading at the time indicated and open areas the initial grading for that group. The figures indicate the number of patients examined at each time point.

Figure 2.3

Table 2.7

Mean grading of disc new vessels for both right and left eyes

Year	Mean Grading	SD	No of Patients
Initial	2.7	1.6	98
One	1.8	1.4	89 **
Three	1.2	1.3	72 **
Five	0.8	1.2	55 **
Ten	0.2	0.5	42 **
Fifteen	0.0	0.0	16 **

** denotes $p<0.01$ comparing the same eye at initial and subsequent visits

N.B. In the following tables it will be seen that the number of patients at each visit varies from one table to the next. This is because in some cases the photographs of particular areas of the eye were ungradable secondary to vitreous haemorrhage, retinitis proliferans or cataract.

Table 2.8

Mean grading of peripheral new vessels for both right and left eyes

Year	Mean Grading	SD	No of Patients
Initial	2.3	1.1	99
One	1.4	0.8	90 **
Three	1.0	0.7	71 **
Five	0.7	0.7	54 **
Ten	0.2	0.4	42 **
Fifteen	0.2	0.4	16 **

** $p<0.01$ comparing the same eye at initial and subsequent visits

Table 2.9

Mean grading of retinitis proliferans for both right and left eyes

Year	Mean Grading	SD	No of Patients
Initial	1.0	1.3	98
One	1.6	1.4	90 **
Three	1.7	1.4	72 **
Five	1.7	1.4	54 **
Ten	1.8	1.3	41 **
Fifteen	1.9	1.5	17 *

* $p<0.05$
** $p<0.01$ comparing the same eye at initial and subsequent visits.

those who had not undergone photocoagulation but 0.1 ±0.2 in those who had (NS).

Microaneurysms and haemorrhages declined significantly during the first year whether the patients had undergone photocoagulation or not (Table 2.11).

Photocoagulation

Fifteen patients who had previously had photocoagulation and were 'treatment failures' were admitted to the pituitary ablation series. After ten years 29 out of the 49 patients remaining in the study had undergone either xenon or argon photocoagulation (this includes nine of the original fifteen). There was a trend for those who had undergone photocoagulation, in addition to pituitary ablation, to lose neovascularisation more rapidly than those who had undergone pituitary ablation alone. For instance at five years the mean grading of disc new vessels in the right eye in those patients who had undergone photocoagulation had fallen from an initial grading of 2.7±1.9 to 0.3±0.7, whereas in those who had not had photocoagulation it had fallen from 2.7±1.4 to 1.1± 1.4. By ten years there was no difference between the two groups.

Effect of Age at entry

As it has been suggested that pituitary ablation is more effective in the young, patients were also studied by age of entry into the study. The population was divided into those under the age of forty years at entry (69 patients) and those forty years or over at entry (31 patients).

Table 2.10

Mean grading of hard exudates for both right and left eyes

Year	Mean Grading	SD	No of Patients	
Initial	0.68	0.73	99	
One	0.60	0.57	89	NS
Three	0.44	0.50	71	*
Five	0.33	0.48	54	**
Ten	0.15	0.28	38	**
Fifteen	0.18	0.22	15	**

* $p<0.05$
** $p<0.01$ comparing the same eye at initial and subsequent visits

Table 2.11

Mean grading of microaneurysms and haemorrhages for both right and left eyes

Year	Mean Grading	SD	No of Patients	
Initial	2.3	1.1	99	
One	1.3	0.7	90	**
Three	1.0	0.6	71	**
Five	0.7	0.5	56	**
Ten	0.5	0.4	42	**
Fifteen	0.5	0.3	16	**

** $p<0.01$ comparing the same eye at initial and subsequent visits.

Mean age at entry of those under 40 was 29±6 years (range 16-39). Mean age at entry of those aged 40 years or more 48±5.8 years (range 40-62). Duration of diabetes at entry was similar in both groups being 17.8±7 years (<40 years), and 16.1±11.6 years (>40 years).

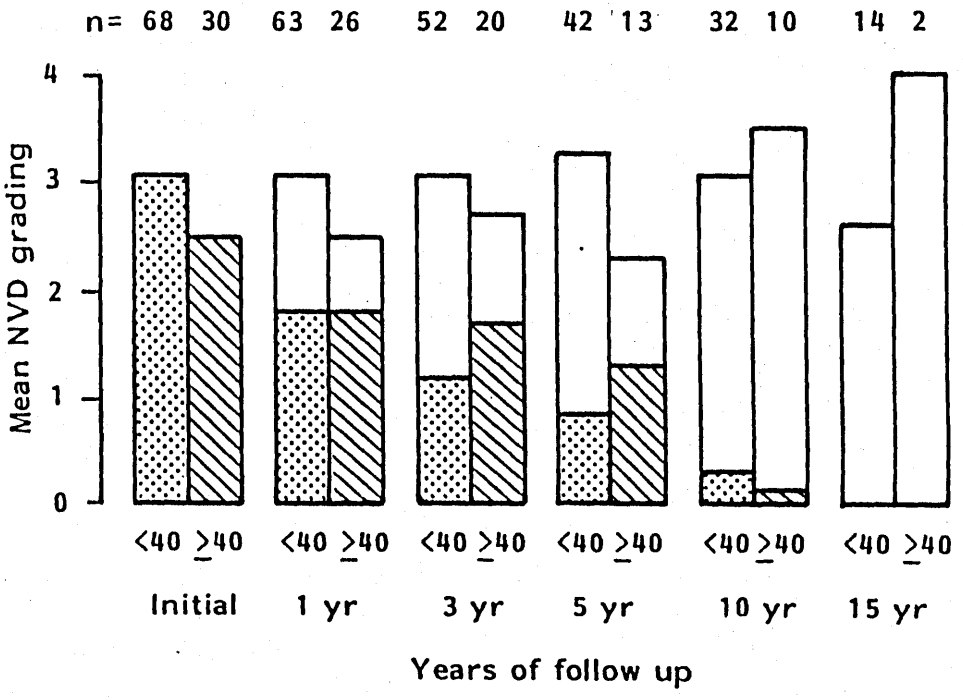
Patients who were younger than 40 years tended to have a more severe degree of neovascularisation (NVE and NVD) than older

patients. The response of patients in this younger group to pituitary ablation appeared to be more rapid than that of the older patients (Fig 2.4). A similar differential response was noted in microaneurysms, haemorrhages and exudates. Hard exudates were more severe in those over forty years (mean R and L 1.1±1.1) than those under forty (mean R and L 0.7±0.5). Consistent with this was the fact that the older age group was more likely to lose vision from maculopathy than the younger age group, who lost vision almost exclusively from the effects of RP/detachment.

2.4 DISCUSSION.

2.4.1 Medical aspects

The initial assessment of the proportion of patients thought to be completely ablated was probably accurate as assessed by current methods, but with the passage of time may have become an underestimate. All patients studied who were thought to be completely ablated were confirmed to be so. In addition, two of those thought to be partially ablated were now found to have no residual pituitary function. This may have resulted from fibrosis of the pituitary gland. Those thought to be partially ablated had variable residual pituitary function. In all of these, GH production is disproportionately reduced compared with other pituitary modalities, in keeping with the findings of Shalet et al. (1977) who found that irradiation of the hypothalamic-pituitary axis resulted in a greater reduction in GH than other pituitary hormones. If indeed GH is the important pituitary hormone in the pathogenesis of diabetic microvascular disease, then ^{90}Y implantation was theoretically successful in nearly all patients in whom the procedure was



Disc new vessel grading from initial assessment to 15 years, comparing those <40 years at implant with those >40 years. Stippling represents patients <40, cross hatching patients >40, open areas initial grading for that group. The figures indicate the number of patients examined in each group.

Figure 2.4

carried out.

In some situations, the combination of hypopituitarism and diabetes would appear to have an additive effect in terms of complications. Thus, hypotension can be aggravated by diabetic autonomic neuropathy, and osteoporosis, despite replacement with sex steroids, has been a significant problem. While most patients have managed to lead a normal life, the effects on the life of a sizeable minority have been devastating. Impotence and inability to produce children led to several episodes of depression requiring psychiatric help, and suicide in at least one case. Lethargy was a common complaint despite full replacement therapy. Interestingly, while men are happy to take replacement therapy with androgens, the women are reluctant to take oestrogens, despite a full explanation of the reasons for doing so. Fear of hypoglycaemia dominates the lives of many patients, and the advent of home blood glucose monitoring has been a help in this direction. Asymptomatic hypoglycaemia has become a major problem in the surviving patients, but is most likely due to long duration of diabetes and associated autonomic neuropathy rather than hypopituitarism since lack of hypoglycaemic symptoms is also seen in non-ablated diabetic subjects. It is surprising that there have been no episodes of ketoacidosis. It has, however, been previously reported (Barnes et al., 1978) that hypopituitary diabetic patients are surprisingly slow to demonstrate a rise in plasma glucose and ketone bodies following insulin withdrawal, especially in association with steroid deficiency.

Mortality rates reported for patients with proliferative retinopathy vary. Two large studies have reported figures of approximately 50% at five years (Caird et al., 1969, Davis et

al., 1979), and rather more among the diabetic blind (Rogot et al., 1966). A further study, selecting patients with 'a good prognosis for survival', reported a 5 year mortality of 25% amongst the patients with proliferative retinopathy (Knatterud, 1983). Thus, the 5 year mortality of 17.9% found in the present study is seen to be very low. This is presumably in part because the patients were a selected group with no significant evidence of renal dysfunction at the time of implant. A small clinical study of 51 patients with proliferative retinopathy who were selected to exclude patients with renal failure at the outset found a mortality rate of approximately 10-15% over a mean follow up time of 6 years (Deckert et al., 1967). This seems to confirm that the low mortality rate found in this study is largely due to the selection criteria. Nevertheless, the figure is still remarkably low when one considers that the combination of diabetes and hypopituitarism carries its own risks. Although the question is open to debate, it could be argued that those patients who died of hypoglycaemia died as a consequence of their hypopituitary state as it is not common for diabetic subjects to die of hypoglycaemia. In addition, there are a number of patients in whom clinical details suggest that they were admitted to hospitals where they were not known, and the dose of adrenal steroid was omitted or not increased to cover the intercurrent illness.

The large proportion of patients who died from myocardial or cerebral vascular disease is in keeping with the figures of other studies in diabetic subjects (Entmacher et al., 1964, Keen et al., 1965). It is now accepted that diabetes predisposes to atheromatous disease. Pituitary ablation would obviously not be expected to have any effect on this.

The reports of Ireland et al.(1967) and Greenwood et al.(1975) of thinning of the glomerular basement membrane after pituitary destruction suggests that pituitary implantation may have beneficial effects on microvascular disease in kidney. Arriving at a figure indicating the numbers in this study who developed renal failure has been difficult. Taking all patients who had an elevated plasma urea is likely to be an overestimate but including only those patients who developed renal failure secondary to diabetes is perhaps making too fine a point. However, in this group of 108 patients, followed for a mean of 10 years, between 7 and 13% developed renal failure. It is difficult to find data with which to compare this figure. In two large series from the Joslin clinic looking at all diabetics (Entmacher et al., 1964), and those with proliferative retinopathy (Root et al., 1959), some 50% of patients of comparable age and duration of diabetes died of renal failure. In a study by Deckert et al. (1978) of patients diagnosed as diabetic before the age of 31 years, 31% died of renal failure. Even in the small study of patients with proliferative retinopathy in whom patients with renal failure were excluded, 24% of patients died of or developed uraemia over the mean follow up period of 6 years (Deckert et al., 1967). This is approximately double the figure found in the present report. However, a study examining the prognosis for renal function in otherwise fit patients with proliferative retinopathy is needed.

The fall in plasma creatinine with time following pituitary ablation reported by Lundbaek has not been confirmed (Lundbaek et al., 1969). In addition, there was a pronounced rise in plasma urea at 6 months post implant which subsequently

returned to normal. The cause of this is not clear. Presumably by 6 months all patients had recovered from the acute effects of the procedure. Possible causes are a sudden fall in glomerular filtration rate, loss of muscle mass or possibly dehydration due to diabetes insipidus which occurred in a number of patients as an early complication.

In conclusion, these patients with pituitary ablation and diabetes seem to have done remarkably well with regard to survival and renal function. The results reported here do not refute the belief that pituitary ablation had beneficial effects on the course of diabetic microvascular disease.

2.4.2 Ophthalmological Aspects

The results presented here are in accord with the findings of short term assessments of pituitary ablation in the treatment of diabetic retinopathy (Bradley et al. 1965, Kohner et al., 1972), confirming it as an effective method of treatment. To find suitable conrtol series, it is necessary to examine the literature prior to the use of photocoagulation as it is would now be considered unethical to withold this treatment in a patient with neovascularisation. The first important point to note is that spontaneous regression of untreated proliferative retinopathy is uncommon, being less than 10% in one early study (Beetham 1963). Since the new vessels regressed in all patients in the present study, the benefit of pituitary ablation can be readily appreciated. In fact as late as 1978, the results of PA in the treatment of 'florid' retinopathy were being compared favourably with those of photocoagulation (Kohner et al., 1976, Valone and McMeel, 1978). The benefit of pituitary ablation is also apparent on assessment of visual outcome. Three studies of

untreated proliferative retinopathy put the progression to blindness at 27-50% (Caird and Garrett, 1963, Deckert et al., 1967, Deckert et al., 1978), whereas in the present study group, the percentage of patients blind in both eyes remained at 12% from 3 to 15 years.

Examining the data by age at entry, separating those under forty from those over forty at implant, it is clear that amongst the older age group, the percentage of blind patients was greater than amongst the younger patients (18% compared with 10% at 5 years, 25% compared with 9% at 10 years). The usual cause of blindness was fibrous retinitis proliferans and its complications. However, the older age group were also more likely to have maculopathy. The different natural histories of the younger and older age groups in terms of visual acuity has been previously noted. Thus, Deckert et al. (1967) in their survey of 51 untreated diabetics with proliferative retinopathy found that patients under 35 years of age had a better prognosis for vision than did the older patients. It appears therefore that the greater improvement in neovascularisation and better visual acuity in the younger age group found in the present study may be unrelated to an improved response to implantation, but rather represents differences in the natural history of the disease.

In the analysis of this data, the role of photocoagulation is a complicating factor, as it played an increasing part in treatment as the benefits of the xenon, and later the argon, photocoagulators became obvious. However, when photocoagulation was first introduced in 1970, the exact indications for its use were not clearly established, nor was the treatment given adequate in the light of later experience.

The precise impact on the present results, therefore, is difficult to assess. For example, two patients had retinal detachment and blindness precipitated by xenon treatment, and some of the 15 patients in whom photocoagulation failed to cause regression of new vessels had inadequate treatment. Nevertheless its influence cannot be ignored, since by the ten year follow-up more than 50% of the surviving 49 patients had received photocoagulation. The main influence seems to have been on the rapidity of resolution of neovascularisation. However, even if the photocoagulated group is removed from the analysis a significant improvement is still apparent in those who had undergone pituitary ablation alone. There was no difference in final visual acuity between the two groups.

In conclusion, the present results indicate that destruction of the pituitary gland is an effective method of treating proliferative diabetic retinopathy.

CONCLUDING REMARKS

The long term follow-up of patients who were treated with ^{90}Y pituitary ablation suggests that such treatment had beneficial effects not only on retinopathy, but also other microvascular complications of diabetes, such as nephropathy. The major problem with the above studies is the lack of control subjects, but comparison with other reports from the literature, often of patients similarly selected, again suggests that the pituitary ablated patients did remarkably well.

Thus far, accepting pituitary ablation or hypophysectomy to be effective in the treatment of retinopathy, it can be stated that removal of some factor produced by the pituitary gland has a beneficial effect on microvascular disease. For reasons outlined

in chapter 1, GH is a good candidate, but the question remains to be resolved.

In the following chapters, possible causes of GH hypersecretion in diabetes will be investigated, and finally, the results of a trial of pharmacological suppression of GH in patients with proliferative retinopathy will be reported upon.

CHAPTER 3

Confirmation of GH hypersecretion in patients with diabetic retinopathy.

INTRODUCTION.

Although elevated diurnal GH levels are well documented in patients with diabetes (Johansen and Hansen, 1971, Hansen, 1973), the following section is a confirmation of the fact that GH hypersecretion can be demonstrated in patients with diabetic retinopathy using the GH assay which will be used throughout this work.

3.1 PATIENTS AND METHODS

Four insulin-dependent diabetic patients (3 female, 1 male) with proliferative retinopathy were selected for study. The mean age of these subjects was 30 years (range 20-45), and the mean glycosylated haemoglobin (HbA1) 11.7% (range 9.6-13.4%, normal range 5-8%). None had any evidence of renal impairment, and none was taking any medication apart from insulin. Two non-diabetic subjects, one male, one female, aged 30 and 31 years, were also studied for comparison. All were admitted to the Metabolic Unit at The Hammersmith Hospital on the morning of the study day, and a cannula was inserted into a forearm vein for the purposes of blood sampling. Subjects were ambulant throughout, and the diabetics had their normal dose of insulin. Hourly blood samples were taken from 8.00am until 10.00pm, and 2 hourly from midnight until 6.00 am. Serum was separated within 2 hours of drawing the blood, frozen at -20^{o}C, and assayed for GH within 3 days.

GH was measured using a double antibody radioimmunoassay, the

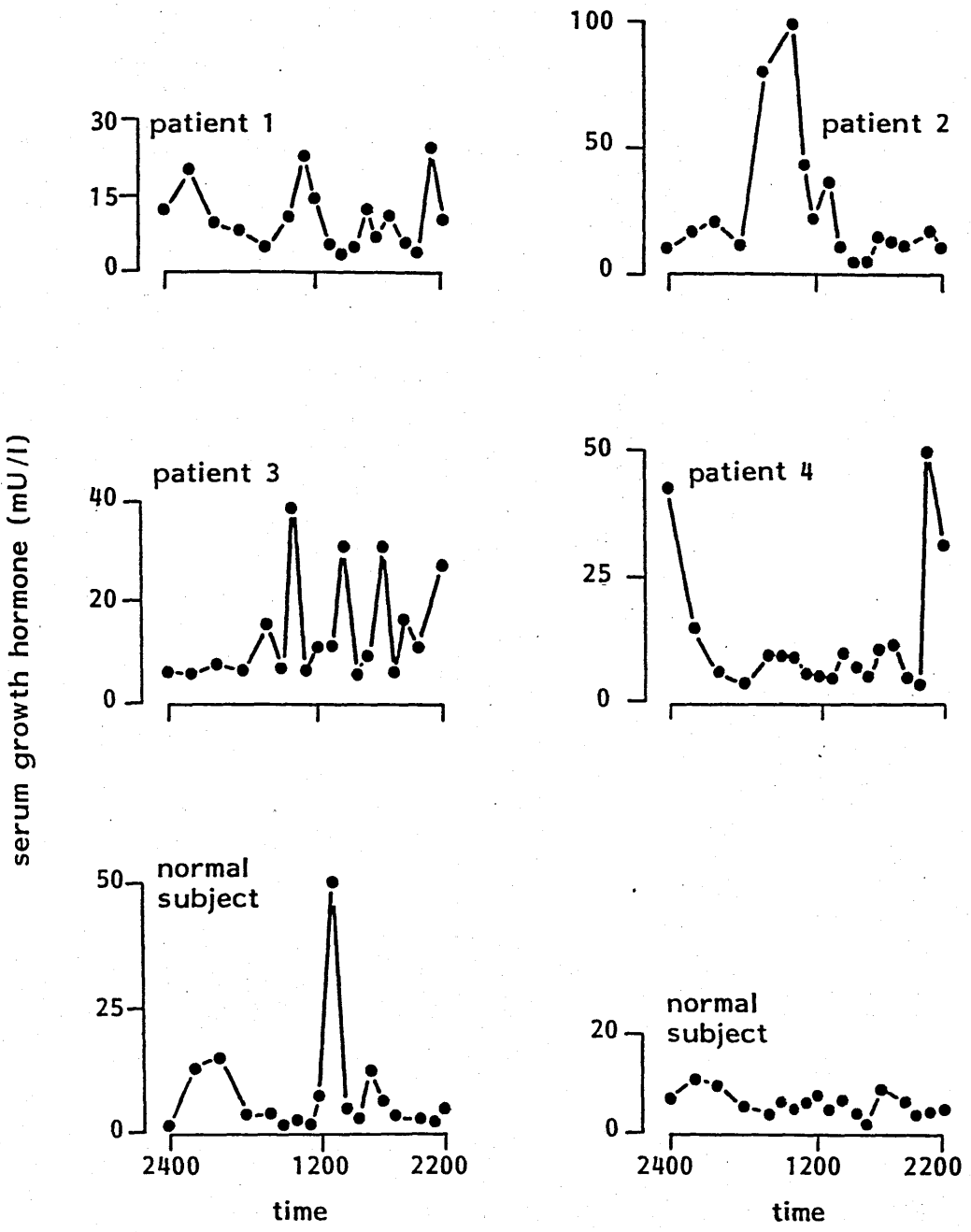
details of which are given in Appendix A.

3.2 RESULTS

The 24 hour GH profiles from the diabetic and normal subjects are shown in figure 3.1.The mean area under the GH curve in the diabetic subjects was 284 mU/l.hr (range 206.2-428.3) whereas the values in the normal subjects were 149.3 and 113.6 mU/l.hr (mean 131.3).

3.3 Comment

This study confirms that GH hypersecretion in patients with proliferative retinopathy is readily demonstrated using the 'in house' radioimmunoassay for GH.



24 hour GH profiles in 4 diabetic patients with proliferative retinopathy and 2 normal subjects.

figure 3.1

CHAPTER 4

Pituitary and hypothalamic regulation of GH in normal and diabetic subjects.

INTRODUCTION

The following chapter outlines studies aimed at assessing hypothalamic and pituitary function in normal and diabetic subjects by extrapolation of data obtained during studies involving the injection of growth hormone releasing factor (GRF) and studies of GH secretion by pituitary cells in culture (Sharp et al., 1984b, Sharp et al., 1984c).

The injection of synthetic GRF can be used as a highly selective pituitary function test, and has found a place in distinguishing pituitary from hypothalamic causes of growth failure in children. It is also a useful research tool in investigating the influence of various factors on pituitary GH secretion, and it was early discovered that age (Shibasaki et al., 1984), obesity (Williams et al., 1984) and atropine (Massara et al., 1984) are potent inhibitors of GRF stimulated GH release. Prior to using this peptide to investigate pituitary function in diabetic subjects, therefore, it is necessary not only to define a normal range for the GH response to GRF, but to investigate the effect of hyperglycaemia in normal subjects. Further, since GRF stimulated GH secretion involves a complex interplay of hypothalamic and pituitary influences, an assessment of the effects of hyperglycaemia on pituitary cells in isolation is a helpful addition. In the following studies, the GH response to GRF was measured in normal subjects under conditions of euglycaemia and hyperglycaemia. Following this preparatory work, similar studies were carried out in diabetic subjects. Lastly, the effects of

hyperglycaemia and hyperinsulinaemia on pituitary cells in culture were assessed.

4.1 NORMAL SUBJECTS

4.1.1 PATIENTS AND METHODS

Six normal subjects, 4 male and 2 female, age range 28-39 years, were selected for study. All had a fasting plasma glucose less than 6mmol/l, and none was on any medication. All subjects were studied under conditions of euglycaemia and hyperglycaemia in a paired study.

euglycaemic study :- study subjects attended the hospital after an overnight fast. A needle was inserted into a forearm vein for sampling purposes, and 3 basal samples were taken for measurement of plasma glucose, serum GH and insulin over a period of 30 minutes. At time zero, a dose of 100ug GRF (1-44) was administered intravenously over 2 minutes, and further blood samples taken at 15 minute intervals for the next 60 minutes.

hyperglycaemic study :- study subjects attended the hospital after an overnight fast. A needle was inserted into a forearm vein in each arm, one for the purposes of glucose infusion and injection of GRF and the other for sampling purposes. A basal sample of blood was taken for measurement of glucose, insulin and GH at times -30 and -15 minutes, following which an infusion of 50% dextrose was given over the next 15 minutes to rapidly elevate the plasma glucose to over 10mmol/l as described for the hyperglycaemic clamp (DeFronzo et al., 1979). The plasma glucose was then measured at 5 minute intervals and maintained at approximately 11mmol/l with a variable infusion of 50% dextrose. At time zero, 100ug GRF was injected as in the euglycaemic procedure, and further samples taken at 15 minute intervals to 60

minutes.

In each case, GRF (Peninsula Laboratories, California) was dissolved in 0.2 mls of 20% human serum albumin, diluted to a volume of 10ml with 0.9% saline, and injected over a period of 2 minutes. A dose of 100ug of GRF was chosen since this has previously been found to produce the maximum GH response (Wood et al., 1983).

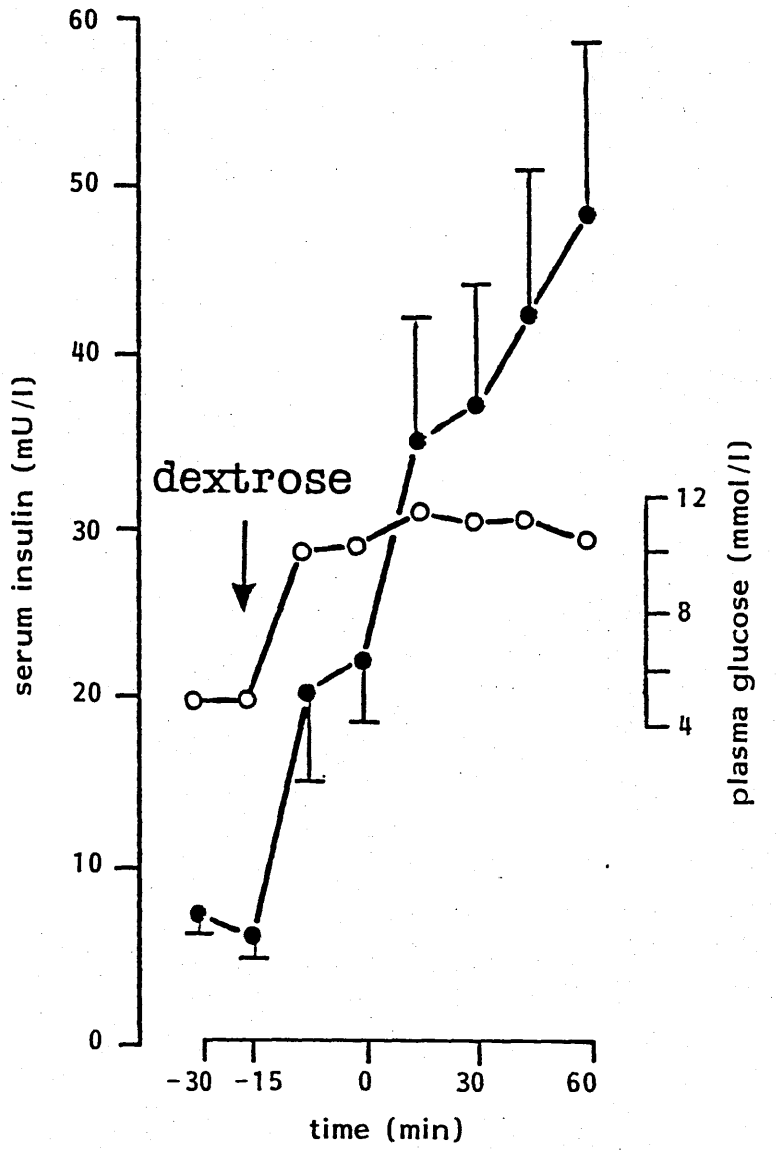
For each individual, no longer than a week was allowed to elapse between the euglycaemic and hyperglycaemic studies, and the GRF was taken from the same batch of material.

Serum insulin was measured by double antibody radioimmunoassay (Morgan and Lazarow, 1963), and plasma glucose was measured by the glucose oxidase method using the Beckman Glucose Analyzer (Beckman, California). Differences between groups were calculated by paired t-test.

4.1.2 RESULTS

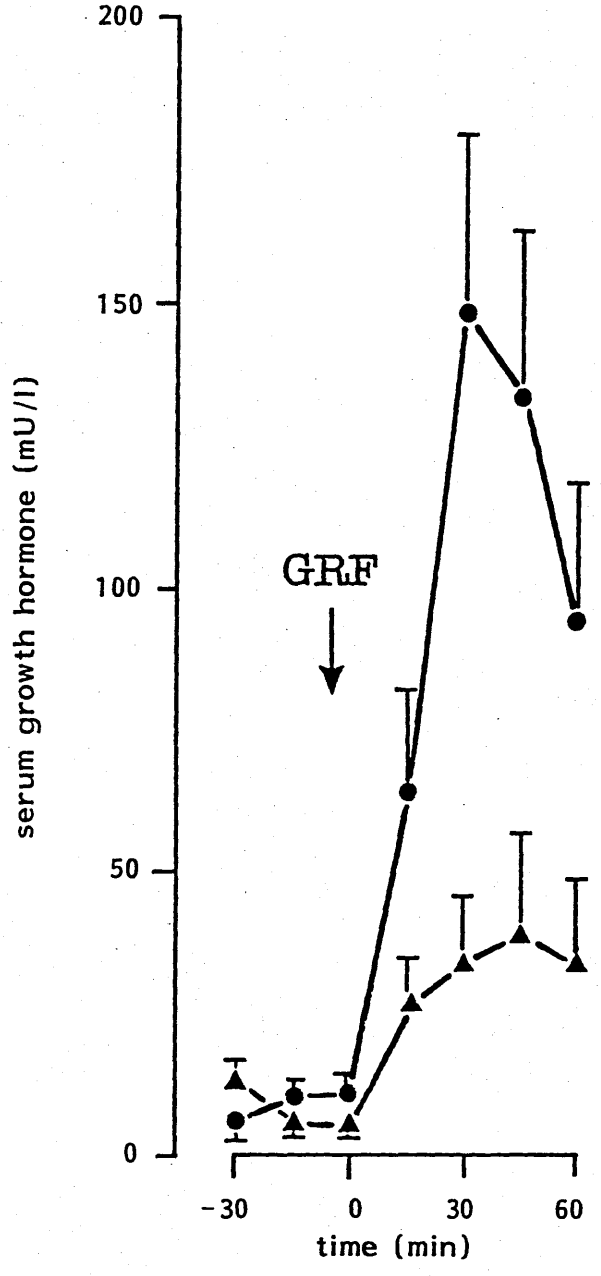
During the euglycaemic study, the mean plasma glucose was 5.1±0.4 mmol/l (±S.D.) and serum insulin 5.6±2.2 mU/l. In no individual did the plasma glucose change by more than 0.5 mmol/l after administration of GRF. Plasma glucose and serum insulin levels during the hyperglycaemic studies are shown in figure 4.1. Figure 4.2 shows the mean serum GH response to GRF in all subjects under conditions of euglycaemia and hyperglycaemia. It can be seen that under conditions of hyperglycaemia, the GH response to GRF is significantly blunted ($p<0.01$ on comparison of peak responses). Individual peak GH responses under conditions of euglycaemia and hyperglycaemia are shown in figure 4.3.

There was no correlation between the change in serum insulin from basal during the hyperglycaemic study and the difference in GH



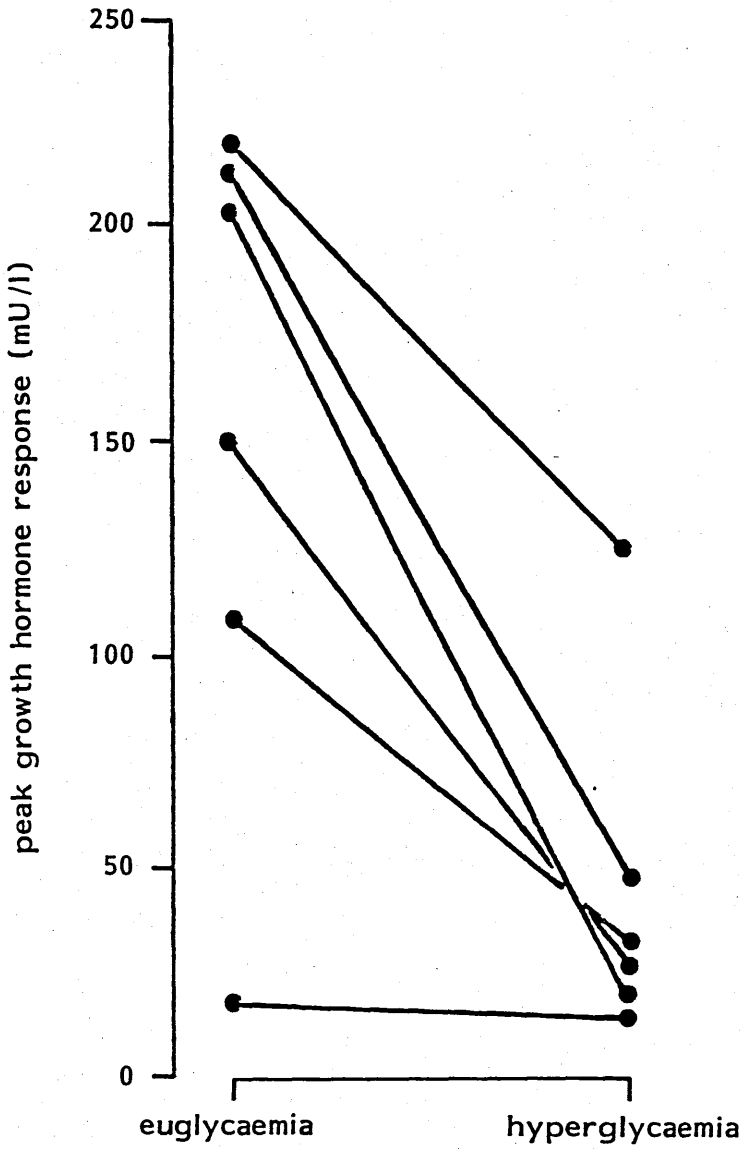
Serum insulin (●) and plasma glucose (○) in the 6 normal subjects during the hyperglycaemic study.

figure.4.1



Serum GH response to 100µg GRF (1-44) in 6 normal subjects studied under conditions of euglycaemia (●) and hyperglycaemia (▲).

figure 4.2



The peak GH response to 100µg GRF (1-44) in 6 normal subjects studied under conditions of euglycaemia and hyperglycaemia.

figure 4.3

response on comparison of the 2 procedures (r=0.095).

4.2 DIABETIC SUBJECTS

4.2.1 PATIENTS AND METHODS

Ten insulin-dependent diabetic subjects were studied while euglycaemic and 10 while hyperglycaemic. Six of the diabetic subjects participated in both parts of the study. Ten normal subjects studied at euglycaemia acted as controls. The clinical characteristics of these groups are shown in table 4.1. All subjects were within 110% of ideal body weight, and had normal values for plasma urea and creatinine. None had proteinuria when tested with Albustix (Ames). In both groups of diabetics, 8 out of 10 had some degree of retinopathy.

euglycaemic study :- The euglycaemic studies in the normal subjects were carried out essentially as described above in section 4.1.1 except that sampling was continued for 90 minutes. The diabetic subjects to be studied at euglycaemia were admitted to hospital the evening before the study day. A needle was inserted into a forearm vein in each arm, one for the purposes of infusion, the other for blood sampling. From 10.00pm, an infusion of 0.9% saline containing 10 units of short acting insulin (Actrapid, Novo Laboratories, Denmark) and 5ml Haemacel was administered at a variable rate, aiming to keep the plasma glucose at a target value of 6mmol/l. The plasma glucose was measured hourly throughout. At 8.00am the following morning, while still fasting, the GRF bolus study was carried out as described above.

hyperglycaemic study :- The diabetic subjects to be studied at hyperglycaemia attended the hospital at 8.00am on the morning of the study day after an overnight fast, having omitted their

table 4.1

Clinical characteristics of subjects involved in the diabetic GRF bolus studies. All data expressed as mean ± S.D.

	sex (m/f)	age (years)	HbA1 (%)	plasma glucose (mmol/l)	basal GH (mU/l)
normal subjects (n=10)	6/4	33.1±8.7	7.3±0.5	5.2±0.3	7.2±6.8
diabetics (euglycaemia) (n=10)	6/4	33.5±9.4	10.2±1.8	6.4±1.0	5.3±6.4
diabetics (hyperglycaemia) (n=10)	5/5	35.1±8.4	10.0±2.2	13.1±4.1	6.3±9.9

morning dose of insulin. The GRF studies were carried out as described above.

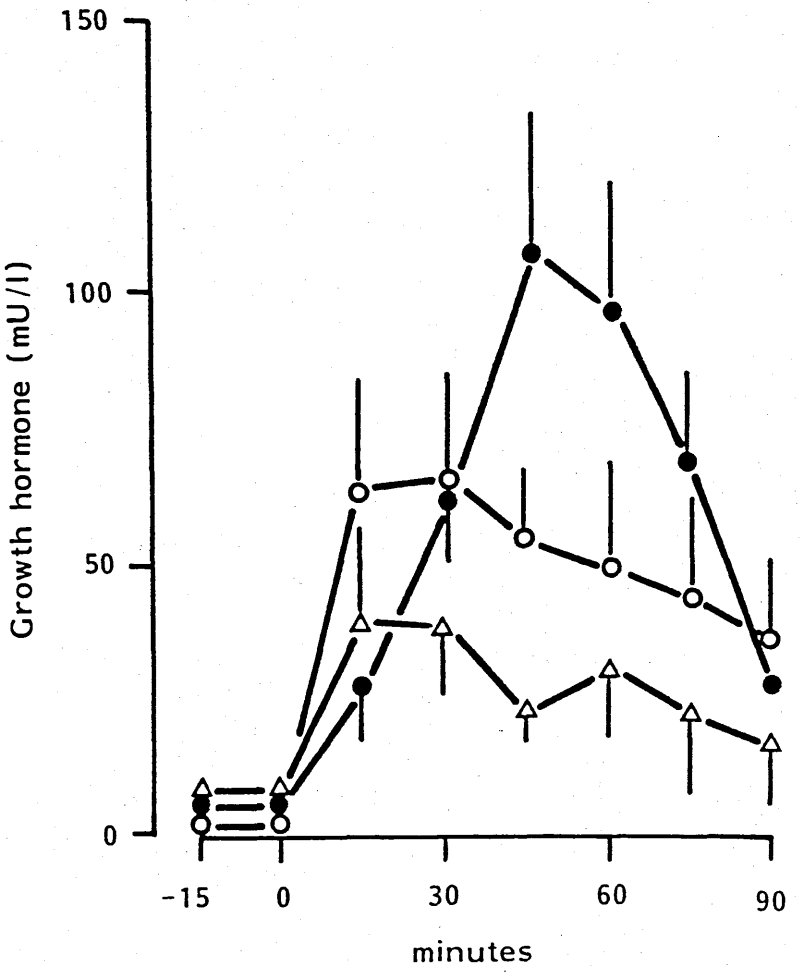
In the 6 diabetic subjects who participated in both the euglycaemic and hyperglycaemic studies, at least a week elapsed between the two procedures.

In these studies, plasma insulin was measured as outlined above with the exception that samples from the diabetic subjects were first extracted with polyethylene glycol (PEG), and free insulin levels measured (Kuzuya et al., 1977). Glycosylated HbA1 was measured by ion exchange chromatography (Bio-Rad kit, Bio-Rad, California).

Differences between groups were calculated by Student's t-test, paired or unpaired as appropriate.

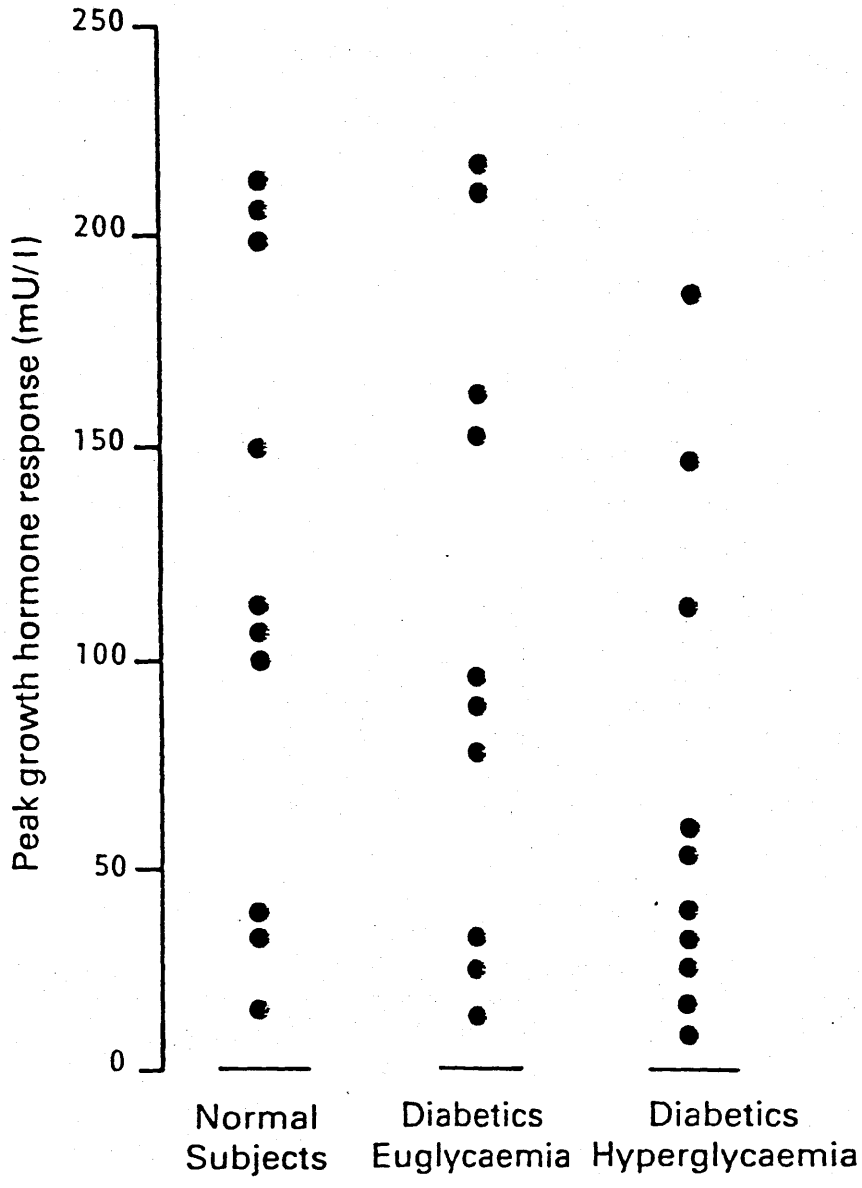
4.2.2 RESULTS

The GH response to GRF in each of the 3 groups is shown in figure 4.4. The shape of the curve differs in the euglycaemic diabetics compared with that of the normal subjects. This occurred due to variability in the timing of the peak GH response in the diabetic group (range 15-90 minutes) which was more consistent in the normal subjects (range 30-45 minutes). The peak GH values achieved in each of the 3 groups studied are shown in figure 4.5 where it can be seen that this did not differ between the euglycaemic diabetics and normal subjects (105.2$\pm$23.9 vs. 118.2$\pm$37.6 mU/l (mean$\pm$ S.E.M.), N.S.). Inspection of figures 4.4 and 4.5 also shows that amongst the diabetics, there was some suppression of the peak GH response in the hyperglycaemic group compared with the euglycaemic group, (105.2$\pm$23.9 vs. 64.7$\pm$19.4 mU/l, N.S.) although again, this was not consistent. Figure 4.6 shows the peak GH response under conditions of euglycaemia and



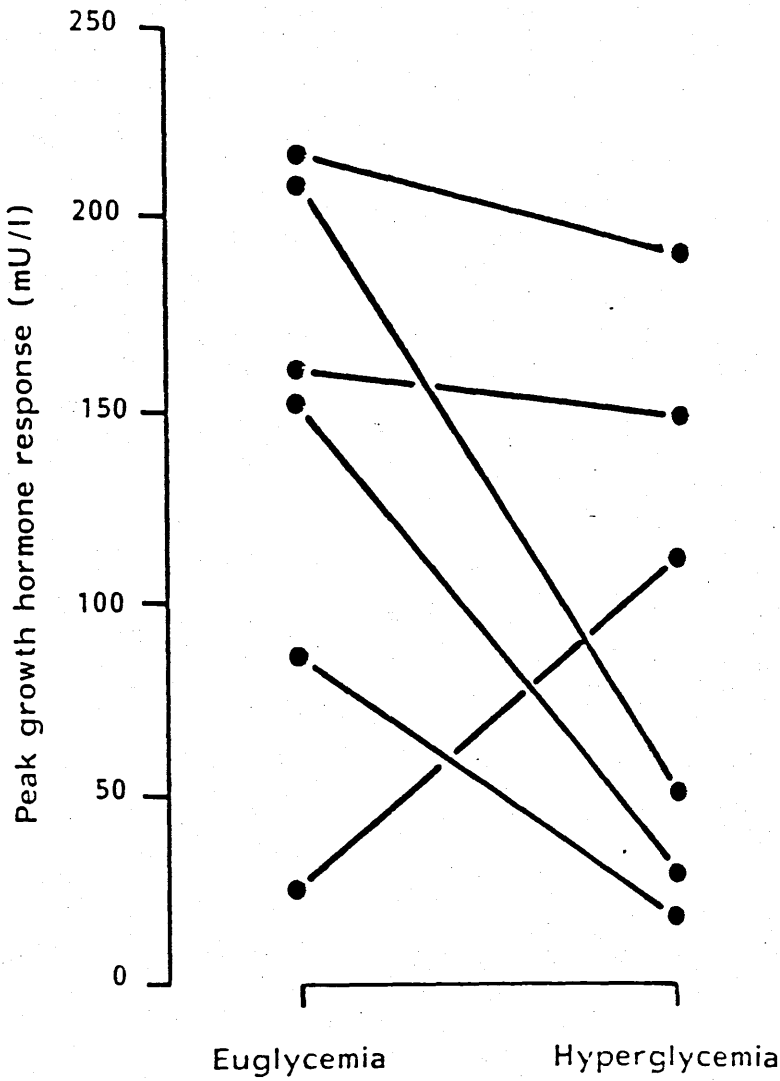
The GH response to 100µg GRF (1-44) in 10 euglycaemic normal subjects (●) and 6 diabetic subjects studied under conditions of euglycaemia (○) and hyperglycaemia (△).

figure 4.4



The peak GH response to an intravenous bolus of 100µg GRF (1-44) in 10 euglycaemic normal subjects, 10 euglycaemic diabetics and 10 hyperglycaemic diabetics.

figure 4.5



The peak GH response to 100ug GRF (1-44) in 6 diabetic subjects studied under conditions of euglycaemia and hyperglycaemia.

figure 4.6

hyperglycaemia in the 6 diabetic subjects who participated in the paired study. Again it can be seen that although there was significant GH suppression in 3 subjects, as a group the difference did not reach significance (140.8±30.9 vs. 90.2±27.7 mU/l, N.S.)

There was no significant correlation between the peak GH level and the serum free insulin, HbA1, age or basal GH in any of the groups studied.

4.3 PITUITARY CELL CULTURES

4.3.1 METHODS

This work was carried out in rat pituitary cells in culture. A limited supply of human pituitary tissue is available intermittently, and in the present studies, it was possible to examine the effect of insulin on GRF stimulated GH secretion in tissue obtained from a human GH secreting tumour. The preparation and treatment of the cultures is identical whether human or rat with the exception that rat GH is measured using a specific radioimmunoassay. This is similar to that described for human GH although the antiserum is raised against rat GH.

Rat anterior pituitary tissue was obtained by dissection from unanaesthetised decapitated laboratory rats. Human tissue was obtained at the time of transsphenoidal hypophysectomy. In each case, immediately after removal, tissue was placed in culture medium (10ml of Eagles Minimum Essential Medium containing 10% fetal calf serum (Grand Island Biological Co., Glasgow), 100U/ml penicillin, 100ug/ml streptomycin (Glaxo, Greenford, Middlesex) and 2.5ug/ml Fungizone (Grand Island Biological Co.) buffered with Hepes (Hopkin and Williams, Chadwell Heath, Essex)). Each tumour was washed with phosphate buffered saline (PBS) (Oxoid,

Basingstoke, Hampshire), divided into small pieces with a sterile scapel, and dissociated by incubation in 40ml 0.02% EDTA, 0.25% trypsin (Flow Laboratories, Irvine, Ayrshire) in PBS for 20-30 minutes at 37°C in a 100ml conical flask continuously stirred by a rotating magnet. The resultant cell suspension was centrifuged at 500g for 4 minutes at 4°C and the supernatant removed. The cell pellet was washed with 10ml of culture medium and resuspended in 2ml of fresh medium.

Cell number and viability were determined using a haemocytometer and trypan blue stain, with inspection under a microscope. The suspension was diluted to a concentration of 2.0×10^5 cells/ml. Aliquots of 1ml were then distributed into glass screw cap culture tubes to which cells were allowed to attach during the following 24 hours. After an incubation period of a further 24 hours, the attached cells were washed with culture medium and subsequently maintained in 2ml of medium at 37°C in closed tubes.

control experiments :- cells were washed in culture medium and reincubated for a further 2 hours in fresh medium.

GRF :- after washing, cells were incubated in 2ml of fresh medium containing 10ng/ml GRF for 2 hours.

insulin :- after washing, cells were incubated with 2ml of fresh medium containing 100uU/ml human insulin (Human Actrapid, Novo Laboratories) in the case of human cells, and 200uU/ml of human insulin in the case of rat cells.

glucose :- after washing, cells were incubated in 2ml of fresh medium containing 5-25mmol/l glucose for 2 hours.

In all of the above studies, statistical analysis of GH data was carried out on log transformed data using Student's t-test, paired or unpaired as appropriate.

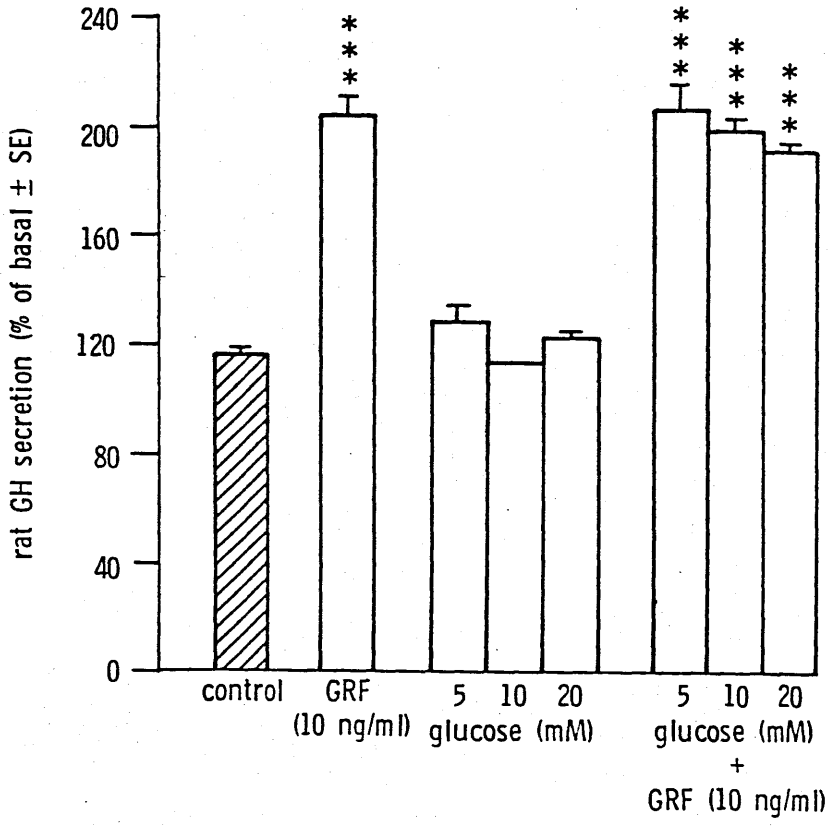
4.3.2 RESULTS

The effect of glucose :- figure 4.7 shows the effect of glucose on GRF stimulated GH secretion in rat anterior pituitary cells. GRF produced a significant increase in GH compared with the control study, whereas glucose alone was without effect. Further, increasing glucose concentration had no effect on GRF stimulated GH secretion.

The effect of insulin :- figure 4.8 shows the effect of insulin on GH secretion in both rat and human pituitary cells in culture. It can be seen that insulin alone had no effect on GH secretion, and produced no change in GRF stimulated secretion in either rat or human tissue.

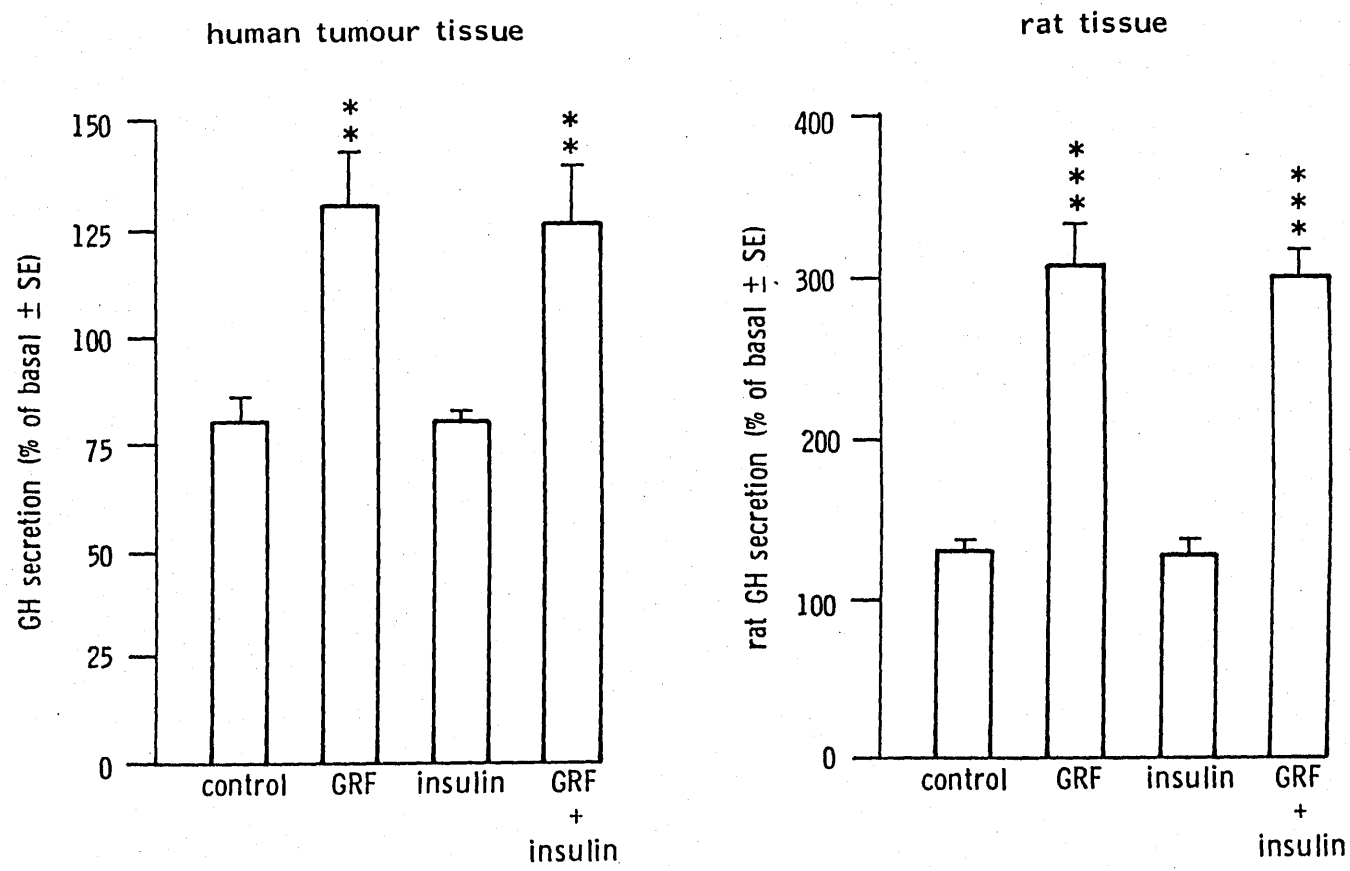
4.4 DISCUSSION

The studies in normal subjects very clearly demonstrate that hyperglycaemia is a potent suppressor of GRF stimulated GH secretion. This finding was subsequently extended by other workers to show that the effect is also found in response to an oral glucose load in normal subjects (Davies et al., 1984), and is in keeping with the well recognised observation that GH levels are suppressed during an oral glucose tolerance test (Glick et al., 1965). Possible mechanisms include a direct action of glucose on the pituitary gland, or on the glucose sensitive cells of the hypothalamus. Since hyperglycaemia induces a rise in serum insulin in normal subjects, it is also possible that insulin itself inhibits GH secretion, either at pituitary or hypothalamic level. Some attempt was made to resolve these possibilities in the studies of pituitary cells in culture. Increasing glucose concentrations failed to affect GH secretion from rat pituitary cells. It has, however, recently been demonstrated that



The effect of growth hormone releasing factor (1-44) (GRF) and glucose, alone and in combination, on GH secretion by rat anterior pituitary cells in culture.
(*** $p<0.001$)

figure 4.7



The effect of growth hormone releasing factor (1-44) (GRF, 10ng/ml) and insulin, alone and in combination, on GH secretion by human and rat anterior pituitary cells in culture. The insulin concentration was 100μU/ml in the human experiment, and 200μU/ml in the rat experiment. (** $p<0.01$, *** $p<0.001$)

figure 4.8

hyperglycaemia fails to suppress the GH response to GRF in rats (Imaki et al., 1986), and therefore the possibility that glucose acts directly on the pituitary gland to suppress GH secretion in man must remain open. There is, however, no precedent in the literature to suggest this possibility. More helpful are the studies of the effect of insulin on GH secretion in culture since these studies were carried out on both rat and human tissue, no effect being demonstrated. There is one report in the literature of insulin inhibiting GH secretion from human pituitary cells in culture (Ceda et al., 1985), but the doses of insulin used were of such magnitude that this is unlikely to be of physiological significance, the insulin probably acting through the IGF-I receptor (see section 1.11).

Based on the present studies and the current knowledge of the regulation of GH secretion, the most likely mechanism of GH suppression by hyperglycaemia is that the elevated blood glucose level is detected by the glucose sensitive cells of the hypothalamus resulting in an increased production of somatostatin. This, in turn, would result in blunting of the GH response to GRF.

A number of conclusions can be drawn from the studies in the diabetic subjects. Firstly, the GH response to GRF, although quantitatively similar, is irregular when compared to that of the normal subjects. This finding is reminiscent of the irregular TSH response to TRH found in subjects with hypothalamic disease (Faglia et al., 1973), and is perhaps indicative of a hypothalamic disorder in the regulation of GH secretion in diabetes. Secondly, while individual diabetic subjects may show normal glucose mediated suppression of GH secretion, as a group, suppression of GH secretion in response to hyperglycaemia in the

diabetic subjects is impaired. This has previously been described in insulin dependent diabetes using arginine as the stimulus (Burday et al., 1968). However, the advantage of using GRF is that the stimulus is well characterised, with a known site of action, whereas the the GH stimulatory action of arginine is unclear. What can be deduced from the GRF studies in the diabetic subjects is that there is no defect in the response of the pituitary gland to direct stimulation. The abnormality rather lies in the response to hyperglycaemia, either failing to produce a rise in somatostatin, or lack of sensitivity to the somatostatin so produced. Somatostatin was not measured in the above studies since plasma measurements do not reflect events at hypothalamic level.

In conclusion, the above studies suggest a possible hypothalamic abnormality in the regulation of GH secretion in diabetes.

Changes in serum GH during studies involving insulin and glucose infusion.

INTRODUCTION

To further examine central regulation of GH secretion, and to expand on certain observations in the previous chapter, the effects of manipulation of plasma glucose and serum insulin on GH secretion in normal and diabetic subjects are examined. The following studies have been selected from a number performed (Sharp et al., 1986a, Sharp et al., 1986b) to illustrate certain important points.

In the first study, the change in serum GH in normal subjects during a fall in plasma glucose from a high to a normal level is described. Since glucose levels in treated diabetic subjects are often variable, albeit within the normal range, it is important to assess the effect of such variations on GH secretion in normal man. In the second study, changes in serum GH during the course of a hyperglycaemic clamp procedure at the time of diagnosis of diabetes and after 6 months insulin therapy are described.

5.1 reduction in plasma glucose within the normal range in normal subjects.

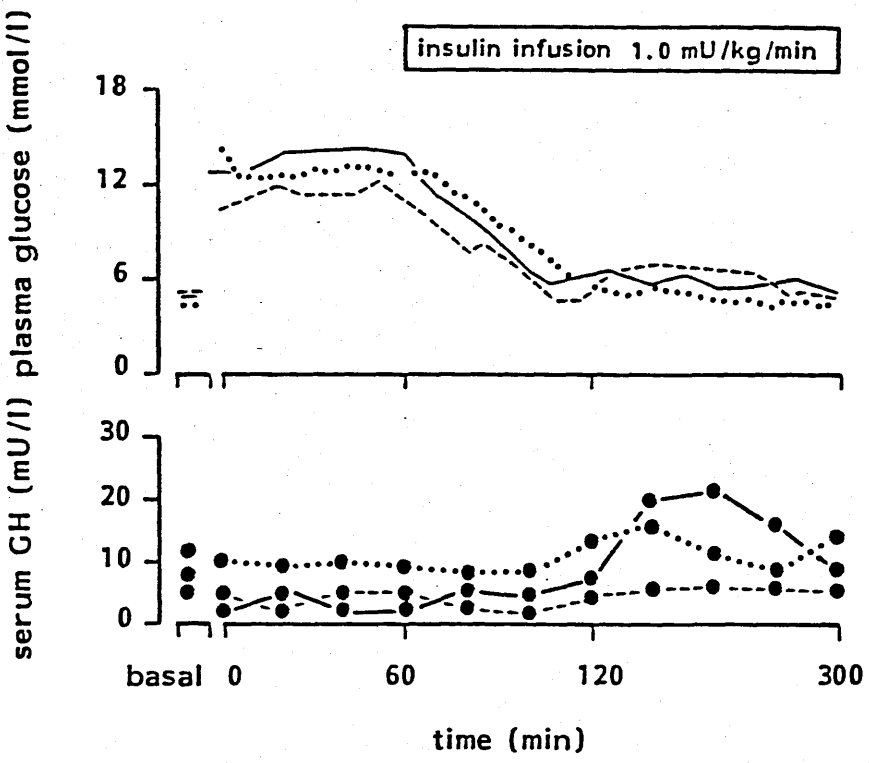
5.1.1 PATIENTS AND METHODS

Three non-diabetc subjects, aged 31, 28 and 55 years, all of whom were within 120% ideal body weight and none of whom was on any medication, were studied. Each subject attended the hospital on the morning of the study day after an overnight fast. An 18cm cannula was inserted through a forearm vein of one arm for the purposes of glucose and insulin infusion, and a 19g 'butterfly'

needle inserted into a vein in the other arm for sampling purposes. Baseline samples were taken for the measurement of plasma glucose and serum GH, following which an infusion of 50% dextrose was given over a period of 15 minutes, aiming to rapidly elevate the plasma glucose to over 10mmol/l (DeFronzo et al., 1979). Thereafter, the plasma glucose was measured at 5 minute intervals, and maintained at approximately 10mmol/l with a variable infusion of 50% dextrose for the next 60 minutes. At the end of this time, the dextrose was discontinued. An infusion of short-acting insulin (Human Actrapid, Novo Laboratories) was given over 10 minutes to rapidly elevate the serum insulin to approximately 100mU/l, followed by a constant infusion at a rate of 1.0mU/kg/min. The plasma glucose was allowed to fall to 6mmol/l over the next 60 minutes, following which the dextrose infusion was recommenced to maintain the plasma glucose at this level. A steady state period of glucose and insulin was maintained for the next 80 minutes. Throughout the procedure, plasma glucose was measured at 5 minute intervals, serum insulin at 10 minute intervals, and serum GH at 20 minute intervals.

5.1.2 RESULTS

The changes in plasma glucose and serum GH during the study are shown in figure 5.1. During the period of hyperglycaemia and reduction in plasma glucose to euglycaemia, serum GH remained below the basal value. In 2 out of the 3 study subjects there was a modest rise in serum GH above basal during the steady state period, after the plasma glucose had been reduced to 6mmol/l.



Changes in serum GH induced by manipulation of the plasma glucose, from hyperglycaemia to euglycaemia, in 3 normal subjects.

figure 5.1

5.2.1 Changes in serum GH during the glucose clamp procedure.

The glucose clamp procedure (DeFronzo et al., 1979) was first described as a method of measuring peripheral insulin sensitivity, measuring glucose uptake in response to a constant insulin infusion. During the course of such studies, it was noted that although the plasma glucose and serum insulin levels are constant, serum GH in diabetic subjects is extremely variable, often rising above the basal value (Sharp et al., 1984a, Sharp et al., 1986a, Sharp et al., 1986b). The cause of such variability in serum GH levels in diabetic subjects is not known, but may relate to prior manipulations of the plasma glucose, or to the high insulin levels employed. The mechanism will not be further discussed since it would not further the arguements presented here. However, this phenomenon has all the features of disturbed GH regulation of diabetes, being absent in normal subjects, more marked in diabetic subjects with retinopathy and not abolished by hyperglycaemia. In the following section, such changes in GH levels, observed during the course of one study, will be described since certain observations on GH secretion in diabetes can be made, and advantage can be taken of the fact that measurements of insulin sensitivity are simultaneously available.

5.2.2 PATIENTS AND METHODS

Thirteen newly diagnosed diabetic subjects were studied. Clinical characteristics are given in table 5.1. Five of these subjects were classified as insulin-dependent, and 8 as non-insulin-dependent according to the criteria of the National Diabetes Data Group (1979). Patients with severe metabolic decompensation were

table 5.1

Diabetic patient characteristics and changes with insulin therapy

SUBJECT NO.	SEX (M/F)	AGE (YRS)	BMI AT DIAGNOSIS	WEIGHT (KG) PRE-	WEIGHT (KG) POST-	HBA1 (%) PRE-	HBA1 (%) POST-	FASTING INSULIN (MU/L)	INSULIN DOSE (U)	DIABETES TYPE
1	M	50	29.7	82.9	88.0	13.1	10.1	17.1	24	NIDDM
2	M	29	23.8	71.4	75.3	9.6	5.9	6.4	30	IDDM
3	M	25	22.8	69.2	69.4	11.3	8.0	5.1	44	IDDM
4	M	30	19.4	54.0	67.9	13.9	10.1	5.1	47	IDDM
5	F	35	25.5	61.4	59.5	12.0	7.7	13.7	39	NIDDM
6	M	25	20.4	59.0	61.0	13.8	8.4	4.7	36	IDDM
7	M	53	23.0	65.8	71.2	13.4	10.0	15.9	28	NIDDM
8	M	32	29.1	83.2	84.0	16.4	8.7	10.6	24	NIDDM
9	M	30	23.2	67.0	73.0	14.4	8.5	6.6	30	IDDM
10	M	64	23.0	66.5	82.1	14.5	6.4	7.8	38	NIDDM
11	F	29	20.1	51.0	58.0	13.8	8.8	6.0	32	NIDDM
12	M	51	24.8	69.2	75.6	11.5	9.0	4.0	36	NIDDM
13	M	30	21.1	64.0	75.0	14.5	7.8	6.0	32	NIDDM

$BMI = Wt\ (kg)/Ht^2\ (m)$

excluded from the study. Hence, none of the study subjects had had significant weight loss, and none had more than a trace of ketonuria.

At the time of diagnosis, and before the commencement of any treatment, all patients underwent a glucose clamp procedure as follows. The patient attended the hospital on the morning of the study day, after an overnight fast. A needle was inserted into a forearm vein of each arm, one for the purposes of glucose and insulin infusion, the other for sampling purposes. The sampling arm was placed in a glove in a water bath at 40^{o}C to obtain 'arterialised' venous blood. Two basal samples were taken over 20 minutes for measurement of plasma glucose, serum insulin and GH. An infusion of short-acting insulin was then given over 10 minutes to acutely raise the serum insulin to approximately 100mU/l, as described for the euglycaemic clamp (DeFronzo et al., 1979), followed by a continuous infusion at a rate of 1.0mU kg/min. This was continued for 180 minutes, during which time the plasma glucose was stabilised and maintained at 12mmol/l with a variable infusion of 50% dextrose. After the stabilisation period of 180 minutes, a steady state of glucose and insulin was maintained for the next 80 minutes, during which time glucose uptake was measured based on the amount of dextrose infused in response to the constant insulin infusion. At the end of this period, a further infusion of insulin was given over 10 minutes to raise the serum insulin in a square wave to approximately 1000mU/l, and this was followed by a constant infusion of 10mU/kg/min. A stabilisation period of 30 minutes was allowed, during which time the plasma glucose was again maintained at 12mmol/l with a variable infusion of 50% dextrose. A further steady state was then maintained for the next 80 minutes, during

which time glucose uptake at the higher insulin infusion rate was again measured. At the end of the experiment, the patients' urine was collected, and glucose uptake measurements corrected for urinary glucose losses and deviation from the target value of 12mmol/l.

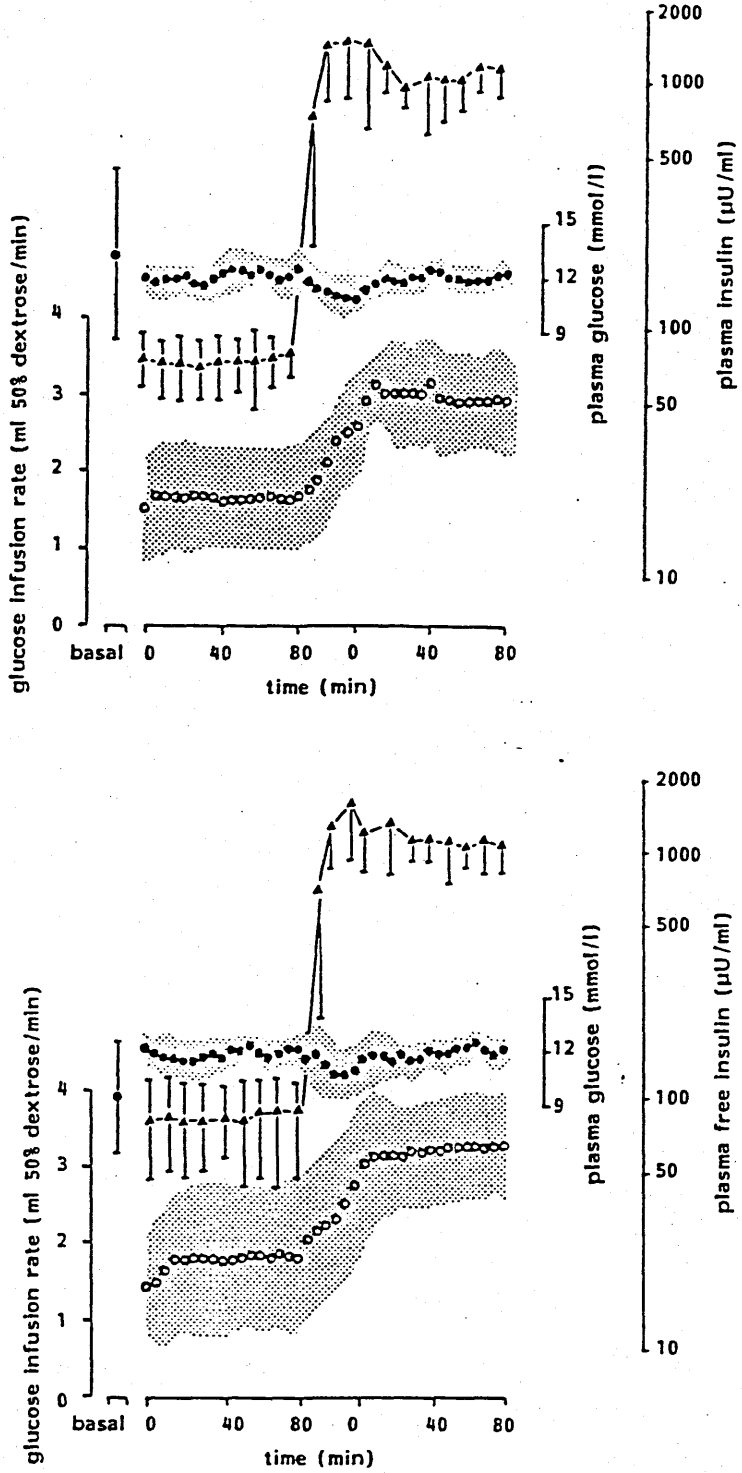
Throughout the whole procedure, plasma glucose was measured at 5 minute intervals, serum insulin at 10 minute intervals, and serum GH at 20 minute intervals.

After these studies had been carried out, each subject was commenced on twice daily insulin, with improved metabolic control. The glucose clamp procedure was repeated as described above after 6 months of insulin therapy. On this occasion, patients missed their morning dose of insulin on the study day, and their long-acting insulin the night before the study day. Free insulin was measured during the repeat study since patients were insulin treated, and may have developed antibodies.

5.2.3 RESULTS

Insulin therapy lead to a significant improvement in fasting plasma glucose (13.2±1.1 vs. 8.9±0.8mmol/l (mean±S.E.M.), $p=0.03$) and HbA1 (13.2±0.5 vs. 8.4±0.4%, $p<0.001$) from diagnosis to 6 months.

Figure 5.2 illustrates the serum insulin, plasma glucose and glucose infusion rates during the glucose clamp experiments at diagnosis and after 6 months. At the lower insulin infusion rate, the plasma glucose was kept at a mean value of 11.95mmol/l, coefficient of variation (CV) 6.3%, and at the higher insulin infusion rate 12.1mmol/l, CV 5.5%. Insulin infusion rates 1.0 and 10mU/kg/min resulted in a serum insulin levels of 86.8±5.1 and 1129±54mU/l respectively.

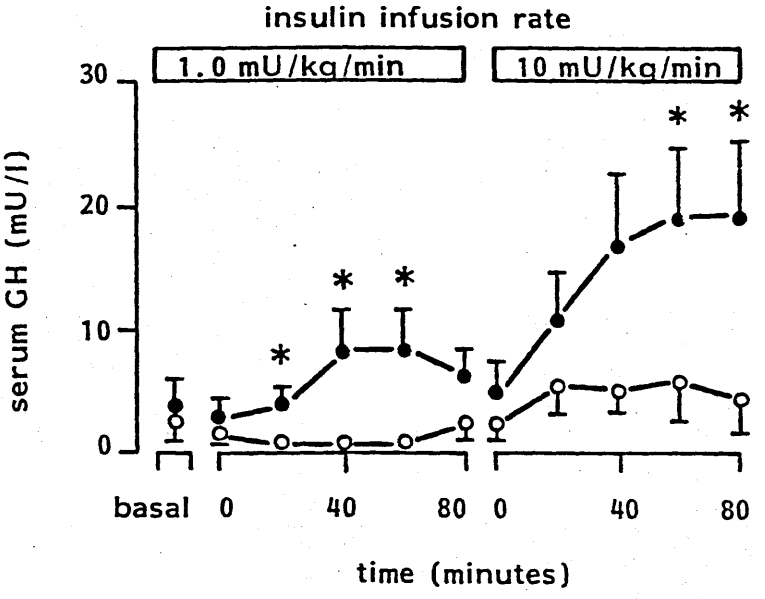


Changes in plasma glucose (●) plasma insulin (▲) and glucose infusion rate (O) during the glucose clamp studies in 12 diabetic subjects studied at diagnosis of diabetes and after 6 months insulin therapy. Shaded areas and error bars represent the standard deviation.

figure 5.2

Changes in serum GH during the glucose clamp procedures at diagnosis and after 6 months insulin therapy are shown in figure 5.3. At diagnosis, the mean serum GH at insulin infusion rate 1.0mU/kg/min was slightly above basal (6.4±2.2 vs. 3.8±1.8mU/l, N.S.), with a further rise at insulin infusion rate 10mU/kg/min (16.8±4.8 vs. 3.8±1.8mU/l, $p<0.002$). At the time of the glucose clamp studies after 6 months insulin therapy, there was no longer any significant change in serum GH from basal during either the lower or higher insulin infusion rate (3.3±1.5 (basal) vs. 1.4±0.4 vs. 5.3±1.9mU/l, N.S.).

Changes in glucose uptake from diagnosis to 6 months varied greatly within the study subjects, but the insulin-dependent group showed a significant increase at both the lower and higher insulin infusion rate (11.0±1.4 vs. 15.9±1.8 mg/kg/min, $p=0.04$ and 22.5±0.9 vs. 26.4±1.7 mg/kg/min, $p=0.025$). In contrast, the non-insulin dependent group showed a decrease in glucose uptake from diagnosis to 6 months at the lower insulin infusion rate (13.0±1.6 vs. 9.3±1.5 mg/kg/min, $p=0.02$), but no significant change at the higher insulin level (22.8±1.3 vs. 22.1±1.5 mg/kg/min, N.S.). Further, at the lower insulin infusion rate, where the serum insulin was in the high physiological range, there was a significant correlation between change in glucose uptake from diagnosis to 6 months and fasting serum insulin at diagnosis ($r=-0.5$), age ($r=-0.62$) and diabetes type, insulin dependent or non-insulin-dependent ($r=-0.79$). There was no significant correlation between change in glucose uptake and serum GH, either absolute levels or change with insulin therapy.



Changes in serum GH during the steady state periods of the glucose clamp studies in 12 diabetic subjects at diagnosis of diabetes (●) and after 6 months insulin therapy (○). (* $p<0.05$)

figure 5.3

One hypothesis to account for GH hypersecretion in diabetic subjects proposes that the plasma glucose level below which GH release is triggered is higher in diabetes than in normal subjects. Thus, it has been claimed that a fall in plasma glucose within the normal range will stimulate GH release in diabetic, but not normal subjects (DeFronzo et al., 1977, DeFronzo et al., 1980, Grecu and Walter, 1983). The results reported here demonstrate that lowering of the plasma glucose within the normal range can cause a delayed GH release in normal subjects, perhaps overlooked by some workers since the study subjects were not followed for a sufficient time after euglycaemia had been achieved. Thus, without the need to propose any abnormality in GH regulation, a fluctuating plasma glucose level in treated diabetic subjects is likely to lead to GH release, even in the absence of hypoglycaemia. While this is not the cause of GH hypersecretion in diabetes, it is likely to be a contributory factor, especially in insulin treated subjects. Further, it is only pathological in as much as plasma glucose levels are likely to be more variable than normal in treated diabetes.

The glucose clamp experiments were carried out to assess changes in insulin resistance in a group of diabetics before and after long-term insulin therapy with improved metabolic control. Serum GH was measured since levels of this hormone could contribute to insulin resistance. As discussed in section 5.2.1, it was found that during the steady state periods of the glucose clamp at diagnosis, there was a rise in serum GH above basal, but this was no longer apparent after insulin therapy and improved glycaemic control. Although not the primary objective of the study, and although the stimulus is unusual, these GH changes are

presented as illustrative of certain points relevant to the discussion of GH secretion in diabetes.

Firstly, there was no correlation between GH levels and measurements of insulin sensitivity, diabetes type being a much stronger factor in this respect. This an important consideration since it has been proposed that elevated GH levels in diabetic subjects are responsible for poor glycaemic control (Press et al., 1984). In the studies of Press et al., GH was infused into well controlled diabetic subjects to obtain plasma GH levels similar to those seen in poorly controlled diabetes. Since GH antagonises the effects of insulin (MacGorman et al., 1981, Bratusch-Marrain et al., 1982), there was a predictable deterioration in glycaemic control in the subjects thus treated, and it was concluded that the elevated GH levels seen in some diabetics are primary in causing poor glycaemic control. This arguement fails to take account of the fact that patients with poorly controlled diabetes may be resistant to some of the effects of GH (see section 1.12). Thus, mimicking the elevated GH levels of poorly controlled diabetes in subjects with good glycaemic control is an entirely artificial situation, and erroneous results could be drawn. The observation here, that there was no correlation between serum GH and insulin sensitivity, suggests that GH hypersecretion is a feature of, and not the cause of, poorly controlled diabetes.

The difference between GH levels at the time of diagnosis and after insulin treatment also serves to demonstrate that improved glycaemic control leads to a reduction in GH levels in diabetes. This point has previously been demonstrated on measurement of 24 hour GH levels in insulin-dependent diabetes (Hansen, 1971, Johansen and Hansen, 1971, Vigneri et al., 1976).

Finally, the fact that at diagnosis, during studies at a plasma glucose of 12mmol/l, the diabetic subjects were still capable of producing serum GH levels above basal, serves as a reaffirmation of the observation in the previous chapter that hyperglycaemia fails to suppress GH secretion in diabetes. Possible causes of this phenomenon have been discussed. A further possibility which has been advanced is that subjects with diabetes have a defect of glucose transport across the blood brain barrier, which results in neuroglycopaenia in the presence of a normal plasma glucose level. This possibility will be considered in the following chapter.

Hexose transport across the blood brain barrier in diabetic subjects.

INTRODUCTION

In the previous chapter, the possibility was raised that GH hypersecretion in diabetes is related to a defect of glucose transport across the blood-brain barrier (BBB). The work in this chapter examines this possibility.

Certain patients with diabetes claim to suffer from symptomatic hypoglycaemia when the plasma glucose is within the normal range (Harris and Prout, 1970). To account for this, it has been suggested that these patients have a defect in glucose transport across the BBB such that they experience central glucose depletion in the presence of normoglycaemia (Gjedde and Crone, 1981). This theory has also been proposed as a cause of GH hypersecretion in diabetes since lack of glucose transport into the brain would lead to neuroglycopaenia in regulatory centres and consequent GH release (DeFronzo et al., 1980). In this chapter, hexose transport across the BBB in diabetic subjects, including those with severe microvascular disease, is examined using positron emission tomography (Brooks et al., 1986a).

6.1 Positron emission tomography

The recent development of the positron camera, coupled with emission computerised axial tomography, has seen the birth of an extremely powerful research tool in the form of positron emission tomography (PET). While imaging in medicine is a rapidly advancing field, and other techniques such as nuclear magnetic resonance may eventually prove superior, at present, PET offers several advantages over single photon imaging (Jones, 1982).

Firstly, it allows accurate measurement of tissue isotope tracer concentrations and their distribution in the body. Thus, a positron emitting isotope can be coupled to a naturally occurring biochemical substrate, and its progress accurately followed and quantitated throughout its metabolic pathways. In the instance of glucose transport in the brain, while tracer dilution techniques allowed the measurement of whole brain glucose uptake, using PET, glucose uptake in particular brain areas of interest can be measured. Further, this information can be converted into visual images. Secondly, the use of positron emitting isotopes has greatly enhanced the range of isotopic tracers at our disposal (Comar et al., 1982). Isotopic tracers used in medicine have to fulfill several narrow criteria. For instance, they should have a suitably short half-life, and tissue damage should not out-weigh the benefit of the test. Further, a good radionuclide should be an isotope of the component elements entering into the composition of the biological tissue of interest. The radioisotopes generally used do not usually meet this criterion. For instance, ^{99m}Tc, which is used in 80% of tests in nuclear medicine, has ideal physical characteristics but is not a good biochemical tracer since its presence can modify the chemical nature, and hence the metabolism of, a labelled organic substrate. In attempts to provide radionuclides which better fulfill the needs of modern medicine, the positron emitters occupy a prominent position. For instance, it is possible to produce positron emitting isotopes of carbon, nitrogen, oxygen and fluorine, all of which produce ideal amounts of energy and which have short half-lives. The half-life of ^{11}C is 20.4 minutes. The short half-life of the positron emitting isotopes, while desirable, limits the use of PET to centres which have

facilities, such as a cyclotron unit, capable of producing these isotopes on site and incorporating them into the substrate. Hence the confinement of PET to research centres at present.

In basic terms, PET relies on the energy released when a positron combines with an electron. ^{11}C for instance, has 5 neutrons and 6 protons, as opposed to 6 of each in the stable carbon isotope. To achieve stability, ^{11}C emits a positron which travels a short distance, combines with an electron, and produces annihilation energy in the form of 2 photons of 511keV each which travel in nearly opposite directions. It is this energy which is measured by coincidence detection, resulting in high spatial resolution and detection efficiency.

To study glucose transport using PET, ^{11}C glucose was first used, but because of its rapid metabolism to $^{11}CO_2$, this molecule was not the ideal radiopharmaceutical to measure glucose metabolism in vitro. The deoxyglucose analogue, labelled with ^{11}C or ^{18}F was found to be superior since it enters the cell and is trapped as deoxyglucose-6-phosphate which cannot go further into the catabolic chain of glucose. 3-O-methyl-D-glucose (MeG) is an attractive form of glucose for measuring hexose transport across the BBB since the affinity of MeG and D-glucose for D-hexose carriers have been found to be very similar in rat brain (Partridge and Olendorf, 1975). It has recently been demonstrated that MeG is also phosphorylated by the myocardium (Gatley et al., 1984). Work from the MRC Cyclotron Unit at the Hammersmith Hospital has suggested that this also occurs in human brain tissue, and that the use of ^{11}C labelled MeG and PET could be used to study D-glucose transport across the BBB in man (Brooks et al., 1986b). This methodology was used in the following study to measure glucose transport across the BBB in diabetic subjects.

6.2 PATIENTS AND METHODS

Five diabetic subjects, 3 with microvascular complications and 2 without, took part in 8 PET studies. The clinical details of these subjects is given in table 6.1. Subject 3 demonstrated the phenomenon of 'relative hypoglycaemia', with symptoms of hypoglycaemia in the presence of a normal plasma glucose. These symptoms are relieved by glucose. Fearful of hypoglycaemia, the patient keeps her insulin dosage low, resulting in poor glycaemic control as evidenced by the high HbA1.

Four normal, normotensive subjects, aged between 23 and 65 years, with normal glucose tolerance, acted as controls.

tracer preparation

[^{11}C]MeG was prepared as follows :- the sodium salt of commercially available diacetone-D-glucose was prepared by reacting this substrate with sodium hydride in diethyl ether. This salt was methylated in the 3 position with $^{11}CH_3I$ and then hydrolysed to [^{11}C]MeG with HCl. Synthesis time, including purification time using high pressure liquid chromatography, was approximately 50 minutes. Yields of up to 12.5mCi were obtained in injectable solution. The absence of impurities after chromatography was confirmed by preparing cold MeG using the same synthetic route and comparing its proton nuclear magnetic resonance spectrum with that of commercially available compound.

scanning procedures

Subjects were scanned at an axial level of 6.5 cm above the orbitomeatal line. All subjects had an initial transmission scan using an external ^{68}Ge ring source so that tissue attenuation of 511 keV radiation could be determined (Frackowiak et al., 1980).

Table 6.1

clinical characteristics of the diabetic study subjects.

patient number	sex (m/f)	age (yrs)	duration of diabetes (yrs)	diabetic complications	HbA1 (%)	insulin dose (U)
1	m	44	5	background retinopathy, neuropathy	12.5	48
2	m	40	18	proliferative retinopathy	9.0	62
3	f	38	23	proliferative retinopathy, neuropathy	16.3	34
4	m	28	9	none	9.4	48
5	m	23	16	none	9.3	50

Regional cerebral blood flow (rCBF) was then determined using steady state inhalation of $C^{15}O_2$ (Frakowiak et al., 1980).

After cerebral ^{15}O activity had cleared, [^{11}C]MeG was administered by intravenous infusion while serial PET scanning at the fixed tomographic level was performed. The [^{11}C]MeG was infused over 4 minutes using a Harvard pump. At the onset of infusion, PET scanning was started, ten 120 second and four 300 second scans being performed. During scanning, arterial blood samples were withdrawn, initially at 30 second intervals, and arterial plasma and whole blood [^{11}C]MeG levels were measured in a well counter cross-calibrated against the PET scanner.

Finally, regional cerebral blood volume (rCBV) was measured following $C^{15}O$ inhalation (Lammertsma et al., 1983).

A complete description of data analysis is given in Brooks et al., 1986b.

experimental conditions in the diabetic subjects

Of the 8 PET scans in the 5 diabetic subjects, 4 were carried out at euglycaemia with physiological insulin levels, 2 at hyperglycaemia with physiological insulin levels, and 2 at hyperglycaemia with no insulin. Three of the diabetic subjects had paired PET studies and 2 had single studies only. The 3 protocols are described below.

euglycaemia with insulin infusion :- patients attended the hospital on the afternoon of the study day, having omitted the long-acting component of their morning insulin dose, and having fasted for a minimum of 4 hours. A 19G cannula was inserted into a forearm vein of one arm for the purposes of insulin, dextrose and tracer infusion. An arterial line was inserted into the radial artery of the other arm for sampling purposes. An infusion

of short-acting insulin (Humulin S, Eli Lilly and Co. Ltd.) was commenced at a rate of 0.3mU/kg/min, and continued throughout the procedure. Arterial plasma glucose was measured at 5 minute intervals throughout, and was held constant at a level of approximately 5mmol/l with a variable infusion of 20% dextrose. A run-in period of at least 90 minutes was allowed in each subject prior to commencing the scanning procedures to allow a steady state in serum insulin and plasma glucose to prevail. This steady state was maintained throughout the scanning period.

hyperglycaemia with insulin infusion :- the procedure was carried out exactly as described above with the exception that the arterial plasma glucose was maintained at a target level of 12mmol/l with the dextrose infusion.

hyperglycaemia with no insulin infusion :- in the 2 subjects studied under this protocol, the arterial plasma glucose was elevated to approximately 12mmol/l with a bolus of 5% dextrose. No insulin was infused. The plasma glucose was monitored at 5 minute intervals throughout, but in neither case did the plasma glucose vary by more than 1 mmol/l from the target value of 12mmol/l.

In all of the above experiments, serum free insulin (Kuzuya et al., 1977) was measured at 20 minute intervals

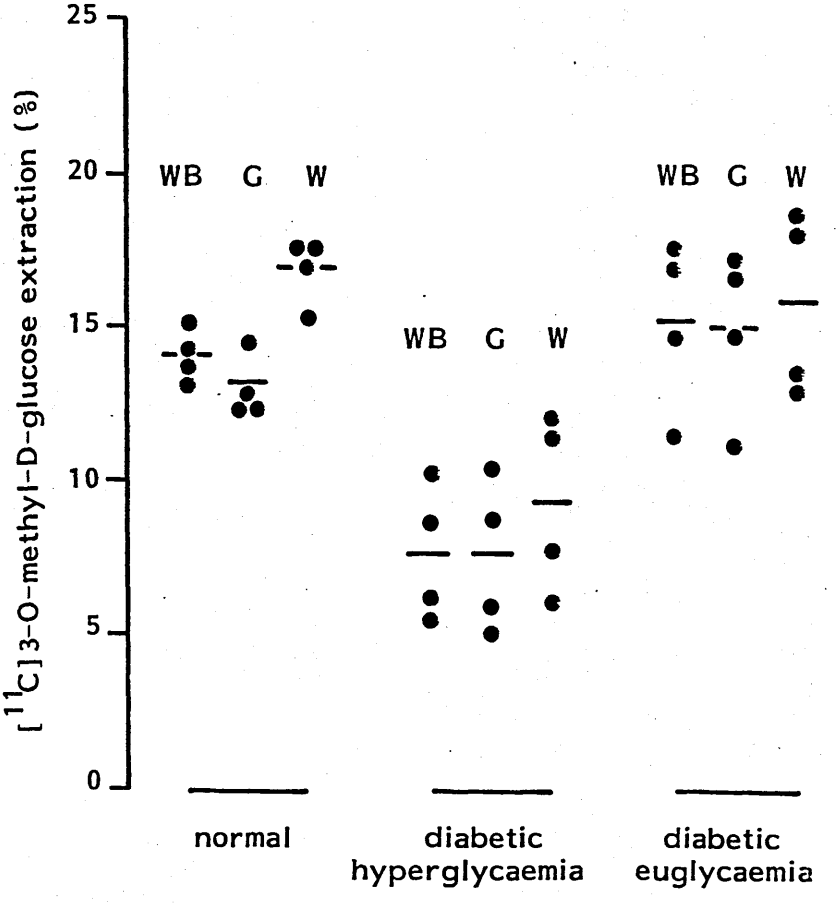
6.3 RESULTS

During the euglycaemic procedures, the plasma glucose was 3.8±0.9 mmol/l (mean±S.D.), and during the hyperglycaemic procedures 13.1±3.5 mmol/l. Insulin infusion rate 0.3mU/kg/min resulted in a mean serum free insulin level of 48±13mU/l in the 6 relevant scans. During the 2 scans where insulin was not infused, serum free insulin levels were <2mU/l.

Details of regional cerebral [^{11}C]MeG unidirectional extraction fractions are shown in figure 6.1 for each diabetic and normal subject. Whole brain, white and grey matter are shown separately. Mean values for the parameters of whole brain [^{11}C]MeG uptake are shown in table 6.2. Extraction fractions did not differ significantly between normal subjects and diabetic subjects studied at euglycaemia. In contrast, [^{11}C]MeG extraction was significantly reduced in the diabetic subjects studied at hyperglycaemia. However, when [^{11}C]MeG influx is measured in mmol/min, taking the plasma glucose into account (table 6.2), it can be seen that there was no significant difference between diabetic subjects studied at hyperglycaemia and normal subjects at euglycaemia. Lastly, there was no difference between extraction fractions in the diabetic subjects studied with and without insulin infusion.

6.4 DISCUSSION

The results presented here provide no evidence for the hypothesis that GH hypersecretion in diabetes is related to defective glucose transport across the BBB. D-glucose transport across the BBB is a passive facilitated process (Crone, 1965). The affinity of MeG and D-glucose for the D-hexose carrier is similar in rat brain (Partridge and Olendorf, 1975). The use of [^{11}C]MeG and PET has been validated as a means of studying D-glucose transport across the BBB in humans. Using this technique, the work in the present chapter demonstrates that in insulin treated diabetic subjects, even those with indifferent or poor glycaemic control, glucose transport across the BBB is normal. The subjects selected for the study included those with severe retinopathy. Since the BBB and the blood-retinal barrier are in continuity, it might be



Individual values for unidirectional regional cerebral [^{11}C]3-O-methyl-D-glucose extraction in diabetic patients at hyperglycaemia and euglycaemia and normal subjects. (WB, G and W denote whole brain, grey and white matter values respectively)

figure 6.1

table 6.2

mean parameters of whole brain [^{11}C]MeG uptake in diabetic and normal subjects.

	plasma glucose (mmol/l)	extraction (%)	rCBF (ml/100ml/min)	rCBV (ml/100ml)	influx (mmol/min)
normal subjects (n=4)	4.4±0.4	14.1±0.8	35±2	4.3±0.5	0.22
euglycaemic diabetics (n=4)	3.8±0.9	15.2±2.8	30±5	3.8±0.9	0.18
hyperglycaemic diabetics (n=4)	13.1±3.5	7.7±2.2	29±3	4.1±0.6	0.29

all data are expressed as mean±S.D.
rCBF regional cerebral blood flow
rCBV regional cerebral blood volume

expected that patients with proliferative retinopathy would also demonstrate some evidence of compromised BBB function, but this is certainly not the case with regard to glucose transport. Further, one subject demonstrated the phenomenon of 'relative hypoglycaemia' (Harris and Prout, 1970), but this individual did not differ from normal with respect to glucose transport across the BBB. It has been suggested that the BBB in diabetic subjects with microvascular disease is defective (Lorenzi et al., 1980). However, since the specialised function of glucose transport is unimpaired, it is hard to believe that other, cruder, functions are lost.

Several workers have studied glucose transport across the BBB in rats rendered diabetic by administration of streptozotocin (Gjedde and Crone, 1981, McCall et al., 1982, Di Mattio et al., 1984). The first two of these noted a significant reduction in BBB glucose transport in rats that had been hyperglycaemic for up to 4 weeks. This suggested that there had been a reduction in the number of carriers for passive facilitated transport of D-glucose across the BBB. This defect could be abolished by both treatment with insulin and reduction of hyperglycaemia by starvation, suggesting that chronic hyperglycaemia can lead to a loss of BBB glucose carriers. The present work indicates that this is not the case with insulin treated diabetics even in the presence of indifferent or poor glycaemic control.

This work also demonstrates that the brain regulates its glucose supply by adjusting glucose transport into the brain according to the prevalent plasma glucose concentration. Hyperglycaemia in the diabetic subjects resulted in a reduction in the percentage extraction of glucose from the blood, but in quantitative terms, the amount of glucose reaching the brain remained constant. An

interesting point is that this is not mediated by insulin since the presence or absence of insulin did not affect extraction. It has been claimed that the BBB in man is an insulin sensitive organ (Hertz et al., 1981). However, since this conclusion was based on work carried out at the time of cerebral angiography, and used plasma insulin levels in the region of 1500mU/l, the physiological relevance of the findings must remain in some doubt. Certainly the present work suggests that the BBB is not insulin sensitive.

Thus, there is no evidence for the existence central neuroglycopaenia in the presence of a normal plasma glucose in diabetes, and no evidence to suggest that excess GH is secreted in diabetic subjects due to glucose depletion in GH regulatory centres of the brain.

Chromatographic separation of circulating GH forms in diabetic subjects.

INTRODUCTION.

Examination of central regulation of GH secretion in diabetes in the previous chapters has identified a number of abnormalities, and excluded others. At circulatory level, another possible cause for GH hypersecretion in diabetes exists.

As outlined in chapter 1, GH is a heterogenous family of peptides, and represents an attractive target for chromatographic analysis. Some work has been carried out in normal subjects on the composition of the GH forms produced in the basal and stimulated state (Stolar et al., 1984) and during spontaneous secretory bursts (Baumann et al., 1985). GH forms produced by subjects with acromegaly have also been examined (Baumann et al., 1983). To date, circulating or pituitary forms of GH in diabetic subjects have totally escaped examination. This is a potentially interesting field, firstly in view of the fact that the 20k form of GH is said to lack the early insulin-like effects of other GH forms (Frigeri et al., 1979). Thus, if diabetic subjects produced an increased proportion of this form compared with normal subjects, it would obviously have implications for glycaemic control. Secondly, and of relevance here, if a bioinactive form of GH were produced by subjects with diabetes, this would provide some explanation for GH hypersecretion in the condition since GH levels would be elevated by negative feedback. There have been reports of bioinactive forms of GH being produced in children with growth failure and high GH levels (see section 1.7). Studies of prolactin, a peptide hormone with structure and characteristics similar to that of GH, has also revealed a

The following chapter describes work attempting to characterise circulating mass variants of GH in diabetic subjects.

7.1 Chromatographic methos of separating GH.

One of the best characterised chromatographic methods of separating GH forms is by means of Sephadex (Pharmacia, Sweden) gel chromatography. For the purposes of GH separation, this is carried out at pH 8-9, using one of the higher grade preparations (Sephadex 100-200). This method relies on the 'molecular sieving' effect of the gel. The resolution is relatively poor, but it is useful as a preparative method, separating monomeric from larger aggregated forms of GH. A representative profile of plasma GH separated on Sephadex is shown in chapter 1 (figure 1.2). This technique is not capable of separating the various monomeric forms, and would not distinguish between the 20 and 22k variants, for instance.

A variety of forms of gel electrophoresis, including native polyacrylamide gel electrophoresis (native PAGE), SDS-PAGE (where the substrate is coupled with sodium lauryl sulphate), and isoelectric focusing, have also been used to separate GH variants, and the results are well characterised (Skyler et al., 1977). Although SDS-PAGE is useful for molecular weight separations, native PAGE achieves adequate resolution of monomeric forms of GH, and was used in the following work. Polyacrylamide gel as a support medium for electrophoresis has the advantage that it is entirely synthetic and can be constructed reproducibly with differing pore sizes, allowing a combination of both molecular sieving and electrophoresis for molecular separation. Moreover, unlike starch and agar gels,

which carry a negative charge at neutral pH, polyacrylamide is entirely devoid of charged groups, which has led to its superseding other gel media. It has been extensively investigated with respect to GH separation, and will separate, among others, the 20, 22 and 24k varieties of GH.

The use of reverse phase high pressure liquid chromatography (HPLC) for the separation of GH variants has not been described. Only one reference appears in the literature of this technique being used in the study of GH, in which synthetic 22k human GH was seen to come off the column in a single peak (Kohr et al., 1982). There have been no reports of separation of plasma or pituitary GH by this means. Nevertheless, the method is not without its possibilities. The technique is a relative new-comer amongst chromatographic methods. The process of liquid chromatography involves the partitioning of a solute between a stationary and a mobile phase. Reverse phase chromatography can be compared with normal phase adsorption chromatography, which is performed on a polar stationary phase such as a silica gel, and uses a non-polar mobile phase. In normal phase chromatography, polar solutes are tightly adsorbed to the polar stationary phase and are therefore eluted later than non-polar solutes. Polar compounds can be eluted by increasing the polarity of the mobile phase. In reverse phase chromatography, as implied, the process is reversed. The stationary phase is non-polar and the mobile phase is polar. It is now the non-polar, or hydrophobic, interactions which determine the migration rate along the stationary phase, and, as one expects, polar solutes are eluted from the column earlier than non-polar solutes. Elution of a particular compound is effected by reducing the polarity of the aqueous mobile phase, usually by the addition of organic

chromatography such that it now holds a prominent place in routine laboratory practice both as a preparative and analytical method. There are now available a wide variety of commercially manufactured columns. These are made from steel, and tightly packed with a hydrocarbon of known chain length, different column packings having differing properties. By varying the column packing and the make-up of the mobile phase, high resolution of many compounds according to hydrophobicity can be achieved. The fact that the process is carried out at high pressure in a tightly packed column makes the process of separation very rapid. It is, however, very difficult to predict how any given compound will behave on reverse phase HPLC, and deciding on the best column type and mobile phase can involve a lot of trial and error.

7.2 preparation techniques

Many chromatographic media require the sample to be suitably prepared prior to loading onto the column. In the case of GH analysis by PAGE or HPLC, the peptide must be extracted from pituitary or serum and concentrated in the appropriate form. Pituitary GH has been most extensively studied since large amounts are present in the gland, whereas in studying plasma GH forms, large volumes of plasma must first be extracted and concentrated. There are obviously a large variety of preparatory techniques. Most commonly used to extract GH from plasma is immunoadsorbent chromatography, where antiserum to GH is adsorbed to an inert medium. The plasma is then run over this preparation, and the GH is bound by the antibody, and subsequently eluted with a suitable agent. This method requires

affinity which will allow binding of the GH from the plasma but which will also allow later dissociation of the antigen-antibody complex. The antiserum should also bind all forms GH equally so that none are lost in preparation. Apart from these theoretical difficulties, this method is extremely expensive, and was not feasible in a routine hormone assay laboratory. Therefore other methods had to be developed. In the following work, the use of the C18 SEP-PAK (Waters Associates, Massachusetts) and acid ethanol stripping was tried. The SEP-PAK is a small column filled with a hydrocarbon similar to that used in the HPLC column. The peptide is first adsorbed on to this and subsequently eluted with an organic solvent. This method has been found to be effective in extracting small peptides from plasma prior to HPLC. Acid-ethanol stripping simply precipitates a number of proteins from plasma leaving a relatively clean sample. This process was tried to see if GH remains in solution after treatment with acid-ethanol, and whether the resulting solution was suitable for chromatographic separation.

7.3 PATIENTS AND METHODS

Serum from diabetic subjects with proliferative retinopathy and documented GH hypersecretion, demonstrated on 24 hour sampling profiles, was studied. Serum was obtained from normal subjects who were age matched with the diabetic subjects and were selected from amongst hospital staff members. GH rich serum was obtained by intravenous administration of 100ug of growth hormone releasing factor (Sigma), with 20 ml samples of blood being drawn at times 15, 30, 37, 45 and 60 minutes. The blood was immediately separated and serum stored at -20^{o} C.

material was a gift from the National Institute for Biological Standards and Control (Holly Hill, Hampstead, London NW3 6RB), and synthetic 22k human GH was a gift from Eli Lilly and Co. Ltd..

extraction of plasma using the C18 SEP-PAK :- this was carried out according to the manufacturer's instructions. The SEP-PAK was first activated by pushing 5ml of 100% acetonitrile or propan-1-ol through the column, and then washed with 10ml of distilled water. The serum sample was then loaded onto the column which was then again washed with 10ml of distilled water. The GH was then eluted from the column over 5-15 minutes with 1.5ml of either 70% acetonitrile or 60% propan-1-ol. The eluent was evaporated to dryness under nitrogen and reconstituted to the appropriate volume with the relevant diluent at the time of sample analysis.

acid-ethanol stripping :- this was carried out according to the method of Daughaday et al. (1980). Serum was added to a mixture containing 12.5% 2N HCl and 87.5% absolute ethanol (1 part serum to 4 parts acid-ethanol). This was vortexed and left to stand at room temperature for 30-60 minutes. During this time, a cloudy precipitate formed, which was spun down by centrifugation for 30 minutes. The supernatant was removed and neutralised with 0.855M Tris-base (5 parts acid-ethanol stripped serum to 2 parts Tris-base). This mixture was left overnight at $4^{o}C$, during which time a second precipitate formed. Samples were again centrifuged for 30 minutes and the supernatant removed. The resulting clear sample was evaporated to dryness and reconstituted appropriately at the time of analysis.

Sephadex chromatography :- this was carried out in a cold room at

glass column. The sample consisted of either 1-2ml of unextracted serum or standard dissolved in 1ml of buffer. The column was run through with barbitone buffer, ph 8.9, using 2 markers, ^{125}I and bromophenol blue. The flow rate was approximately 6ml/hour, 100 2ml samples being collected. The void volume was marked by the appearance of bromophenol blue, and the total volume by the appearance of ^{125}I.

HPLC :- this was carried out on a 2 pump system, capable of generating a gradient of solvent (Waters Associates, Milford, Massachusetts), using a C-18 column with a pore size of 100um (HPLC Technology Ltd., UK). The mobile phase consisted of solution A (99.95% distilled water, 0.05% trifluoro-acetic acid (TFA)) and solution B (either propan-1-ol or acetonitrile, 99.95%, 0.05% TFA). The dried sample or standard was reconstituted in 1.5ml solution A and injected onto the column. A linear gradient of a combination of solutions A and B (increasing solution B by 1%/min) was then run down the column (flow rate 1ml/min to 70% in the case of acetonitrile and flow rate 0.5ml/min, to 50% and held at that level for a further 20 minutes in the case of propan-1-ol). Fractions were collected on a fraction collector (1ml in the case of acetonitrile and 0.5 ml in the case of propan-1-ol), and each fraction assayed for GH by RIA, the standard curve being made up in 60% acetonitrile or 50% propanol as appropriate.

native PAGE :- the lower, or resolving gel was made up from a mixture of 23.5g/100ml acrylamide (A) and 0.5g/100ml bisacrylamide (Bis) such that T (total percentage concentration of A + Bis) was equal to 24% (w/v) and C (Bis/T expressed as a

part of 36.4ml 1NHCl + 9.23g Bis/100ml, pH 5.8 and 1 part 60mg/100ml potassium persulphate, giving a final T value of 12%. Instigator NNN´N´ tetramethylethylenediamine (TD) was added (10ul/ml of gel), and the resultant mixture poured into 12cm by 4mm glass columns and allowed to set at a temperature of 4°C. The upper, or stacking gel was made with a T value of 6.25%, C value 20%, diluted 1:1 by adding 2 parts to 1 part acetic acid 1.25g + Bis 3.95g/100ml, pH 5.43 and 1 part potassium persulphate 60mg/100ml, giving a final T value of 3.125%. Instigator TD was added (10ul/ml of gel), and poured onto the top of the resolving gel such that the stacking gel was approximately 0.5cm in length. The columns were then placed into a Shandon Southern rod gel electrophoresis chamber. The lower buffer consisted of, per litre, 50ml 1NHCl and 13.08 g Bis, pH 6.9. The upper buffer consisted of, per litre, 9.16 g N-tris-aminoethane-sulphonic acid (TES), 7.36 g Bis, pH 6.9. The sample diluent consisted of a 1:6 dilution of the upper gel buffer, 9ml and 1ml of glycerol to which 0.2mg bromophenol blue and 0.1mg bovine serum albumin (fraction V) was added. The dried sample was reconstituted in 100-200ul of this mixture, and layered onto the top of the stacking gel. The columns were then connected to the power unit (Evans Electroselenium Ltd., Holstead, Essex), and a current of 2mA/gel applied. In general, the dye front took 3 hours to reach the bottom of the gel, after which the gel was stripped from the column, and sliced into 100 discs using a microtome. Each gel segment was soaked overnight in RIA buffer and subsequently assayed for GH.

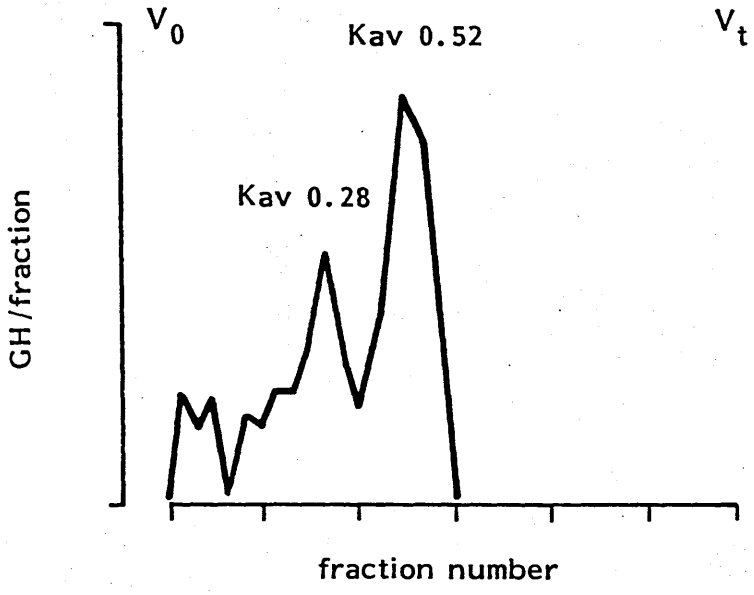
Sephadex chromatography :- both the synthetic 22k GH and the purified human pituitary GH extract, when run on Sephadex, came off the column in a single peak with a Kav of approximately 0.5, indicating that both standard preparations contain only monomeric GH. One serum sample from a diabetic patient with proliferative retinopathy was run on Sephadex (figure 7.1), producing a profile similar to that shown in chapter 1, figure 1.2.

sample recovery from the SEP-PAK :- using acetonitrile to elute the column, only 5% of GH immunoreactivity could be recovered from any given serum sample. Using propan-1-ol, this figure could be increased to approximately 50%.

sample recovery from acid-ethanol stripped serum :- using the method outlined above, 100% of GH immunoreactivity was retained in the clear supernatant. However, when this material was dried down, several samples pooled in order to obtain sufficiently large quantities of GH, and reconstituted in a small volume, the resulting mixture was relatively viscous.

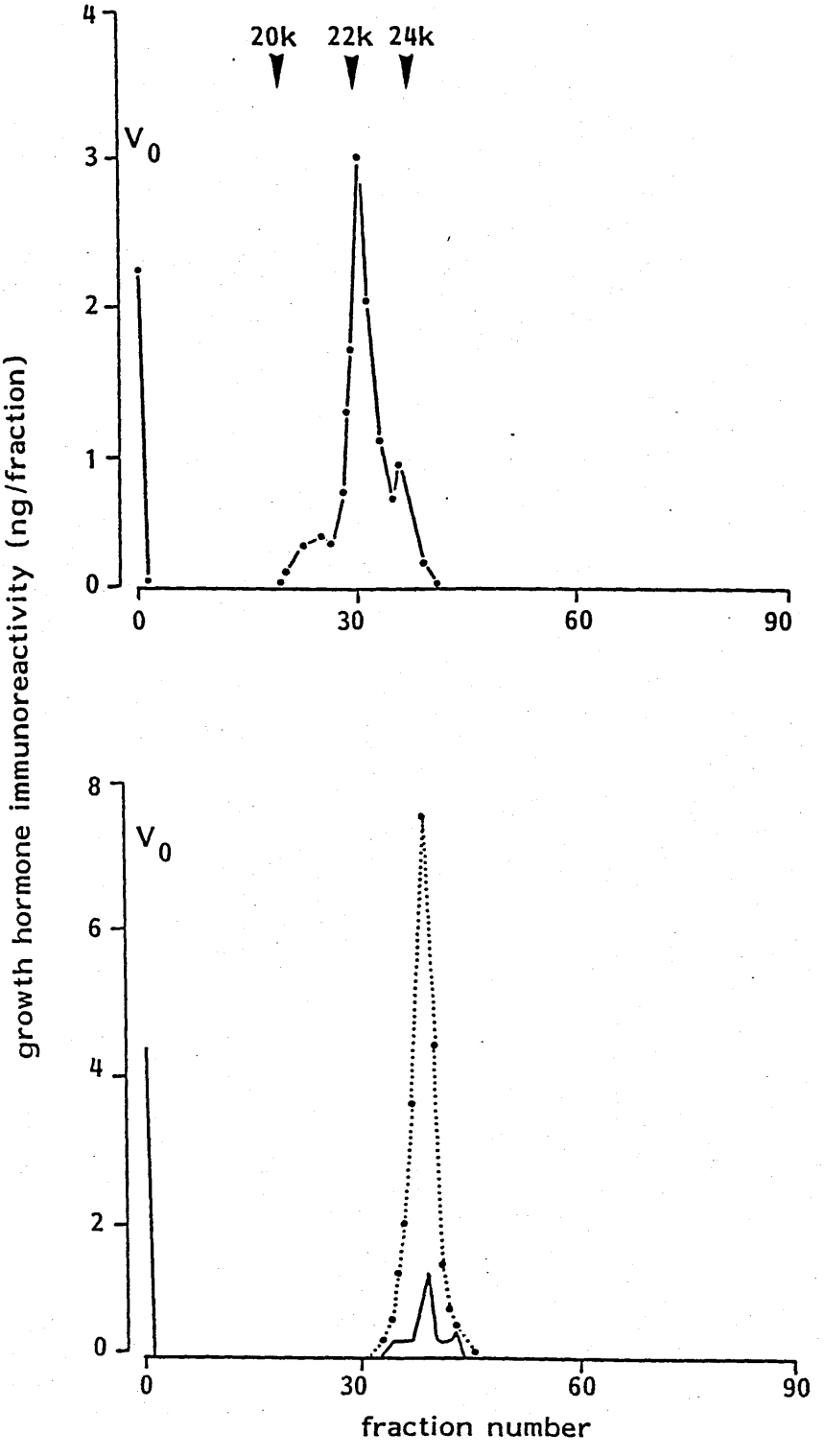
native PAGE :- synthetic 22k GH produced 1 clear peak on PAGE. Pituitary extract produced a major peak corresponding to that seen on the 22k GH profile, and a smaller earlier peak (figure 7.2).

Problems were experienced in attempting to run serum samples on PAGE. It was calculated that approximately 100uU of GH immunoreactivity would have to be run on each gel to produce meaningful results, given that 22k GH makes up 90-95% of circulating GH forms. This material has to be concentrated into a sample volume of 100-200ul. When acid-ethanol stripped serum is concentrated in this fashion, the resultant sample is of such



Profile of serum growth hormone separated on Sephdax G-100.

figure 7.1

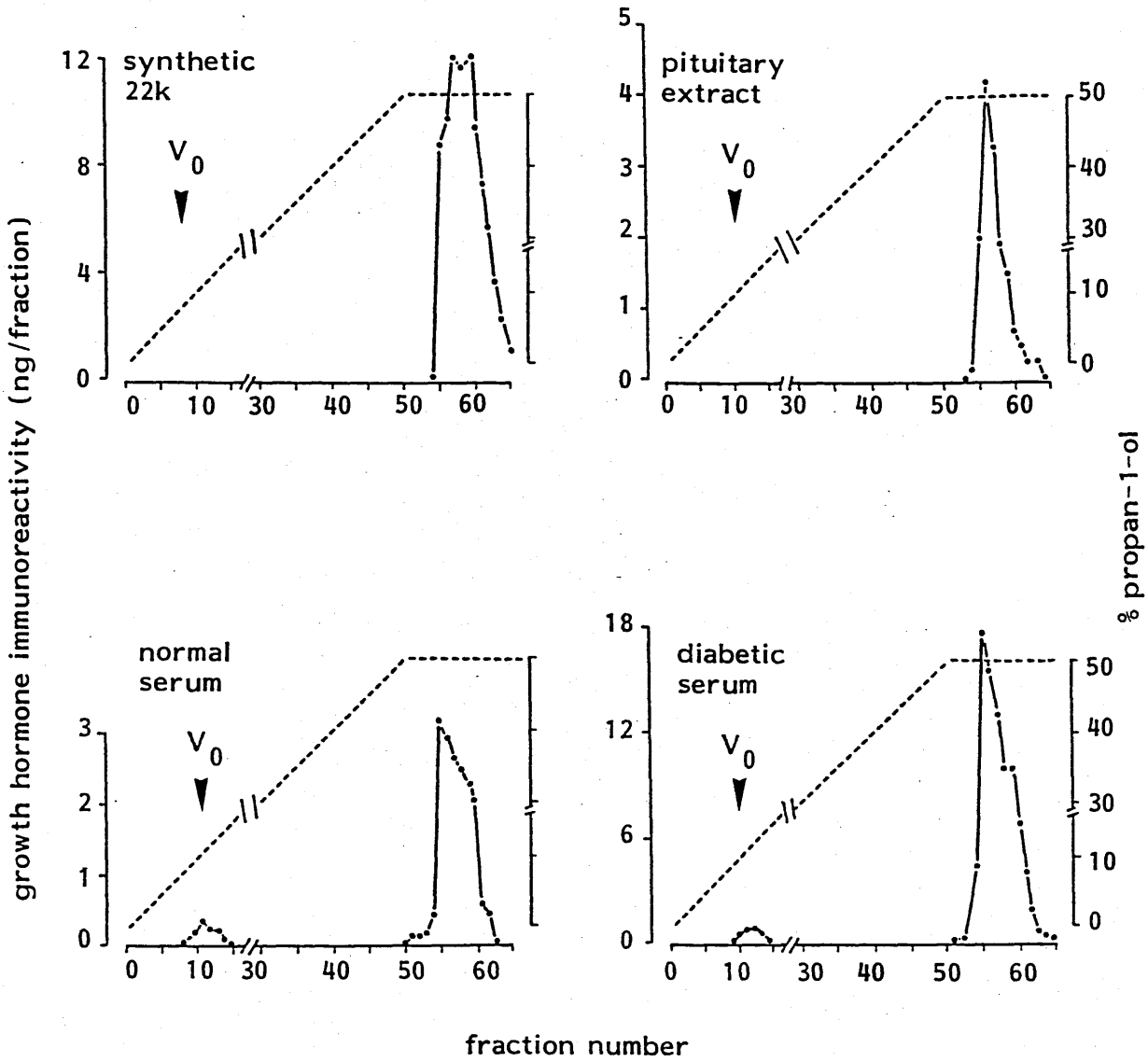


Composite native PAGE profile of plasma GH from 4 diabetic subjects with proliferative retinopathy (——) compared with pituitary extract (upper panel •—•) and synthetic 22k GH (•···•). The arrows in the upper panel represent the position of the GH forms (Stolar et al., 1984).

figure 7.2

The profile of diabetic serum extracted on a SEP-PAK eluted with propanol is shown in figure 7.2, but as the recovery from the SEP-PAK is poor, with further losses on the gel, the resultant peak is of small magnitude.

HPLC :- a practical advantage of using HPLC is that the sample volume can be up to 1.5ml, thus obviating the need to use highly concentrated samples. It was therefore possible to use acid-ethanol stripped serum, dried down and appropriately reconstituted, for the HPLC separation. Using this material, there was an early rise in the pressure in the system which quickly dropped once the void volume had been passed. Initial attempts were made to use acetonitrile as the mobile phase since this solvent has ideal viscosity characteristics, and the pressure in the system remains low. However, the GH immuno-reactivity did not come off the column until a concentration of 70% acetonitrile had been achieved, this concentration of solvent totally inhibiting binding in the GH RIA. It was therefore decided to try propan-1-ol in view of the findings of others (Kohr et al., 1982). Using this system it was found that the GH came of the column after 10 minutes at a propanol concentration of 50%. It was possible to assay the fractions in propanol since binding in the RIA was not significantly affected. However, the disadvantage of using propanol is that it is more viscous than acetonitrile, and it was necessary to reduce the flow rate to 0.5ml/min to keep the operating pressure within safe limits.
Using either acetonitrile or propanol, the GH activity in both standard preparations and serum from normal and diabetic subjects came off the column in a single peak (figure 7.3). The recovery



Representative profiles of synthetic 22k GH, pituitary extract, normal serum and serum from a diabetic subject with proliferative retinopathy run on HPLC with an increasing gradient of propan-1-ol (- -).

figure 7.3

had been stored for 6 months or more revealed an earlier peak which came off the column at about 40% propanol or 55% acetonitrile, but since this was not present in fresh samples, it was concluded to be an artifact of storage.

Since all GH immunoreactivity comes off the HPLC column in a single peak with excellent recovery of the loaded material, an attempt was made to use HPLC as a preparative step, first acid-ethanol stripping the serum, running this on HPLC, drying down the appropriate fractions and subsequently running these on PAGE. However, since the GH immunoreactivity comes off the HPLC column over 3-5 fractions of 0.5ml each, and the maximum volume which cam be loaded onto a PAGE gel is 200ul, it was not found possible to obtain good recovery of GH immunoreactivity from the dried HPLC fractions.

7.5 DISCUSSION

An attempt was made in this chapter to examine mass variants of GH in diabetic and normal serum. Only one sample diabetic serum was run on Sephadex, but as expected, this did not differ from normal. More importantly, the use of Sephadex demonstrated that both standard preparations consisted of only monomeric GH, thus simplifying subsequent analysis of results.

The use of HPLC appeared to be an attractive prospect since it is rapid, reproducible and allows a relatively large injection volume. This is particularly important since the greatest problem experienced was the extraction and concentration of GH from plasma. However, GH would appear to be an extremely hydrophobic molecule, and does not come off the column until very high organic solvent concentrations have been reached. Since the

monomeric GH of the standard preparations, it is probable that the low pH and high solvent conditions used in the HPLC system breaks down the aggregated forms of GH found in plasma. Of more importance is the fact that HPLC does not resolve the various forms of monomeric GH. It is possible that with extensive experimentation with the solid and mobile phases, the resolution could be improved, but the deathly hush in the literature regarding HPLC as a means of separating GH forms suggests that other workers have had no more success. However, the analysis of circulating GH forms using HPLC suggests that there is no major difference between diabetic and normal subjects since in both instances a single peak was produced.

The use of PAGE as a means of separating GH forms is better characterised. Comparing the PAGE profile of the pituitary extracted GH standard preparation with the results of others (Stolar et al., 1984, Baumann et al., 1985), it is apparent that this form of chromatography is capable of separating the 20 and 22k forms of GH. Analysis of serum demonstrated no difference between the profiles of normal and diabetic subjects. However, since PAGE is very fastidious in that the sample volume has to be small and highly concentrated, a very efficient extraction system has to be employed. The results obtained here are based on the use of SEP-PAK extraction which produces only 50% recovery from a loaded serum sample. With this recovery, it is not possible to say that some GH forms were not lost. To obtain a definitive answer as to whether GH forms are identical in normal and diabetic sera using PAGE, a better extraction procedure would need to be used. As outlined in section 7.2, immunoadsorbent chromatography has been successfully used, but was not possible

in the present work.

The results presented here have not demonstrated any difference in the nature of circulating GH in normal and diabetic subjects. This project has not been without its problems, but until one of the groups of workers specialising in analysis of GH forms undertakes to examine the question, it must be said that there is no evidence for an abnormal GH form or proportion of forms contributing to GH hypersecretion in diabetes.

CHAPTER 8

Insulin-like growth factor-I and diabetes

INTRODUCTION

In the previous chapter, GH was examined at circulatory level. With the measurement of insulin-like growth factor-I (IGF-I), it is possible to assess the effects of GH at tissue level.

IGF-I is the major circulating GH dependent growth factor in adult man. It is of potential interest in the present work for two reasons. Firstly, it represents a tissue response to GH and contributes to the feedback mechanism. If, as discussed in chapter 1 (section 1.12), IGF-I levels in diabetes are low, this would provide a possible cause for GH hypersecretion since GH levels would be increased by negative feedback at pituitary and hypothalamic level. Secondly, since GH has been proposed as a permissive factor in the genesis of diabetic microvascular complications, information on levels of IGF-I in diabetic subjects with microvascular disease is of importance in attempting to verify the hypothesis.

In the following chapter, the results of measurements of IGF-I in blood and vitreous of diabetic subjects with retinopathy will be described, and their relevance discussed.

8.1 Radioimmunoassay for IGF-I

Radioimmunoassay (RIA) of IGF-I is a recent development since pure forms of the peptide are required for labelling and to define a standard curve for quantification of results. Initially, its use was limited to laboratories which had the facilities to isolate IGF-I from serum, but since this peptide is now made synthetically and is commercially available, the RIA is becoming increasingly available to clinicians. Preliminary work has shown

that accurate measurement of IGF-I is of practical use in the assessment of acromegaly and growth failure, and it is likely to become a routine measurement in clinical endocrinology. The assay itself is straightforward, but the one unusual feature of IGF-I is that in the circulation, it is almost totally bound to its binding protein. The relevance of the bound and free components is unclear, but all RIAs to date measure total IGF-I which seems to provide clinically meaningful results. Certain antisera to IGF-I are capable of binding the peptide without prior stripping of the binding protein, but the majority require prior separation of IGF-I from from its binding protein by treatment with acid.

The antiserum used in the present work was a gift provided by Dr R.C.Baxter (Sydney, Australia) and distributed through the National Hormone and Pituitary Program (University of Maryland). It is a monoclonal antiserum, and its specificity has been fully characterised (Baxter et al., 1982). It has a high degree of specifity for IGF-I, little or no crossreactivity with human insulin or C-peptide, and 7% crossreactivity with IGF-II. Its use requires the prior removal of IGF-I binding protein, and this was carried out using acid-ethanol stripping (Daughaday et al., 1980). The standard curve was made up using the IGF-I recombinant analogue (Amersham International plc, Buckinghamshire, England), which differs from the native peptide only by the substitution of threonine for methionine in position 59. The label was made from the same material iodinated with chloramine-T. Since the assay of serum samples was carried out in neutralised acid-ethanol, the standard curve was made up in similar salt and ethanol concentrations. Using this system, binding of 35% was routinely obtained. The acid-ethanol stripping procedure results in a 1:7 dilution of the original sample. Measuring this material

directly, the sensitivity of the assay expressed as a concentration was 80ng/ml, assessed by the measurement of 20 'zero' tubes, and taking the lower limit of sensitivity of the assay as 2 standard deviations from the zero value. Lower values, such as those found in vitreous, could be measured by drying down the acid-ethanol and reconstituting the sample in its original or smaller volume. The upper limit of the assay, measuring samples in acid-ethanol, was approximately 5000ng/ml. Since patients with active acromegaly have values of 700-1000ng/ml, further dilution of samples was never required. The recommended assay volume was 10ul, but using this volume, adult values for IGF-I fell on the upper part of the standard curve. Therefore, while the assay volume for the standard curve was kept at 10ul, the sample volume was increased to 20ul to put adult values on the most sensitive part of the curve. Within and between assay coefficient of variation was assessed by the use of 2 standard samples, a normal adult with a value of approximately 200ng/ml, and an untreated acromegalic with a value of 700ng/ml. At the dose level of 200ng/ml, the intra and inter assay coefficients of variation were 5 and 10% respectively. The details of the IGF-I assay and iodination of the analogue are given in appendices B and C.

It is common to express values for IGF-I in Units/ml, 1 Unit being the concentration of IGF-I in pooled serum. However, there is as yet no standardisation, and in this work, results are expressed in ng/ml as read from the standard curve.

8.2 Serum levels of IGF-I in patients with varying degrees of retinopathy

8.2.1 PATIENTS AND METHODS

Clinical characteristics of the 61 normal and 121 diabetic study subjects are given in table 8.1. The normal subjects were chosen from amongst staff members. The diabetic subjects were selected from the Diabetic Retinopathy Clinic. The only selection criteria were that the study subjects should not have renal failure, as indicated by an elevated serum creatinine level, and that the diabetic subjects should have some degree of retinopathy. No distinction was made between subjects with insulin-dependent and non-insulin-dependent diabetes. Broadly similar numbers were included within each decade over the age range 20-60 years. Using direct and indirect ophthalmoscopy and colour photography, the diabetic subjects were divided into those with background retinopathy, those with previously proliferative but now quiescent retinopathy, and those with new vessels (proliferative retinopathy) at the time of blood sampling. Patients in the latter 2 groups had had or were undergoing photocoagulation at the time of the study.

Four patients with active proliferative retinopathy and 2 normal subjects were admitted to hospital for 24 hour profiles of GH and IGF-I (see chapter 3). The purpose of this was to assess the variability of serum IGF-I in patients with GH hypersecretion to ascertain the validity of measuring a single sample as representative of 24 hour IGF-I levels in such patients.

All other subjects had a single non-fasting sample taken for measurement of glycosylated haemoglobin A1 (HbA1) and serum IGF-I.

All statistical calculations on levels of IGF-I were carried out

TABLE 8.1

CLINICAL CHARACTERISTICS OF STUDY SUBJECTS

	number of subjects	age (years)	body mass index	HbA1 (%)
Normal subjects	61	37.1±12.6	22.9±2.4	5-8
Diabetic Subjects	121	41.1±11.8	25.8±4.0	9.9±2.4
background retinopathy	47	39.7±12.3	25.9±4.1	9.9±2.8
previous proliferative	40	42.8±10.9	26.2±4.5	10.2±2.1
active proliferative	34	42.1±11.9	25.2±3.0	9.6±2.2

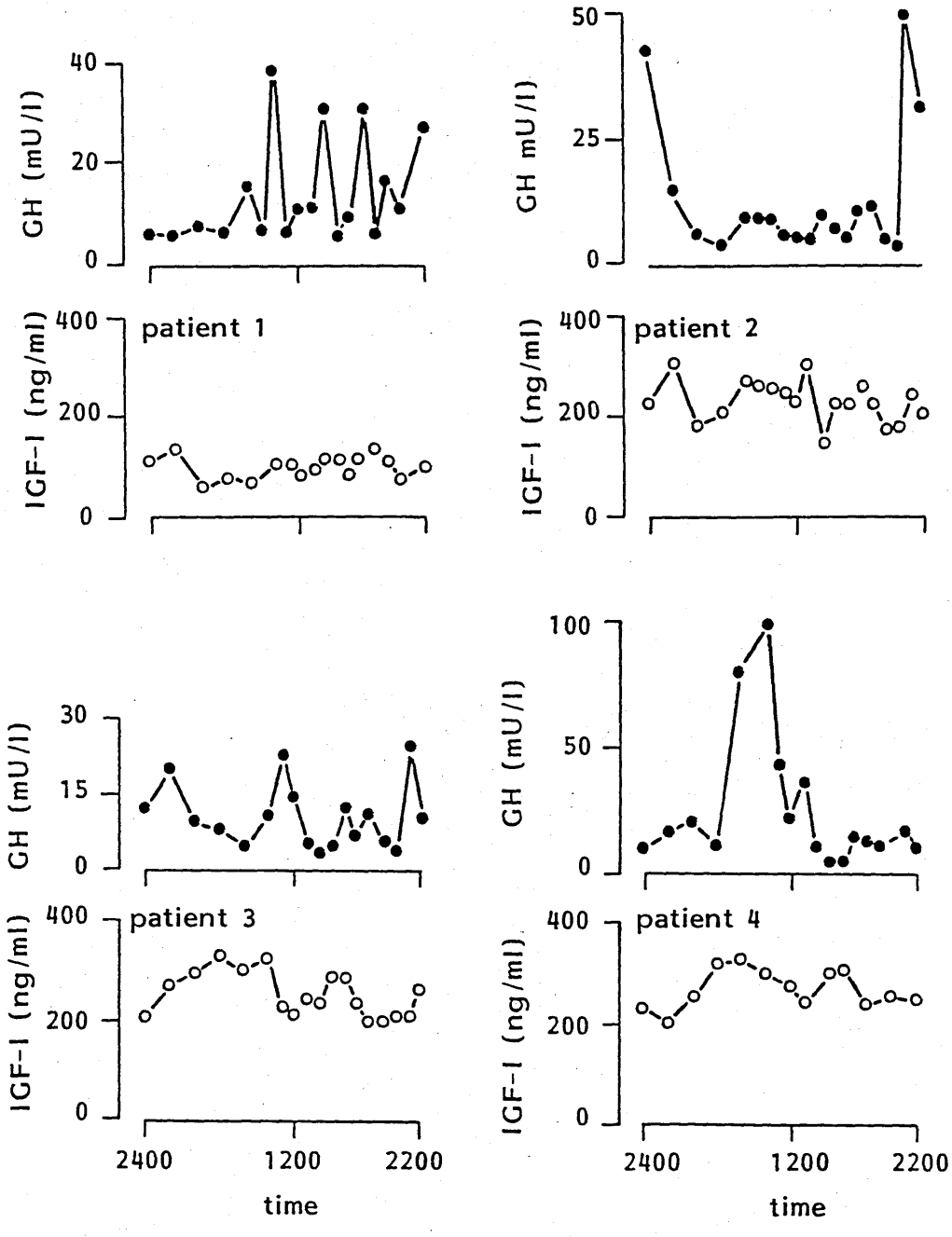
all data are expressed as mean ± S.D.

on log transformed data. Correlations were carried out using stepwise multiple regression analysis. Comparisons between multiple groups were carried out using one way analysis of variance. Comparisons between 2 groups were carried out using a two-tailed unpaired t-test. Body mass index (BMI) (Bennett, 1979) was calculated according to the formula weight (kg)/height $(m)^2$. All data are quoted as mean±S.E.M. unless otherwise stated.

8.2.2 RESULTS

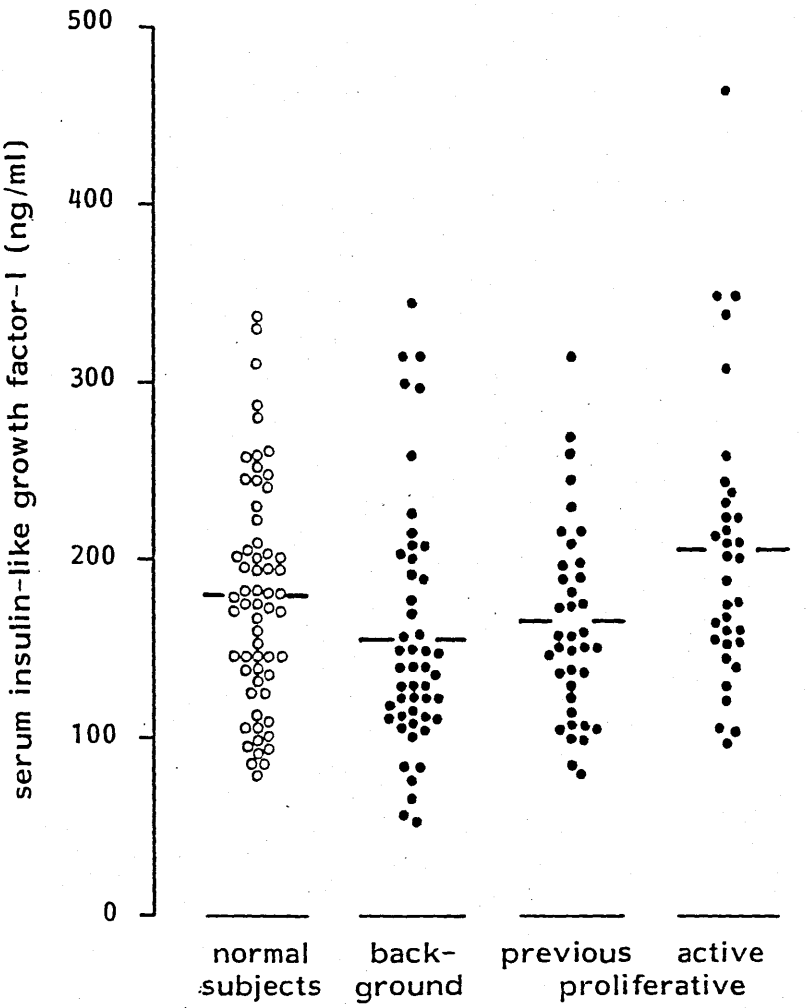
Of the 4 patients admitted for 24 hour profiles of serum GH and IGF-I the mean area under the 24 hour GH curve was elevated (284±24mU/l.hr, normal subjects 114 and 149mU/l.hr). Despite a mean 24 hour coefficient of variation (CV) for serum GH of 84% in the diabetic subjects, the mean CV of the IGF-I values was 19.7% (normal 15.9%) (figure 8.1).

The mean IGF-I level in the normal and diabetic subjects did not differ significantly (180 ± 8 vs. 173 ± 6 ng/ml, NS). A scatterplot of individual values of normal subjects and diabetic subjects divided according to retinopathy grade is shown in figure 8.2. Mean IGF-I in subjects with background retinopathy was 156 ± 10 ng/ml, in those with previously proliferative retinopathy 166 ± 9 ng/ml, and in those with active proliferative retinopathy 206 ± 14 ng/ml. Analysis of variance revealed a significant difference between IGF-I values in the 3 diabetic groups (p=0.003), but no significant difference in age or HbA1. Pooling IGF-I values for those with background and previously proliferative retinopathy, and comparing values with those with active proliferative retinopathy showed the active group to be significantly higher (206±14 vs. 161±7 ng/ml, p<0.001). Those



24 hour profiles of serum GH (closed circles) and IGF-I in four patients with proliferative retinopathy. The mean coefficient of variation (CV) of serum GH was 80% while that of IGF-I was 19%.

figure 8.1



Scatterplot of individual IGF-I values in the normal subjects (open circles) and the diabetic subjects divided according to retinopathy type. The bar represents the mean for each group.

figure 8.2

with active retinopathy also had a higher mean IGF-I than the normal subjects ($p<0.05$).

On further analysis of data from the normal subjects by stepwise multiple regression analysis, with IGF-I as the dependent variable versus BMI and age, only age entered into the equation ($r = -0.52$, $p<0.001$). Similar analysis of data from the diabetic subjects, with IGF-I as the dependent variable and entering age, HbA1, BMI and retinopathy type, retinopathy type ($r = 0.32$, $p<0.001$) and HbA1 ($r = -0.31$, $p<0.001$) entered the equation with a similar contribution. The correlation with age was much weaker than that of the normal subjects ($r = -0.18$, $p=0.05$). The total r for these variables was 0.45. As with the normal subjects, BMI did not enter the equation.

8.3 measurement of IGF-I in vitreous

8.3.1 PATIENTS AND METHODS

Seven patients with proliferative retinopathy, mean age 44 years (range 20-63 years) and 3 control subjects, mean age 37 years (range 11-50 years) were included in the study. The group with proliferative retinopathy all had active new vessels and were undergoing vitrectomy for recurrent vitreous haemorrhage or to divide fibrous retinitis proliferans. Of the control subjects, one had diabetes with an exudative retinopathy, and was undergoing vitrectomy to divide fibrous tissue. The other two were non-diabetics with rhegmatogenous retinal detachment.

At the time of operation, a vitreous sample was taken from each subject. This was immediately frozen at -20^0C. Samples were submitted to acid-ethanol stripping prior to assay as above. The resultant material was dried down, and reconstituted in its original volume in assay buffer. IGF-I was measured by RIA as

outlined above and detailed in appendix B.

8.3.2 RESULTS

The mean IGF-I value in the vitreous of the patients with proliferative retinopathy was 46.7 ±3.3 ng/ml (mean±S.E.M.), and that of the control group 44.9±9.3 ng/ml (N.S.).

8.4 Serial IGF-I measurements in a diabetic subject with rapidly worsening retinopathy.

A 25 year old female with insulin dependent diabetes of 15 years duration and a history of non-compliance with her insulin therapy, was referred to the Diabetic Retinopathy Clinic at the Hammersmith Hospital. Her glycosylated haemoglobin (HbAl) one month prior to referral was 16% (normal range 5-8%), indicating very poor glycaemic control. Diabetic retinopathy was noted, and on learning of this, the patient improved her diabetic control by regularly taking her insulin and home monitoring of her blood glucose with appropriate adjustment of insulin dosage. At the time of her first visit to the Retinopathy Clinic one month later, her HbAl had improved to 5.2%. Ophthalmological examination revealed early background retinopathy in both eyes. Routine measurement of her serum IGF-I revealed a value of 270ng/ml (normal range 80-300ng/ml) When seen 3 months later, her glycaemic control remained excellent. Her retinopathy, however, had deteriorated and she now had pre-proliferative changes with multiple cotton wool spots and intra-retinal microvascular abnomalities. Her serum IGF-I had risen to 350ng/ml. She has been followed monthly since that time, and 5 months after the discovery of background retinopathy, has developed new vessels requiring photocoagulation. Her serum IGF-I has stabilised at

227ng/ml. Changes in HbA1 and IGF-I are outlined in table 8.2. All IGF-I values were measured in the same assay (within assay coefficient of variation 5.3%).

table 8.2

Changes in glycosylated haemoglobin and IGF-I

	prior to referral	at time of referral	3 months	4 months	5 months	6 months
HbA1 (%)	16	5.2	6.6	6.7	8.3	10.0
IGF-I (ng/ml)	-	270	350	196	227	227

8.5 DISCUSSION.

The results presented in this chapter confirm that serum levels of IGF-I are normal in diabetic subjects as a group. However, within the group, there appear to be several factors operating. A number of studies have suggested that serum IGF-I levels in patients with proliferative retinopathy are higher than in patients with inactive retinopathy (Merimee et al., 1983, Ashton et al., 1983), but this finding was not confirmed by others (Lamberton et al., 1984). The results presented here are in agreement with those who have found elevated IGF-I levels in diabetic patients with proliferative retinopathy. This observation has prompted some to suggest that IGF-I and hence GH are involved in the genesis of proliferation, but this is taking the arguement too far too quickly. Elevated serum levels of IGF-I could be the result of, as much as the cause of proliferation. The fact that the subjects with previously proliferative but now quiescent retinopathy have normal levels of IGF-I, while of interest, does not resolve the question since this observation could mean one of two things. It could imply that the stimulus to

proliferation, perhaps elevated IGF-I, is no longer present, and hence the lack of activity in the eye. However, it could also mean that elevated levels of IGF-I are produced by the proliferating vasculature of the diseased eye itself, returning to normal with treatment by photocoagulation. This is possible in view of the autocrine or paracrine model proposed for IGF-I production (Underwood et al., 1986).

Attempts have been made to measure IGF-I levels in the eye more directly. Vitreous is a convenient ocular fluid in which to measure potential vasculogenic factors, but the significance of results obtained are difficult to interpret. In the present studies, we did not find increased levels of IGF-I in the vitreous of diabetic patients with proliferative disease. There has, however, been a report to the contrary (Grant et al., 1986). This suggests that in some patients with proliferative retinopathy, blood, and hence IGF-I, leaks into the vitreous, but whether this is of significance is not clear.

An important question to be answered prior to assigning a role to IGF-I in the development of neovascularisation is whether the rise in IGF-I antedates the onset of vascular proliferation. The case report in this chapter suggests that this is true since the patient's IGF-I levels started rising while she had only background retinopathy, but the sudden improvement in glycaemic control is a complicating factor. Sudden improvement in glycaemic control is known to cause deterioration in retinopathy (Kroc Collaborative Study, 1979) and is also known to cause a rise in IGF-I (Tamborlane et al., 1981). Whether the rise in IGF-I and deterioration in retinopathy are related cannot be stated with certainty. A prospective study, involving serial measurement of IGF-I in patients with background retinopathy, is required to see

if patients who go on to develop proliferative retinopathy demonstrate a prior rise in IGF-I.

There are a number of correlations of interest within the data on serum measurements of IGF-I. The well recognised negative correlation between age and serum IGF-I levels is seen, but is less marked in the diabetic patients. This presumably arises since other confounding factors are present in the diabetics. As discussed above, there is a correlation with activity of retinopathy. Of particular interest is the negative correlation between glycaemic control, as assessed by HbA1, and IGF-I. This has been described in some studies (Winter et al., 1979, Blethen et al., 1981, Amiel et al., 1984, Tan and Baxter, 1986) but was not observed by others (Merimee et al. 1983, Merimee et al., 1984). However, although a weak correlation, it has been described sufficiently frequently to suggest that poor glycaemic control leads to a failure of IGF-I generation in response to GH as discussed in chapter 1 (section 1.12). This may be relevant to the cause of GH hypersecretion in diabetic subjects since such a defect would lead to a rise in GH by negative feedback. That this may be the case is suggested by the findings of Tamborlane et al. (1981) who found that improvement in glycaemic control in children and young adults resulted in a rise in serum IGF-I but a fall in GH.

In conclusion, the results presented here demonstrate that IGF-I levels are elevated in patients with proliferative retinopathy, but the significance remains to be assessed. Evidence is presented to suggest that poor glycaemic control leads to a failure of IGF-I production which may contribute to elevation of GH levels in diabetes.

CHAPTER 9

A trial of long-acting somatostatin analogue SMS 201-995 in patients with proliferative retinopathy.

INTRODUCTION.

A final answer to the question of whether GH and/or IGF-I play a role in the genesis of diabetic microvascular disease will only be achieved by selective suppression of GH in diabetic patients with assessment of parameters relevant to microvascular disease. Early attempts to use somatostatin for this purpose (Lundbaek and Hansen, 1980), although promising, were not therapeutically feasible because of the short half life of this peptide which therefore had to be given by continuous infusion. Recently, a long-acting analogue of somatostatin has been developed (SMS 201-995, Sandoz). This is an octapeptide which has a biological half-life of about 3 hours (Bauer et al., 1982). It has been shown to be about 20 times more potent than native somatostatin in inhibiting GH secretion. In this chapter, the results of a pilot study to assess the feasibility of GH and IGF-I suppression in patients with proliferative retinopathy using this agent are reported.

9.1 PATIENTS AND METHODS

Four patients with insulin dependent diabetes and proliferative retinopathy which had not responded adequately to photocoagulation were selected for study. Serum creatinine was within the normal range although one subject had proteinuria. Clinical details are given in table 9.1.

All subjects were admitted to the Metabolic Unit at the Hammersmith Hospital for baseline studies and were given a full medical examination to ensure good general health. They were also

Table 9.1.

clinical characteristics of the study subjects

Patient	sex (m/f)	age (yrs)	body mass index	duration of diabetes (years)	proteinuria (yes/no)	initial HbA1 (%)
1	f	20	22.6	19	no	13.4
2	m	26	23.6	19	yes	12.5
3	f	45	23.3	30	no	11.3
4	f	30	26.2	20	no	9.6

body mass index = weight (kg)/height $(m)^2$

examined ophthalmologically, including colour retinal photography and fluorescein angiography. Blood was taken for measurement of glycosylated haemoglobin A1 (HbA1). An indwelling catheter was inserted into a forearm vein and blood taken for measurement of serum GH at hourly intervals from 0800 to 2200 and at 2 hourly intervals from midnight to 0600 the following morning. Blood for measurement of IGF-I was taken at 6 hourly intervals. Two normal subjects (male age 31 and female age 30) were also studied in a similar fashion for comparison. Patients were then commenced on a low dose of SMS 201-995 three times daily by subcutaneous injection. This dose was maintained for 3 days, and the 24 hour blood sampling repeated. The patient was instructed on self-administration and discharged home. Telephone contact was maintained and follow-up varied from 1-6 weeks. During that time, the patients were asked to gradually increase the dose of SMS 201-995 to the next dose level, which depended upon the results of the previous 24 hour GH profile and the patient's tolerance of the therapy. The 24 hour profile was repeated at each dose level.

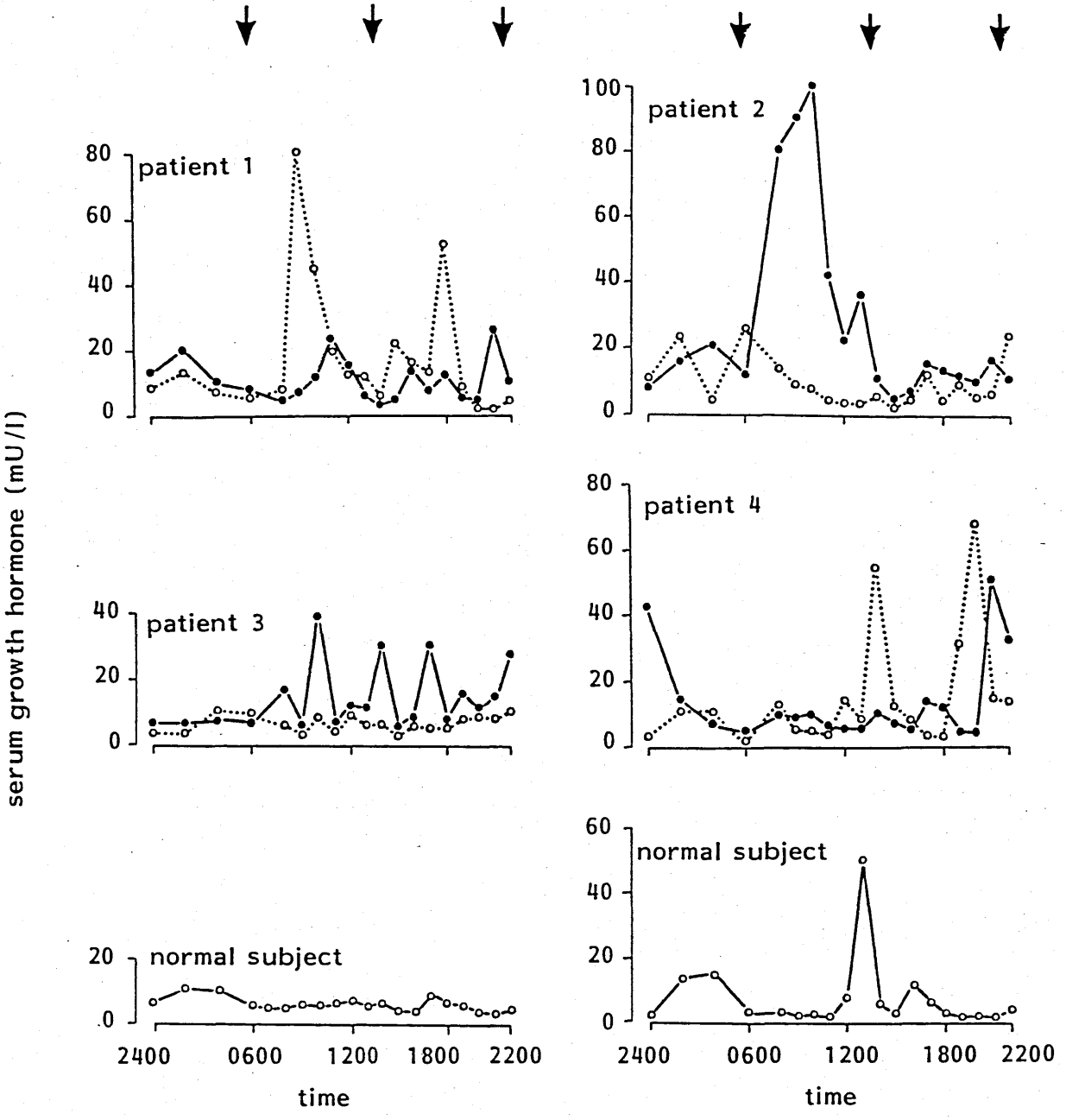
9.2 RESULTS

The response to SMS 201-995 is summarised in table 9.2. Patient 1 was commenced on a dose of 10ug thrice daily, increasing to 25, 50, 100, 200 and 500ug thrice daily. The lack of response or untoward effects in this subject prompted a more rapid increase in dosage in subsequent study subjects. Patient 2 was started on 25ug thrice daily, and the dose increased to 50, 100 and 200ug thrice daily. The patient defaulted from the study at this stage. Patients 3 and 4 were commenced on a dose of 50ug thrice daily, which dose was increased to 200 and 500ug thrice daily.

Table 9.2.

Principle results prior to and after stated doses of SMS 201-995.

dosage of SMS 201-995	daily insulin dosage (Units)	HbA1 (%)	24 hour GH (area under curve) (mU/l.hr)	mean IGF-I ng/ml)
Patient 1				
basal	58	13.4	206.2	233.0
200ug 3x daily	55	10.5	212.9	240.0
500ug 3x daily	45	12.0	283.7	193.4
Patient 2				
basal	68	12.5	428.3	271.0
200ug 3x daily	68	12.1	182.5	269.0
Patient 3				
basal	30	11.3	258.1	97.1
200ug 3x daily	30	7.4	241.3	109.2
500ug 3x daily	20	11.7	138.0	87.5
Patient 4				
basal	50	9.6	246.9	230.2
200ug 3x daily	40	9.1	182.6	162.7
500ug 3x daily	30	8.9	281.3	171.5
Normal Subject 1	--	--	149.3	141.7
Normal Subject 2	--	--	113.6	205.9



24 hour serum GH profiles in 4 diabetic subjects with proliferative retinopathy before treatment (solid lines) and after treatment with the maximum dose of SMS-201-995 (dotted line). The profiles from two normal subjects are shown for comparison. The arrows at the top of the figure represent the time of administration of SMS 201-995 where relevant.

figure 9.1

Individual 24 hour GH profiles before therapy and on maximum dosage of SMS 201-995 are shown in figure 9.1. Profiles from the 2 normal subjects are shown for comparison. Two of the 4 study subjects demonstrated some diminution in 24 hour GH, one on a dose of 200ug thrice daily, the other on a dose of 500ug thrice daily. Of the 3 patients studied on a dose of 500ug thrice daily there was a decrease in the level of IGF-I which was accompanied by a fall in the total daily dose of insulin. These patients reported an increased incidence of hypoglycaemia, sometimes severe and slow to respond to oral carbohydrate ingestion. Patients manipulated their own dosage of insulin, and there was no change in HbA1. All patients experienced abdominal discomfort, diarrhoea, and in patient 3, vomiting. Patient 2 experienced uncomfortable abdominal distension at a dose of 100ug thrice daily and above. Abdominal X-ray demonstrated gaseous small bowel distension. In the remaining 3 patients, gastrointestinal symptoms became a problem with doses in excess of 200ug thrice daily, but generally improved within 1 week on any particular dose.

9.3 DISCUSSION

Information to date indicates that SMS 201-995 will be useful in the treatment of acromegaly (Daughaday, 1985, Editorial, Lancet 1985), having been shown to decrease GH levels both in the short and long term (Lamberts et al., 1985, Ch´ng et al., 1985). It is thus surprising that there was no consistent GH suppression in this group of diabetic patients with proliferative retinopathy using doses 5 times greater than those reported to be successful in acromegalic patients. This is disappointing since we are thus not only deprived of a further method of treatment for

proliferative retinopathy, but also of the opportunity to re-assess the contribution of GH and GH related factors in the pathogenesis of diabetic retinopathy. The results do, however, allow us to speculate further on the cause of GH hypersecretion in diabetic subjects as follows. The normal response to hyperglycaemia is production of somatostatin which suppresses GH secretion. In chapter 4, it was noted that, unlike normal subjects, diabetic subjects fail to demonstrate glucose mediated suppression of GRF stimulated GH secretion. This finding suggests that in diabetic subjects, hyperglycaemia either fails to elicit somatostatin production, or that they are resistant to somatostatin so produced. The results presented here favour the latter argument, and suggest that administration of a long-acting analogue of somatostatin is not the correct approach to suppression of GH secretion in diabetes.

SMS 201-995 is also a potent suppressor of other hormones in pathological conditions, having been found to be effective in cases of VIPoma (Clements and Elias, 1985, Kraentzlin et al., 1985), Zollinger Ellison syndrome (Bonfils et al., 1986), GRFoma (Von Werder et al., 1984), carcinoid syndrome (Kvols et al., 1985) and insulinoma (Osei and O´Dorisio, 1985). In view of the gastrointestinal symptoms reported by the patients in this study, it is probable that gut hormones were suppressed by SMS 201-995. The fact that hypoglycaemia was a problem in these patients with insulin-dependent diabetes is of importance. This presumably arises as a result of gut hormone suppression leading to slowing of absorption of ingested carbohydrate, and, as a result of glucagon suppression, slow recovery from established hypoglycaemia. That this is probable is suggested by a recent study examining the effect of bolus doses of SMS 201-995 given to

patients with insulin-dependent diabetes (Spinas et al., 1985).

In conclusion, in patients with proliferative diabetic retinopathy, treatment with SMS 201-995 caused no appreciable drop in 24 hour GH levels, and only a modest decrease in IGF-I. This suggests that patients with diabetes and GH hypersecretion are somatostatin resistant. Further, since the success of pituitary ablation was thought to depend on the degree of ablation (Joplin et al., 1967, Wright et al., 1969), it is unlikely that SMS 201-995 will be useful in the treatment of proliferative retinopathy.

CHAPTER 10

Concluding remarks

INTRODUCTION

In this work, an attempt was made to answer two questions. The first, why diabetics secrete excessive amounts of GH, is the more easily approached since it has never been the subject of systematic investigation. The second, whether elevated GH levels are a permissive factor in the genesis of microvascular complications of diabetes, is more difficult since the idea that it is has already taken root in popular opinion. To break new ground, therefore, the onus is on the investigator to either conclusively prove or disprove the theory. Either course is equally difficult, but there is scope for considering new and established data with a view to forming an objective opinion as to whether the balance of evidence is for or against the hypothesis.

10.1 GH hypersecretion and diabetes

Prior to considering the results relevant to the cause of GH hypersecretion in diabetic subjects presented in this work, the nature of the abnormality should be considered. GH hypersecretion in insulin-dependent diabetic subjects is easily demonstrated and has been well documented. In non-insulin-dependent diabetes, although subjects may demonstrate elevated GH levels relative to their plasma glucose and age, GH is not elevated in absolute terms. The cause of the abnormality is not clear, but it is worth pointing out that it is exclusive to GH and does not extend to other pituitary hormones. As a fundamental point, it is possible to suggest that the primary abnormality does not lie at pituitary level, since any pituitary abnormality would be unlikely to

affect the somatotroph alone. The defect is therefore more likely to reside in the GH regulatory mechanisms. Due to a peculiarity of human physiology, GH secretion in man is more closely related to circulating glucose levels than in other animal species. Whether this is of any practical advantage is doubtful since the rat, for example, manages to survive without the benefit of this mechanism. Further, since diabetic rats do not demonstrate GH hypersecretion, it is reasonable to assume that GH hypersecretion in diabetic man is related to the abnormal glucose levels acting via the hypothalamic glucose sensitive cells. Certainly, as demonstrated in chapter 5, improvement in glycaemic control in diabetes leads to a normalisation of abnormal GH secretion.

Beyond these basic observations, little more can be said regarding the cause of GH hypersecretion in diabetes. Various stimuli such as TRH, arginine and dopamine have been found to stimulate GH release in diabetic but not normal subjects, but the significance of these observations is obscure since the mechanism of action of these stimuli is not known. It has been proposed that the GH response to TRH in diabetic subjects is caused by over-sensitivity of a normal pathway (Chiodera et al., 1984), and the same may apply to the other stimuli. In other respects, GH regulation in diabetes is normal. Bromocriptine has a GH stimulatory effect as in normal subjects (Cassar et al., 1975), and a normal response to hypoglycaemia indicates that the feedback loop is intact, if over-sensitive.

The present work has been an attempt to examine the various components of the GH regulatory loop in diabetic subjects. Using GRF, it was demonstrated that diabetic subjects have a failure of glucose mediated GH suppression, and work with pituitary cells in culture suggests that this is not mediated at pituitary level.

This indicates that there is a defect in the the normal GH inhibitory mechanism initiated by hyperglycaemia in diabetes. In normal physiology, this is probably mediated by secretion of somatostatin in response to the elevated blood glucose level, and this mechanism is in some way compromised in diabetic subjects. A possible explanation may be derived from the observation in the present work that diabetics with GH hypersecretion are resistant to the GH suppressant effect of long acting somatostatin analogue. This suggests that these subjects are resistant to the effects of hypothalamic somatostatin. The simplest explanation would be that chronic hyperglycaemia leads to chronic over-secretion of somatostatin. This in turn would lead to down-regulation of pituitary receptors and consequent somatostatin resistance. Although confirmation is needed, this theory would neatly account for all the described anomalies of GH secretion in diabetes, including hypersecretion, the abnormal or exaggerated GH response to various stimuli, and for failure of glucose mediated GH inhibition.

Studies of glucose and insulin infusion indicate that even in non-diabetic subjects, lowering of the plasma glucose within the normal range can cause GH release. It should not be forgotten that this mechanism is likely to contribute to GH hypersecretion in treated diabetic subjects, but it is unlikely to be the primary mechanism since GH levels are high at the time of diagnosis of diabetes when blood glucose levels are persistently high.

The studies of insulin and glucose infusion further demonstrate that patients with poorly controlled diabetes are capable of secreting GH in the presence of hyperglycaemia. This has been interpreted by some as indicating that despite elevated

exist in these patients due to an abnormality of glucose transport across the blood-brain barrier (BBB). The work outlined in chapter 6 indicates that hexose transport across the BBB is normal in diabetic subjects, including those with microvascular disease. Thus, there is no evidence to suggest that GH hypersecretion in diabetes is related to central glucose depletion, and no evidence for the existence of the entity known as 'relative hypoglycaemia'.

At circulatory level, it is possible that an abnormal GH form, or proportion of forms, with altered biological activity, could contribute to GH hypersecretion in diabetes by interfering with the normal feedback mechanisms. Although perhaps the least satisfactory of the chapters in this work, the evidence presented suggests that circulating GH forms in diabetic subjects do not differ from normal, and provides no evidence for a cause of GH hypersecretion at this level.

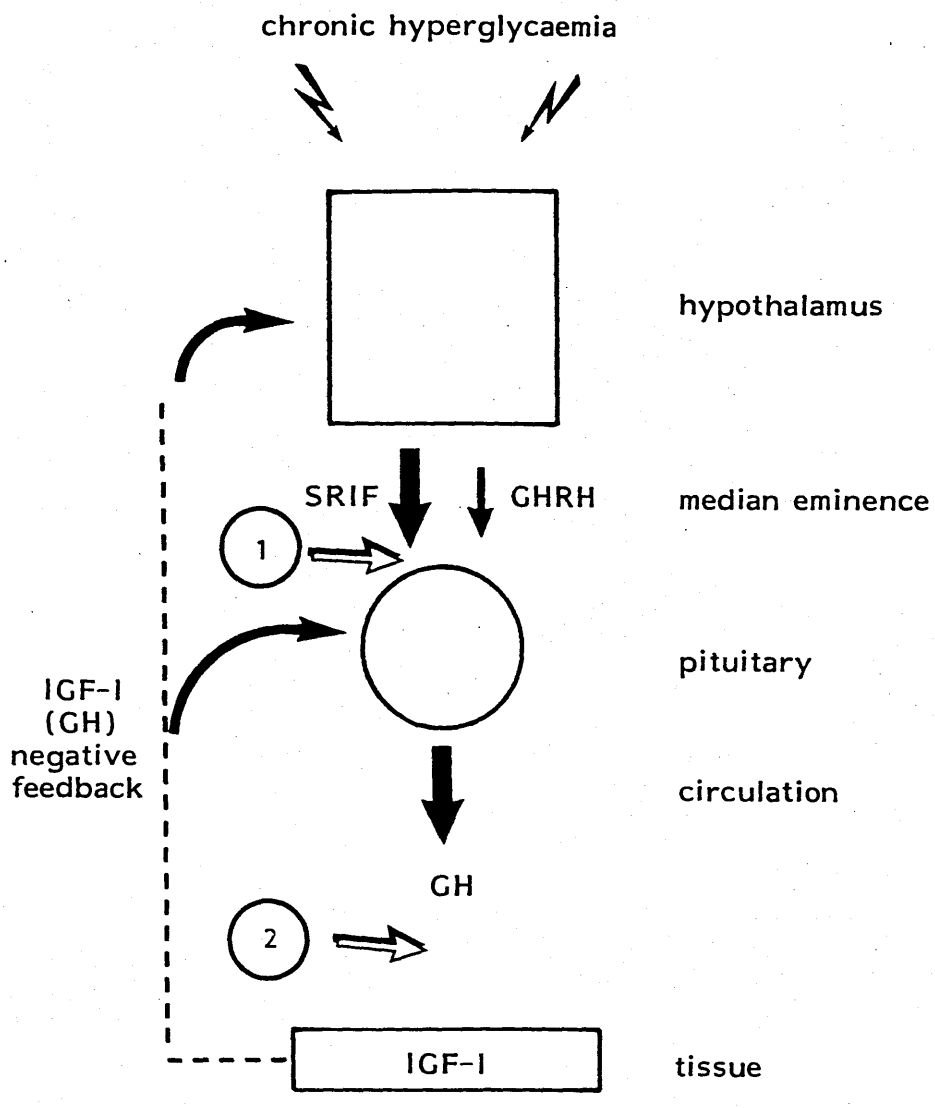
The finding of a negative correlation between serum IGF-I levels in diabetes and glycaemic control may provide further cause for GH overproduction, especially in subjects with poor diabetic control. There is considerable indirect evidence, outlined in chapter 1, section 1.12, to suggest that poor glycaemic control leads to a decrease in IGF-I generation, and the finding of this correlation provides further confirmation. Such a defect would lead to an in increase in GH by negative feedback. However, it is hoped that in insulin treated diabetic subjects, glycaemic control would not be sufficiently poor to make this factor a major determinant of GH levels. It is the opinion of the author, therefore, that this mechanism is likely to be a contributory factor, and not the primary cause of GH overproduction in

diabetes.

In conclusion, regarding the mechanism of GH hypersecretion in diabetes, a number of possible causes have been identified, two of which are shown in figure 10.1. The most attractive theory, which would account for most of the described abnormalities, is somatostatin resistance induced by chronic hyperglycaemia. Limited confirmation of this is obtained from the studies using somatostatin analogue, but further work along these lines in a greater number of patients would be helpful. Fluctuating glucose levels and low IGF-I levels in patients with poor glycaemic control are likely to contribute to high GH levels in diabetes. Certain aspects of this part of the work could be usefully expanded. Further assessment of the integrity of the BBB could be undertaken. In the present work, glucose transport across the BBB was measured and found to be normal, but whether the BBB in diabetic subjects with retinopathy is abnormally permeable as has been suggested (Lorenzi et al., 1980) remains to be assessed. Further work on GH forms and bio-activity in diabetes might be useful, especially the possibility of glycation leading to impairment or alteration of its biological function. It should, however, be pointed out that abnormalities of GH forms are more often proposed than the evidence warrants, but this project could usefully be carried out by groups who are studying GH variants in other conditions and who already possess the necessary techniques.

10.2 GH and diabetic microvascular disease.

The question of whether elevated GH levels are relevant to the development of diabetic microvascular disease is less readily answered. A number of factors suggest that it is not. Firstly,



The 2 major causes of GH hypersecretion in diabetes proposed in this work :- 1. overproduction of somatostatin (SRIF) induced by chronic hyperglycaemia leading to pituitary resistance to the effects of somatostatin. 2. failure of IGF-I generation in response to GH induced by poor glycaemic control.

figure 10.1

the basis of the GH hypothesis, on review of the literature, is thin. Secondly, acromegalic patients, even in the presence of diabetes, do not develop abnormally severe microvascular disease. Lastly, non-insulin dependent diabetic subjects, despite the fact that their GH levels are not elevated in absolute terms, still develop microvascular complications. Undeniably, however, patients who underwent pituitary ablation for proliferative retinopathy seem to have done extremely well with regard to both retinopathy and nephropathy. Nevertheless, this provides no specific evidence that reduction of GH was responsible for this beneficial effect. For instance, pituitary ablation may have had certain haemodynamic effects, as suggested by the plot of blood urea versus time post-ablation. Further, with the passage of time, and the isolation of an increasing number of hormones from the pituitary gland, the GH hypothesis seems increasingly naive. For instance, fibroblast growth factor has recently been identified in the pituitary gland. This growth factor has been shown to stimulate production of extracellular matrix together with vascular proliferation, and has been found in excess in the vitreous of patients with neovascularisation (Baird et al., 1985). Such findings suggest that reduction of GH following pituitary ablation may simply supply a correlation with the reduction of some other factor (as was originally suggested (Wright et al., 1969)). Unfortunately, an attempt to answer the question of whether GH is specifically involved, by selective suppression of GH secretion in diabetics using somatostatin analogue, was unsuccessful.

Part of the allure of the GH hypothesis is that just when the idea is going out of fashion, additional suggestive information is provided. Such is the case in this work with the data of serum

IGF-I levels. In common with other workers in the field, it was found that serum IGF-I levels are elevated in patients with proliferative retinopathy. As pointed out in chapter 8, however, it will be necessary to prove that levels are elevated prior to the onset of proliferation before a role in the genesis of proliferation can be ascribed to IGF-I.

Thus, the question of whether GH and IGF-I are involved in the development of diabetic microvascular disease remains unanswered. Future work should be directed towards more dynamic studies of serum IGF-I in patients with retinopathy, following the progress of the retinopathy together with serial measurements of IGF-I. Concurrently, an open mind should be maintained, and other growth factors should be investigated. To finally answer the question, a method of selectively inhibiting GH in diabetic subjects with retinopathy must be developed. As discussed in chapter 1, anticholinergic agents may provide the best results since somatostatin analogues may not be effective.

If pressed for an opinion, having reviewed the literature and the data included in this work, the author would conclude that there is not yet sufficient evidence to incriminate GH as a permissive factor in the genesis of diabetic microvascular disease.

APPENDIX A

The growth hormone assay

Materials

buffer (i) Stock buffer contains

14.7g barbitone sodium

9.7g anhydrous sodium acetate

Made up to 500ml with distilled water and stored at 4°C.

(ii) Working buffer

100ml stock buffer

15.3g NaCl

Dissolved and made up to 2 litres with distilled water. 4g BSA (fraction V) and 200mg thiomersal added. pH adjusted to 7.4.

Standard

Working standards were obtained from Dr J Seth, Immunoassay Section, Department of Clinical Chemistry, Royal Infirmary, Edinburgh. They contained hGH 25uIU/freeze dried ampoule.

2.5ml assay buffer added to give a concentration of 10mIU/l

Double diluted to obtain a standard curve of 10, 5, 2.5, 1.25 and 0.625 mIU/l

Antiserum

Rabbit anti-HGH was obtained from Wellcome Reagents (cat. RD 16, now discontinued). This consisted of 0.5ml aliquots freeze dried at 1:1000 dilution, stored at 4°C. Each aliquot was made up to 32.5ml in 1:200 non-immune rabbit serum (NIRS) in assay buffer (ie a working dilution of 1:65,000).

Tracer

This was prepared in the laboratory by iodinating hGH using the chloramine-T method, and purified on a Sephadex column.

Non-immune rabbit serum

Obtained from Wellcome Reagents and stored at -20°C in 2ml aliquots diluted 1:10. Each aliquot was made up to 40ml with assay buffer (ie a 1:200 dilution), and used to dilute the antiserum as above.

Second Antibody

Goat anti-rabbit serum was obtained from Guildhay (Surrey, England), and stored in 0.5ml aliquots, neat, at -20°C. Each batch was titrated to obtain maximum binding of zero tubes, and aliquots diluted accordingly.

Quality control

Lypho-Chek pools I-IV were used, appropriately diluted to lie on 4 points on the standard curve.

Method

Day 1.

An assay sheet was prepared, and samples diluted 1:5 and 1:25 in assay buffer. All samples were assayed neat, 1:5 and 1:25.

Standards, quality control sera and antiserum were prepared as above. Sample volume was 100ul to which 100ul antiserum/NIRS mixture and 200ul of buffer was added. Non-specific binding (NSB) tubes contained only NIRS and buffer to a total volume of 400ul. Four blank tubes were left to measure total counts.

Tubes were incubated for 4 hours at room temperature following which 100ul of tracer (10,000 counts per 60 seconds) was added to all tubes (including total counts and NSB). These were incubated overnight at room temperature.

Day 2.

Goat anti-rabbit serum (100ul) was added, and tubes incubated at room temperature for 5 hours. At the end of this time, they were centrifuged at 3000rpm for 20 min at 20°C, aspirated and counted for 60 seconds.

The standard curve was plotted on log-logit paper after correction for non-specific binding, and sample values read from this curve. The sample dilution which fell on the optimum part of the curve was used. The within and between assay coefficients of variation at a dose level of 22.9mIU/l (which required a 1:5 dilution) were 8.9 and 19.8% respectively.

APPENDIX B

The IGF-I assay

Materials

The assay buffer contained :- 4.7g sodium phosphate
200mg protamine sulphate
200mg sodium azide

This was made up to 1 litre with distilled water and the pH adjusted to 7.5. At the time of the assay, 0.25% RIA grade BSA (Sigma) was added.

Monoclonal Antiserum

This was obtained on application to the NIH. Each freeze dried vial is diluted with 2ml of assay buffer, and frozen at -20^{o}C in aliquots of 100ul. At the time of assay, each aliquot was diluted to 10ml with assay buffer (working dilution 1:100,000), which quantity was sufficient for 100 tubes.

Standard

This was obtained from Amersham International plc (Buckinghamshire, England), and consisted of the recombinant analogue of IGF-I, differing from the native peptide by the substitution of threonine for methionine in position 59. The freeze dried material was reconstituted with 0.1M acetic acid, mixed with freeze drying solution, and lyophilised in aliquots of 0.25ug. At the time of assay, each aliquot was made up to 0.5ml in assay buffer. With a standard assay volume of 10ul, this was equivalent to 5ng/tube. This was double diluted down to give a standard curve of 5, 2.5, 1.25, 0.625, 0.312, 0.15 and 0.075 ng/tube.

Tracer

Preparation of tracer is outlined in appendix C.

Non Immune Mouse Serum

Aliquots of 200ul were frozen at -20°C. At the time of assay, this was made up to 10ml, and 100ul added per tube, giving 2ul non-immune mouse serum/tube.

Second Antibody

Donkey anti-mouse serum was titrated to obtain maximum binding of tracer in zero tubes. Suitable aliquots were stored at -20°C.

Quality Control Serum

Two serum samples were used, each being extracted with the samples prior to assay. The lower sample (normal subject) had a value of approximately 200ng/ml and the upper (acromegalic subject) had a value of approximately 700ng/ml.

Sample Extraction

This was carried out according to the method of Daughaday et al. (1980). The procedure was as follows :-

1. 0.2ml serum to 0.8ml of 87.5% ethanol and 12.5% 2N HCl.
2. mix and stand at room temperature for 30 min.
3. centrifuge at 1860g for 30 min at 4°C
4. remove supernatant (0.5ml) and add to 0.2 ml 0.855M Tris base.

After extraction, extracts can be stored for up to a week. The extraction procedure results in a 1:7 dilution of the original sample. Serum samples were assayed directly, but vitreous samples were dried down and reconstituted in the original volume.

Method

Day 1.

The assay sheet was prepared, and samples extracted as above. Antiserum, non-immune mouse serum and standards were prepared

as described. The standard assay volume of 10ul, and sample assay volume of 20ul was added to each tube (the sample assay volume was increased to place values on the centre of the standard curve). For serum samples, 20ul of acid-ethanol/Tris base mixture was added to each standard tube to equalise the salt and ethanol concentrations in standard and unknown tubes. Antiserum and non-immune mouse serum were added to each tube, and the final volume made up to 400ul with assay buffer. Tubes for assessment of non-specific binding contained only non-immune mouse serum and buffer. Tracer (100ul, 10,000cpm) was immediately added to all tubes which were incubated at $4^{o}C$ for 18 hours.

Day 2.

Donkey anti-mouse serum, 100ul of an appropriate dilution, was added and tubes incubated at $4^{0}C$ for 30 minutes. Polyethylene glycol (PEG), 1ml of a 6% solution, was then added to each tube which were then centrifuged and aspirated. The standard curve was then plotted on log-logit paper after correction for NSB (which was typically 4%). Sample values were read from the curve in ng/tube and converted to concentrations in ng/ml (for serum samples, with an assay volume of 20ul, this involved a factor of 50 to express the concentration per ml, and a factor of 7 to allow for the extraction procedure).

Assaying the acid ethanol mixture directly, the sensitivity of the assay was 80ng/ml. The within and between assay coefficients of variation were 5 and 10% respectively.

APPENDIX C

Iodination of IGF-I

Recombinant analogue of IGF-I (Amersham International plc), 10ug, was reconstituted with 1ml 0.1M acetic acid and 50mg lactose. This was freeze dried in aliquots of 1ug for iodination. The iodination procedure was as follows :-

1. a 30 x 1 cm glass column was prepared and siliconised using Sigmacote (Sigma). This was equilibrated with Sephadex G50 (fine) (Pharmacia, Sweden) and run through with 0.1M ammonium carbonate, pH 7.9, containing 0.05% RIA grade BSA (Sigma).
2. the freeze dried peptide was reconstituted with 50ul 0.5M phosphate buffer.
3. 1mCi ^{125}I in 10ul was added.
4. 10ul of 0.5mg/ml chloramine-T was added and mixed for 20 seconds.
5. the reaction was stopped with 10ul of 5mg/ml sodium metabisulphite.
6. this iodination mixture was layered onto the pre-prepared column, and 30 x 1ml fractions collected and counted.

The count profile typically produced 3 peaks, the first representing 'aggregates' and coming off the column from fractions 11-14. The second peak contained the iodinated IGF-I and typically appeared in fractions 17-20. The last, and largest, peak contained the iodine salts and appeared in fractions 23-30. The fractions from the second peak were stored with additional BSA and aliquoted in 50ul volumes. The incorporation was typically 200uCi/ug peptide.

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