

STUDIES ON INSULIN SECRETION AND INSULIN RESISTANCE
IN
NON-INSULIN-DEPENDENT DIABETES IN YOUNG INDIANS

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WILFRID G. OAKLEY (1905 - ?)

"MAN MAY BE THE CAPTAIN OF HIS FATE,
BUT HE IS ALSO THE VICTIM OF HIS
BLOOD SUGAR."

TRANSACTIONS OF THE MEDICAL SOCIETY
OF LONDON

78 : 16, 1962.

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Where use was made of the work of others it has been duly acknowledged in the text.

While this work was in progress, the following papers relevant to this thesis were published or are in the process of being published:

- 1) The Insulin-Receptor: Biochemical and Clinical considerations.
I. Jialal, C. Naidoo, R. Dunn and S.M. Joubert. South African Journal of Science (1985) 81: 453-455.
- 2) 125 Insulin binding to circulating monocytes and erythrocytes in Indian patients with Non-insulin-dependent diabetes in the young.
I.Jialal, C.Naidoo, and S.M.Joubert. Diabetes Research and Clinical Practice (1985) 1 Abstract 701: XII Congress of the International Diabetic Federation.
- 3) The insulin response to glucose, glucagon, tolbutamide and arginine in Indian patients with NIDDM.
C.Naidoo, I.Jialal, and S.M. Joubert. Diabetes Research and Clinical Practice (1985) 1 Abstract 1039: XII Congress of the International Diabetic Federation.
- 4) Demonstration of Sensitivity to the anti-lipolytic action of exogenously administered insulin in non-insulin-dependent diabetes in the young.

C.Naidoo, I.Jialal, S.M.Joubert.

South African Medical Journal (1985) 68: 917-918.

- 5) The insulin and glucose response to an oral glucose load in non-insulin-dependent diabetes in the young (a study of 4 families).

C.Naidoo, I.Jialal, T. Govender, M.A.K. Omar and S.M.Joubert.

South African Medical Journal (1986) 69: 119-121.

- 6) The acute insulin response to glucagon, tolbutamide and glucose in non-insulin-dependent diabetes in the young.

C.Naidoo, I. Jialal, A. Suleman, M.C.Rajput, and S.M.Joubert

Diabetes Care (1986) 9: 57-61.

- 7) Is there a defect in the hepatic extraction of insulin in patients with non-insulin-dependent diabetes in the young?

C.Naidoo, I. Jialal, and S.M.Joubert.

Diabetes Care (1986) 9: 96-97.

- 8) Insulin secretion during intravenous glucose tolerance tests in non-insulin-dependent diabetes in the young.

C.Naidoo, I.Jialal, G. Davies and S.M.Joubert.

Tropical and Geographical Medicine (1986) 38: 16-20.

- 9) Arginine-stimulated acute phase of insulin secretion in non-insulin-dependent diabetes in the young.

C.Naidoo, I. Jialal, and S.M.Joubert.

Diabetes Research (1986) 3: 127-129.

- 10) HLA and Non-insulin-dependent diabetes in the young.,

C.Naidoo, I.Jialal, M.G. Hammond, M.A.K. Omar and S.M. Joubert.

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- 11) Insulin binding to circulating monocytes and erythrocytes in patients with non-insulin-dependent diabetes in the young.

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LIST OF ABBREVIATIONS

| | |
|-------------------|-------------------------------------|
| Bo | Maximum binding |
| β cell. | Beta cell |
| BMI | Body Mass Index |
| BSA | Bovine Serum Albumin |
| C | Centigrade |
| CPAF | Chlorpropamide Alcohol Flush |
| cpm | counts per minute |
| CPR | C-peptide |
| ECG | Electrocardiogram |
| EDTA | Ethylene diaminetetra-acetic acid |
| FFA | Free Fatty Acid |
| g | gram |
| g | gravitational force |
| GFR | Glomerular Filtration Rate |
| HbA _{1c} | Glycosylated Haemoglobin |
| HBB | Hepes Binding Buffer |
| HGH | Human Growth Hormone |
| HLA | Human Leucocyte Antigens |
| IDDM | Insulin Dependent Diabetes Mellitus |
| IGT | Impaired Glucose Tolerance |
| ITT | Insulin Tolerance Test |
| IU | International Unit |
| iv | intravenous |
| IVGTT | Intravenous Glucose Tolerance Test |
| KBq | Kilo Becquerel |
| Kg | kilogram |
| Kgtt | Glucose disposal constant (IVGTT) |
| K _t | glucose disposal constant (ITT) |
| L | Litre |

| | |
|-------|---|
| MBq | Mega Becquerel |
| MEM | Minimal Essential Medium |
| μCi | microcurie |
| μEq | microequivalent |
| μl | microlitre |
| μg | microgram |
| μmol | micromole |
| μU | microunit |
| mg | milligram |
| ml | millilitre |
| mmol | millimole |
| M | molar |
| MODY | Maturity Onset Diabetes in the Young |
| MOHY | Maturity Onset Hyperglycaemia in the young |
| MSI | Modified Seltzer Insulinogenic Index |
| ng | nanogram |
| nmol | nanomole |
| NIDDM | Non-Insulin-Dependent Diabetes Mellitus |
| NIDDY | Non-Insulin-Dependent Diabetes in the Young |
| NSB | Non-Specific Binding |
| OGTT | Oral Glucose Tolerance test |
| PBS | Phosphate Buffered Saline |
| PEG | Polyethylene Glycol |
| pg | picogram |
| RBC | red blood cell |
| Ro | receptor number |
| SD | Standard Deviation |
| TCA | Trichloroacetic Acid |
| Tris | Tris (hydroxymethyl) aminomethane |
| WHO | World Health Organisation. |

CHAPTER ONE

THE CLINICAL AND BIOCHEMICAL PRESENTATION OF NON-INSULIN-DEPENDENT DIABETES IN THE YOUNG

1.1 INTRODUCTION

Over 2000 years ago, two Hindu physicians Charaka and Sushruta commented on "honey urine" of two causes - genetic (passed from one generation to another) and environmental (due to injudicious diet). Although familial aggregation of diabetes has been apparent for some time, there has been little agreement as to the specific nature of the genetic factors involved (Rimoin and Schimke, 1971).

Furthermore, the high prevalence of diabetes in the population presents additional difficulties for the geneticist. Is a relative affected because he has the same genotype, shares the same environment or has a chance occurrence of a common disorder?

Familial aggregation of a trait could have genetic or environmental causes. Studies in twins have proved most valuable in resolving these problems. Such studies have confirmed that the concordance rate of non-insulin-dependent diabetes mellitus, for monozygotic (identical) twins varied from 45-76% whilst that for dizygotic (fraternal) twins ranged from 3-37% (Barnett et al, 1981a and 1981b). These findings suggest that a major factor which determines the onset of clinical disease is primarily the genetic susceptibility of an individual.

The highest prevalence of non-insulin-dependent diabetes mellitus of late onset (NIDDM) is found amongst American Indians, urbanized Pacific Island populations, and migrant Asian Indians (Zimmet, 1982). The characteristic features of insulin dependent diabetes mellitus (IDDM): islet cell antibodies, aggregation of HLA types, insulin dependence and evidence of organ specific cell mediated immunity are absent in NIDDM, confirming that there are two major types of diabetes mellitus.

Although the prevalence of diabetes mellitus is high amongst South African Indians (Marine et al, 1969) the presentation of diabetes is atypical in that IDDM is rare and non-insulin-dependent diabetes in the young (NIDDY) is common (Asmal et al, 1981).

1.2 AN OUTLINE OF THE HISTORY OF NON-INSULIN-DEPENDENT DIABETES IN THE YOUNG.

As early as 1928, Cammidge reported a few cases of NIDDM in the young with strong familial backgrounds and evidence suggesting dominant inheritance in some patients. Nothing of consequence appeared in the literature until Fajans and Conn (1960) published the results of the responses of mild diabetes to tolbutamide therapy in fourteen young patients. At the time hyperglycaemia was diagnosed all patients were under 35 years: all showed marked improvement in glucose tolerance when treated with oral hypoglycaemic agents. Thereafter numerous reports worldwide confirmed the presence of NIDDY in White Caucasoid populations (Campbell, 1960; Johansen, 1973; Tattersall, 1974; Tulloch and Adamson, 1976; Barbosa et al, 1978; Kobberling et al, 1980 and Panzram and Adolph, 1981).

The classical form of NIDDY is a mild non-insulin requiring type of diabetes in which the disorder is inherited as a dominant trait, glucose intolerance does not tend to progress with time, the diabetes is infrequently accompanied by vascular complications. (Tattersall et al, 1980). This subtype is referred to as maturity onset diabetes in the young (MODY) and constitutes a subset of the broad spectrum of NIDDY. However, the term MODY was originally also applied to any patient under the age of 25 years whose diabetes could be controlled for two years or more without insulin. Yet another term, maturity onset hyperglycaemia in the young (MODY) was used for the same condition (Barbosa et al, 1978). Thus there was some confusion in terminology.

1.2.1. Clinical Features in NIDDM With Dominant Inheritance.

In 1974 Tattersall clearly established that a familial form of diabetes existed which was manifest at an early age and was dominantly inherited. In the three families described by Tattersall: there was direct parent to child inheritance through at least three generations; the ratio of affected: unaffected children of diabetic parents was 1:1 and almost all affected individuals had a diabetic parent. Renal glycosuria coexisted with diabetes in two of the three families studied. In a later communication Tattersall and Fajans (1975) reported on the families of 26 patients with MODY: in 46% of patients direct vertical transmission of diabetes through three generations were found; 53% of the siblings had latent diabetes and 85% of the probands had a diabetic parent. The diabetic phenotype was consistent in that most affected individuals had a non-insulin requiring form of diabetes.

Several reports of NIDDM with dominant inheritance followed (Tulloch and Adamson, 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978; Faber et al, 1980; Panzram and Adolph, 1981). Kobberling et al (1980) also demonstrated renal glycosuria in three of the five families studied.

In the South African Indian population Campbell (1960) described NIDDM over two and a half decades ago. Since his original report, nobody had really studied NIDDM in any depth in South Africa until the pioneering work of Jialal et al (1982a). Eighty-two percent of their 85 patients gave a positive family history; 75% of the probands had a diabetic parent; 41% had a diabetic sibling and three generation transmission of the disorder through a single parent occurred in 7% of the patients. In the chapters to follow NIDDM with three generation transmission will be discussed in greater depth.

1.2.2. HLA Status of NIDDM.

The association of histocompatibility antigens and IDDM have been known for some time (Nerup et al, 1974). However the relationship with NIDDM is less clear. Initially in a family with MODY (Barbosa, 1977) HLA A3 and BW15 were reported to be strongly associated with the hyperglycaemic trait. Later, Barbosa (1978a) described a family with four generation transmission of non-insulin-dependent diabetes in which eight of the eleven diabetic subjects shared the haplotype A3 and BW15. It should be noted that the proband's husband was not typed and therefore these results are less conclusive.

Later, Faber et al (1978) studied a family with MODY and dominant inheritance. There was no HLA haplotype demonstrated in the affected members of this family.

Platz et al (1982) investigated a family with MODY previously studied by Johansen and Gregersen (1977). All 53 members were typed for HLA-A, B and C antigens. The lod scores excluded close linkage between MODY and HLA in this family.

Barbosa (1983) corrected his earlier studies (Barbosa, 1977 and 1978a) and confirmed that MODY is neither associated nor linked to specific HLA types.

Thus far all HLA studies in NIDDM were confined to White Caucasoid population groups. The HLA status of non-white Caucasoid populations with this disorder has not been studied to date.

1.2.3. Chlorpropamide Alcohol Flush

Facial flushing after alcohol is a well known complication of chlorpropamide treatment and occurs in 33% of patients receiving the drug. (Fitzgerald et al, 1962). Leslie and Pyke (1978) tested numerous pairs of

identical twins in whom diabetes was transmitted through 3 generations. Since all twins reacted similarly to the test, the authors concluded that the chlorpropamide alcohol flush (CPAF) was a dominantly inherited trait associated with NIDDDY. Later, Pyke (1979) confirmed that the frequency of (CPAF) in NIDDDY was 90%.

Kobberling (1980) studied 14 patients from 5 families with MODY but found no association between the disorder and the CPAF. Similarly Jialal et al (1984a) found in 15 patients with NIDDDY that a mere 40% had positive tests which agreed with the results reported by Fajans (1981) and Panzram and Adolph (1981). It seems clear that the CPAF is not closely associated with all subtypes of NIDDDY.

1.2.4. Complications of NIDDDY

1.2.4.1. Microvascular

Only 5 of the 12 patients studied by Tattersall (1974) had background retinopathy. Yet, all were diagnosed before the age of 30 years and had a mean duration of diabetes of 37 years. Johansen and Gregersen (1977) reported background retinopathy in 2 of 22 diabetic members in a family of NIDDDY with dominant inheritance, despite a mean duration of diabetes of 13 years. In 3 other reports of NIDDDY with autosomal dominant inheritance there was no evidence of vascular complications (Tulloch and Adamson, 1976; Barbosa et al, 1978; Kobberling et al, 1980). In contrast to these reports, Fajans et al (1978) found vascular complications in 35% of 6 pedigrees with 3-4 generations of diabetes. Of the 85 patients studied by Jialal et al (1982b), 14 had retinopathy (17%); 13 of these patients had background retinopathy while the remaining patient had proliferative retinopathy.

Nephropathy was not a feature of NIDDDY with dominant inheritance (Tattersall, 1974; Tulloch and Adamson, 1976; Barbosa et al, 1978;

Johansen and Gregersen, 1977; Kobberling et al, 1980) except for a single patient described by Fajans et al (1978). However, it should be noted that these workers assessed renal function by the presence of proteinuria in 24 hour urine collections and by creatinine clearance. Jialal et al (1982b) using more sensitive techniques to establish nephropathy found that 7% of his 85 patients had evidence of renal disease; 5 of these patients had retinopathy while a sixth patient had bilateral cataracts.

1.2.4.2. Macrovascular complications

No evidence of macrovascular disease was found in the studies of NIDDM reported by Tulloch and Adamson (1976), Barbosa et al (1978) and Kobberling et al (1980). Of the 22 members of a family with MODY, one had a myocardial infarction and a further patient had peripheral vascular disease (Johansen and Gregersen, 1977). In the 3 families reported by Tattersall (1974) four had myocardial infarctions and one patient had an amputation because of peripheral vascular disease.

Fajans et al (1978) recorded macroangiopathy in 16 of 69 patients from 6 families with NIDDM. The presence of macroangiopathy was more frequent than microangiopathy. By contrast Jialal et al (1982b) reported macrovascular disease in 3 of 85 patients (4%) whilst microvascular complications occurred in 18%. Hence it can be seen that the incidence of macrovascular disease in NIDDM is quite low.

1.2.4.3. Neuropathy

In most patients with dominantly inherited NIDDM neuropathy was absent (Johansen, 1973; Tulloch and Adamson, 1976; Barbosa et al, 1978; Kobberling et al, 1980). Neuropathy was recorded in one of Tattersall's series of 22 patients (1974), whilst 20% of Jialal's patients (1982b) had peripheral neuropathy as diagnosed by history and clinical exam-

ination. Thus neuropathy proved to be the commonest complications in NIDDM.

1.2.5. The Counter-Regulatory Hormones

The circulating hormonal insulin antagonists : growth hormonal (GH), cortisol and glucagon were measured in MODY by Barbosa (1978) and found to be within normal limits. This contrasts with the findings of Jialal et al (1982c) who reported that patients with NIDDM had raised glucagon and cortisol and lowered GH concentrations. This aspect of NIDDM needs clarification as the only 2 studies in the literature have conflicting results.

1.2.6. Adipocyte Sensitivity

In an attempt to explain the rarity of ketonuria in Indian diabetics, insulin and free fatty acid (FFA) levels were measured in 35 patients with NIDDM (Jialal et al, 1985) during a 100 gram oral glucose tolerance test. Although the diabetics had a severe degree of hyperglycaemia and fasting hyperinsulinism, their fasting FFA levels were similar to those of their reference subjects. Furthermore, the decrement of the FFA responses were equivalent in the diabetics and control subjects. Thus the adipocyte appeared to be unaffected in its response to inhibition by insulin in NIDDM. This could be one factor accounting for the ketosis resistance in these patients inasmuch as the FFA release from adipose tissue is adequately suppressed by circulating insulin levels.

1.3. A CRITICAL APPRAISAL OF THE LITERATURE OF INSULIN SECRETION AND INSULIN RESISTANCE IN NIDDM WITH SPECIAL REFERENCE TO NIDDM

Patients with NIDDM have defects in both insulin action and insulin

secretion (Reaven, 1983; Beck Nielson, 1984; Reaven, 1984). However, there is considerable debate as to which is the primary lesion responsible for the glucose intolerance in this condition. It may be that impairments in insulin secretion and insulin action are linked to each other or share a common basis.

1.3.1. Biosynthesis of Insulin and Mechanism of Secretion

Insulin is initially elaborated by the β -cells of the pancreas as preproinsulin, a large polypeptide precursor which is rapidly converted to proinsulin in ribosomes associated with the rough endoplasmic reticulum (Rubenstein, 1981). Proinsulin consists of a single polypeptide chain and after synthesis it is transported to the golgi apparatus (Rubenstein, 1981) where it is condensed into membrane enclosed granules. The size of individual granules is very small (0,2 - 0,3 μ m) necessitating the storage of a very large number of granules, about 13000 in each β cell (Dean, 1973). Thereafter the conversion of proinsulin to insulin and C-peptide, entails an excision of the basic residues which link the C-peptide to the A and B chains. This conversion reaction takes place in the Golgi apparatus and following appropriate stimulations, the granules migrate to the plasma membrane whence they are released by a process of emiocytosis. Insulin and C-peptide are released in equimolar quantities with a small amount of unconverted proinsulin, into the portal circulation (Lacy, 1961; Gepts and Pipeleers, 1976; Rubenstein, 1981). The process of emiocytosis involves activation of the microtubular, microfilamentous system and appears to be dependent on intracellular shifts of calcium ions (Malaisse et al, 1975). It is now evident that at least some of the effects of calcium are mediated through calmodulin, since glucose stimulated insulin secretion is inhibited by the calmodulin inhibitor trifluoperazine (Henquin, 1981).

1.3.2. Insulin Secretion in NIDDDY.

Comprehensive studies on insulin secretory responses to oral glucose have been carried out in White Caucasoid and Indian populations with NIDDDY. But, at present, no studies on insulin responses to intravenous (IV) glucose and non-glucose secretagogues e.g. glucagon, tolbutamide and arginine have been undertaken in NIDDDY of any population group.

1.3.2.1. Insulin responses to glucose stimuli.

i. Oral The insulinaemic responses to oral glucose have been studied in great detail previously (Fajans et al, 1969; Fajans et al, 1974; Fajans et al, 1976; Fajans et al, 1978, Fajans 1979; Fajans, 1981). The majority of patients in their series had a delayed and subnormal insulin response to oral glucose. In one of Fajans's studies (Fajans et al, 1978) on 68 non-obese diabetics, it was found that 21 patients had increments in plasma insulin responses to glucose which were less than 1S.D. below the mean of the control subjects; these patients were termed "low insulin responders". Thirteen patients had increments exceeding the mean and 1S.D. of control subjects: these were designated as "high insulin responders". Most diabetic members of NIDDDY families had lower insulin responses than their controls; no families had members with high and low responders appearing together; progression to insulin requiring diabetes only occurred in individuals who had low insulin responses. However, not all "low responders" progressed to insulin dependence as was evidenced by the follow-up of patients for 20 years.

Delayed and attenuated insulin responses in the majority of patients with NIDDDY have been corroborated by previous workers (Thorell et al, 1975; Faber et al, 1978; Barbosa et al, 1978).

In the 85 Indian patients with NIDDDY studied by Jialal (1982d) only one patient was found to be a high insulin responder, all others

displayed subnormal responses. This contrasts with the 12% and 15% of high responders found by Barbosa et al (1978) and Fajans et al (1978). Taking all the above studies in conjunction, one gets a spectrum of insulin responses in NIDDM which apparently conforms to the "horseshoe" concept of Reaven (1980). It is Reaven's view that the basic lesion in NIDDM is insensitivity of the target tissues to insulin. As a result, there is initially an increased insulin response to overcome the resistance in target organs, evoked by the increased hyperglycaemia. This stage corresponds to the phase of high insulin response. As the β cells do not have an unlimited capacity for increased insulin secretion a stage of β cell exhaustion ultimately sets in, leading to hyperinsulinaemic responses.

ii) Intravenous glucose:

Although the IVGTT is unphysiological it has the advantage of avoiding differences in absorption rates of glucose and the interferences evoked by gastro-intestinal tract stimuli affecting insulin release. In response to a maximal stimulatory dose of 20 gram glucose IV (Ward et al, 1984) it was noted that there was a rapid rise in insulin levels within one to three minutes after glucose injection in normal subjects. The first phase of insulin release is proportional to the rate and amount of the stimulus (Chen et al, 1975) and is believed to represent the release of stored insulin. Following the acute phase, there is a second phase of insulin release which rises more gradually and is directly related to the degree and duration of the glucose stimulus (DeFronzo et al, 1979). This second phase is thought to be due to de novo synthesis of insulin (Lerner and Porte, 1971).

All workers who have studied the insulin response to IV glucose in NIDDM, have concluded that the acutely releasable pool of insulin in NIDDM is depleted (Simpson et al, 1966; Perley and Kipnis, 1967;

Brunzell et al, 1976; Savage et al, 1979; Luft et al, 1981). Luft et al (1981) have indicated that the failure of the β cells to respond adequately to glucose is a genetic marker of NIDDM.

1.3.2.2. Insulin responses to non-glucose stimuli

The failure of the β cell to respond adequately to a glucose stimulus does not mean that it will not respond to other stimuli. In patients with NIDDM, it has been demonstrated that there is a selective inability to release insulin in response to a glucose load, whereas the early insulin release is preserved for non-glucose secretagogues (Lundquist et al, 1984). This suggests that the islet β -cell shows a relative blindness to hyperglycaemia.

i) Glucagon

It has been demonstrated that glucagon has an action on insulin secretion in normal human subjects (Samols et al, 1965; Karam et al, 1965). This is independent of the glycogenetic action of glucagon since it also occurs in patients with Type I glycogen storage disease (Crockford et al, 1966). Patients with NIDDM had higher insulin responses to a combined bolus of 25 gram glucose and 1 gram glucagon i.v. than to an intravenous bolus of glucose alone, confirming that glucagon releases insulin via a different mechanism than glucose (Simpson et al, 1966; Simpson et al, 1968).

ii) Sulphonylurea administration: tolbutamide

The hypoglycaemic effect of the sulphonylureas was first discovered in the early 1950's as a chance finding in the course of investigations concerning the anti microbial properties of modified sulphonamides.

Studies have shown that the acute administration of sulphonylureas resulted in increased secretion of insulin (Pfeiffer et al, 1959). However, there have been conflicting results obtained during the IV

tolbutamide administration (1 gram). While Yalow et al (1960) reported a less marked insulin rise as compared with IV glucose administration in NIDDM, Varsano-Aharon et al (1970) showed that their diabetics had a higher insulin response with tolbutamide than with an IV glucose stimulus. Most other workers (Perley and Kipnis; 1966; Deckert and Mogensen 1970) have confirmed that tolbutamide administration stimulated a greater degree of insulin release than glucose in patients with NIDDM. According to Lundquist et al (1984) sulphonylureas are a more potent stimulant of insulin release than glucose and glucagon.

iii) Amino acid stimulation.

Most amino acids have been shown to stimulate insulin release; the two most potent being arginine and leucine (Palmer et al, 1975; Floyd et al, 1968). An optimal stimulatory dose of arginine was found to be 2,5 gram by the intravenous route (Palmer et al, 1975). Patients with NIDDM were found to have apparently normal insulinaemic responses to IV arginine as compared with reference subjects (Palmer et al, 1976; Pfeifer et al, 1981; Savage et al, 1979). These findings are at variance with the findings of Fajans et al (1972) who demonstrated greater insulin responses in healthy controls as compared with mildly non-insulin-dependent diabetic patients.

It has been suggested, that for arginine to adequately release insulin, a pre-existing hyperglycaemia should prevail (Halter et al, 1979). Levin et al (1971) and Palmer et al (1975) have given patients with NIDDM a prior infusion of glucose and this enhanced the arginine induced insulin release. This effect of hyperglycaemia to augment the acute insulin response to a non-glucose secretagogue is termed "glucose potentiation" (Ward et al, 1984). It can be seen that although the β cell is acutely hypo-responsive to glucose stimulation in NIDDM, it remains more responsive to non-glucose secretagogues.

1.3.3. Insulin Resistance in NIDDM

Himsworth (1936) noted that not all patients with diabetes were insulin deficient and that insensitivity to the action of insulin played an important role in the pathogenesis of NIDDM. This was followed by the observation made by Yalow and Berson (1960) who stated that "the tissues of the maturity onset diabetic do not respond to his insulin as well as the tissues of the non-diabetic respond to his insulin". In the broadest sense insulin resistance may be defined as being present whenever normal concentrations of insulin elicit a less than normal biologic response (Kahn, 1978). It is presently known that insulin resistance is a cardinal feature of patients with NIDDM (Olefsky, 1981; Olefsky and Kolterman, 1981; Kolterman et al, 1981; DeFronzo et al, 1979; Beck-Nielsen, 1978; Alford et al, 1971; Ginsberg et al, 1975). Available information indicates that this is largely due to a cellular defect in insulin action, that is, receptor and post receptor defects (Olefsky, 1976).

Whereas insulin resistance is a characteristic feature of patients with NIDDM, in the limited studies to date there are contrasting views regarding insulin resistance in NIDDM. Gelehrter et al (1981) and Thorell et al. (1975) found no evidence of insulin resistance in the patients with NIDDM, while Jialal and co-workers (1984b) demonstrated the presence of insulin resistance in NIDDM subjects.

1.3.4. Measurement of Insulin Resistance

1.3.4.1. In vivo techniques

Various methods have been used to confirm the presence of insulin resistance in NIDDM namely the insulin tolerance test, the quadruple infusion and the insulin clamp technique.

The insulin tolerance test suffers from two main drawbacks: the res-

ponse to insulin-induced hypoglycaemia is unpleasant; the counter-regulatory hormones secreted in response to hypoglycaemia blunt the fall in plasma glucose concentration. Despite these theoretical problems, this method has been used extensively (Stocks and Martin, 1969; Alford et al, 1971; Lebovitz et al, 1977; Beck-Nielsen et al, 1979; Jialal et al, 1984b).

In the quadruple-infusion technique: adrenaline, propanolol, glucose and insulin are administered at a constant rate (Shen et al, 1970). Endogenous insulin secretion is inhibited by adrenaline and the stimulatory effect of adrenaline on hepatic glucose production is assumed to be blocked by propanolol and insulin. However, as adrenaline itself is a potent peripheral insulin antagonist (Altszuler et al, 1967), the validity of these test findings are suspect.

During the insulin clamp test (De Fronzo et al, 1979a) the plasma insulin concentration is acutely raised and maintained at approximately 100 $\mu\text{u/ml}$ above basal by a continuous infusion of insulin and the plasma glucose concentration is held constant at the basal level by a variable glucose infusion. Here the drawback is that hyperglycaemia enhances insulin-mediated glucose uptake (Cherrington et al, 1978).

Despite the criticisms stated above, the fact remains that all methods used to measure insulin resistance have produced the same answer: there is apparently insulin resistance in NIDDM.

1.3.4.2. In vitro methods

The first step in insulin action is binding to its glycoprotein membrane receptor on the surface of target cells (Kahn, 1979). The major functions of the insulin receptor are to recognise and bind insulin with high affinity, specificity and saturability, and to transmit a transmembrane signal to initiate the biological functions

of the hormone. Glucose utilization in its many forms is one result. (Kahn, 1982).

It is currently believed that the insulin receptor is a dimer composed of 2 alpha (α) and 2 beta (β) subunits linked by disulphide bonds as a complex with an estimated molecular weight of 350,000 daltons (Czech et al, 1981; Jacobs and Cautrecasas, 1983). The α subunit is primarily externally orientated and contains the insulin binding site as demonstrated by a number of affinity labelling techniques and has an apparent molecular weight of 135 000 (Czech et al, 1981; Jacobs and Cautrecasas, 1983). The β subunit appears to be a transmembrane protein with kinase activity and an apparent molecular weight of 95 000 (Czech et al, 1981; Jacobs and Cautrecasas, 1983; Kasuga et al, 1982).

The major clinical application of studies of the insulin receptor is to elucidate the mechanisms of insulin action and define the role of insulin receptor aberrations in states of insulin resistance. Although there are numerous causes of insulin resistance, the most extensively studied conditions have been obesity and NIDDM.

Obesity is associated with hyperinsulinism and insulin resistance (Olefsky, 1981; Reaven, 1983; Flier, 1983). The chronic hyperinsulinism leads to a downregulation of the insulin receptors (Olefsky, 1981; Flier, 1983 and Kahn, 1980). In general, raised concentrations of insulin are associated with a reduced number of insulin receptors on the cell surface; this insulin-induced receptor loss is termed down-regulation (Kahn, 1980). Obese subjects with mild insulin resistance and mild hyperinsulinaemia have only receptor defects, manifesting as a decrease in receptor number (Kahn, 1980; Flier, 1983). In subjects with more pronounced hyperinsulinism and insulin resistance both receptor and post-receptor defects co-exist (Kahn, 1980; Olefsky et al,

1982). The affinity of the insulin receptor in the insulin resistant obese individual, is normal. The major factor that appears to be regulating the insulin receptor in obesity appears to be the circulating level of insulin, since correction of the hyperinsulinism by diet, or streptozotocin therapy results in the correction of the receptor defect. (Kahn, 1980.; Flier, 1983; Olefsky et al, 1982). It should be noted that the decrease in insulin receptor number has been demonstrated in a wide variety of tissues: liver, muscle, adipocyte, monocytes and erythrocytes: from both obese humans and animals (Kahn, 1980; Flier, 1983, Olefsky et al, 1982).

Obesity cannot account entirely for the insulin resistance of NIDDM, since not all patients with NIDDM are obese and many of these non-obese patients with NIDDM are also insulin resistant (Kahn, 1980; Olefsky, 1981; Olefsky, 1982; Flier, 1983;.. As with obesity, it appears that the insulin resistance in NIDDM manifests as target tissue defects (Kahn, 1980; Olefsky 1981; Flier, 1983). Despite the greater severity of insulin resistance in NIDDM, the severity of ^{125}I -insulin binding defect is similar in patients with NIDDM and subjects with the milder disorder of impaired glucose tolerance (IGT) (Kahn, 1980; Olefsky et al, 1982; Flier, 1983). In both NIDDM and subjects with IGT, the receptor defect manifests as a decrease in the number of receptors which correlates inversely with the fasting hyperinsulinaemia (Kahn, 1980; Olefsky, 1981; Flier, 1983). Since the degree of insulin resistance, as assessed by *in vivo* techniques, is greater in patients with NIDDM as compared to those with IGT, it is held that the insulin resistance is also related to post-receptor defects (Kahn, 1980; Olefsky, 1981; Olefsky et al, 1982). Support for this contention is the finding that the *in vivo* assessment of insulin resistance correlates poorly with the deficits in the number of cell surface insulin receptors in patients with NIDDM, while in subjects with IGT, the degree of receptor

deficit is directly correlated with the degree of insulin resistance (Kahn, 1980; Flier, 1983; Olefsky et al, 1982; Kaplan, 1984). In NIDDM ¹²⁵I-insulin binding is decreased on adipocytes, circulating monocytes and erythrocytes but normal on fibroblasts in culture (Flier, 1983). This suggests that the insulin resistance is secondary to some in vivo metabolic aberration. As hyperinsulinism significantly downregulates the insulin receptor, this causes major problems in the study of circulating blood cells, namely, erythrocytes and monocytes. On the other hand, fibroblasts in culture are removed from diabetics and are thus not influenced by high circulating insulin levels. This affords one of the major advantages of cultured cells.

A review of the literature confirms that patients with NIDDM have defects in insulin secretion and insulin action. In the discrete genetic syndrome of NIDDM (which is most probably inherited in an autosomal dominant fashion) the situation is less clear. These aspects will be the subject of this thesis.

THE PRESENT STUDY

CHAPTER 2

CLINICAL AND BIOCHEMICAL FEATURES OF INDIAN PATIENTS

WITH NIDBY

2.1 PURPOSE OF STUDY

Nidby as a clinical syndrome amongst South African Indians has been described in detail (Jialal et al. 1982a). The most salient findings to emerge from Jialal's study was the increased prevalence of microvascular complications (19%); the positive family history in 84% and evidence of three generation transmission of the disorder through one parent in 7% of his patients. Three generation transmission of non-insulin-dependent diabetes argues strongly for autosomal dominant type of Mendelian inheritance. Quite obviously, if NIDBY is indeed inherited on this basis, the mode of inheritance is much simplified and hence it should be potentially possible to identify a true genetic marker for the disorder.

It is self evident that one of the great problems in studying diabetes has been the lack of a marker which identifies the potential diabetic. Having identified a diabetic disorder in which the inheritance is apparently autosomal dominant, a golden opportunity presents itself to attempt to identify a genetic marker. Hence in this study, patients with NIDBY who have an unequivocal inheritance of the disorder through one parent for at least three successive generations, will be studied in detail.

2.2 PATIENTS

Patients used in this study were selected from those attending the diabetic clinics of B.K.Khen and King Edward VIII Hospitals to which the author has been attached since 1982.

2.2.1. Criteria for diagnosis of NIDDM and selection of patients.

The patients were categorized as having NIDDM if they met the following criteria: age at diagnosis of diabetes < 30 years; symptomatic but aketonuric presentation; prevention of ketonuria and control of symptoms without insulin therapy; duration of diabetes for ≥ 2 years. Diabetes was diagnosed according to World Health Organisation criteria (WHO 1980): fasting plasma glucose ≥ 8 mmol/l and/or 2 hour plasma glucose ≥ 12 mmol/l after 100 grams oral glucose load. Over a three year period (1982-1985), 12 Indian patients with NIDDM who belonged to six families in which non-insulin-dependent diabetes was transmitted through three generations by one parent, were studied. All assenting living siblings, their parents and grandparents were studied, after obtaining informed consent. In one family, however, the 5 non-diabetic siblings did not agree to testing. They were all non-diabetics, as they had undergone testing in private clinics (Table 2.1).

2.3. STUDY PROCEDURE

2.3.1. History and Clinical Examinations

A careful history was taken from all patients. For the present study, age at which symptomatic diabetes was first positively diagnosed was noted. A detailed family history was taken and only families in which diabetes was transmitted via one parent through three generations were studied. All patients were questioned about symptoms which could suggest the presence of vascular complications of diabetes (angina pectoris, intermittent claudication, strokes, visual disturbances and urinary symptoms).

Full systemic examination was performed on all patients by the author and a physician. The aspects relevant to this study were sex, height (m), mass (kg), systolic and diastolic blood pressure, evidence of

neuropathy, cerebrovascular disease, ischaemic heart disease and peripheral vascular disease. Retinal changes were recorded on fundal examination after full mydriasis and a routine urinary examination for glucose, ketones and protein performed. Furthermore, all patients were typed for human leucocyte antigens (HLA) on the A,B,C and DR loci. HbA_{1c} was used as a cumulative index of diabetic control.

2.3.2. Special Investigations

A 12 lead ECG was recorded in all patients. In addition fundal examinations were performed in the clinic. Renal function was assessed in all patients by 24 hour urine protein excretion, serum creatinine and glomerular filtration rate (GFR).

2.4 METHODS

Methods used to determine plasma glucose, creatinine, urinary protein and HbA_{1c} are described in Appendix A-1 - A-4. GFR was measured by ⁵¹Cr-EDTA technique [Chantler et al, 1969].

Body mass index (BMI) was calculated from the patients weight and height according to Bray (1978). Obesity was defined as a BMI of ≥ 25 in males and ≥ 27 in females (National Diabetes Data Group, 1979).

Diabetic nephropathy was defined as an increase in urinary protein excretion ≥ 0.5 g/24 hours in the absence of cardiac insufficiency or renal tract infection (Mogensen et al, 1981).

The WHO definition of hypertension was adopted (Strasser, 1972):

systolic blood pressure ≥ 160 mmHg and/or diastolic b.p. ≥ 95 mmHg

in subjects over 30 years; in subjects < 30 years, values were taken

as ≥ 150 mmHg systolic and/or ≥ 90 mmHg diastolic. A patient was labeled

hypertensive when increased blood pressure was recorded on 2

different occasions at least one month apart.

The HLA studies were performed in collaboration with the Natal Institute of Immunology (M.G. Hammond). HLA-A, B, and C antigens were measured by the method of Terasaki and McLeod (1964) while the DR antigens were isolated according to Danilovs et al (1980).

2.5. RESULTS

2.5.1. Sex Ratio, Age at onset, Duration of Diabetics, Body Mass

Indices and Family History of Patients with NIDDM.

In all, 13 diabetics and 20 non diabetic family members were studied. The pedigrees are outlined in Table 2.1.

All patients presented with symptomatic diabetes: polyuria, polydipsia, lethargy, skin infections and pruritis vulvae in females. Of the 13 patients studied, 12 were female. The mean age at which diabetes was positively diagnosed was 20,6 years (12-29 years). The mean duration of diabetes was 12,1 years, ranging from 2-40 years at the time of study. The mean BMI in the diabetics was 25,2 kg/m² (19-39). Four of the thirteen diabetics were obese (31%).

In the 20 non-diabetic family members studied (12 males and 8 females) the mean age was 29,7 years (11-58 years) and the mean BMI was 22,3 (19-26 kg/m²). Two of the non-diabetics were obese (10%). These results are set out in Table 2.2.

Of the thirteen patients with NIDDM, 8 were third generation- 4 were second generation- and 1 was a first generation diabetic. It is interesting to note that in four of the six families studied, the mother was the affected parent. Only 36% of the siblings tested were diabetic.

2.5.2. Biochemical Findings

The mean fasting plasma glucose levels in patients with NIDDM were 13,2 \pm 1,4 as opposed to a mean level of 4,6 \pm 0,2 mmol/l ($p < 0.001$).

Table 2.1

Family trees of patients with NIDDM.

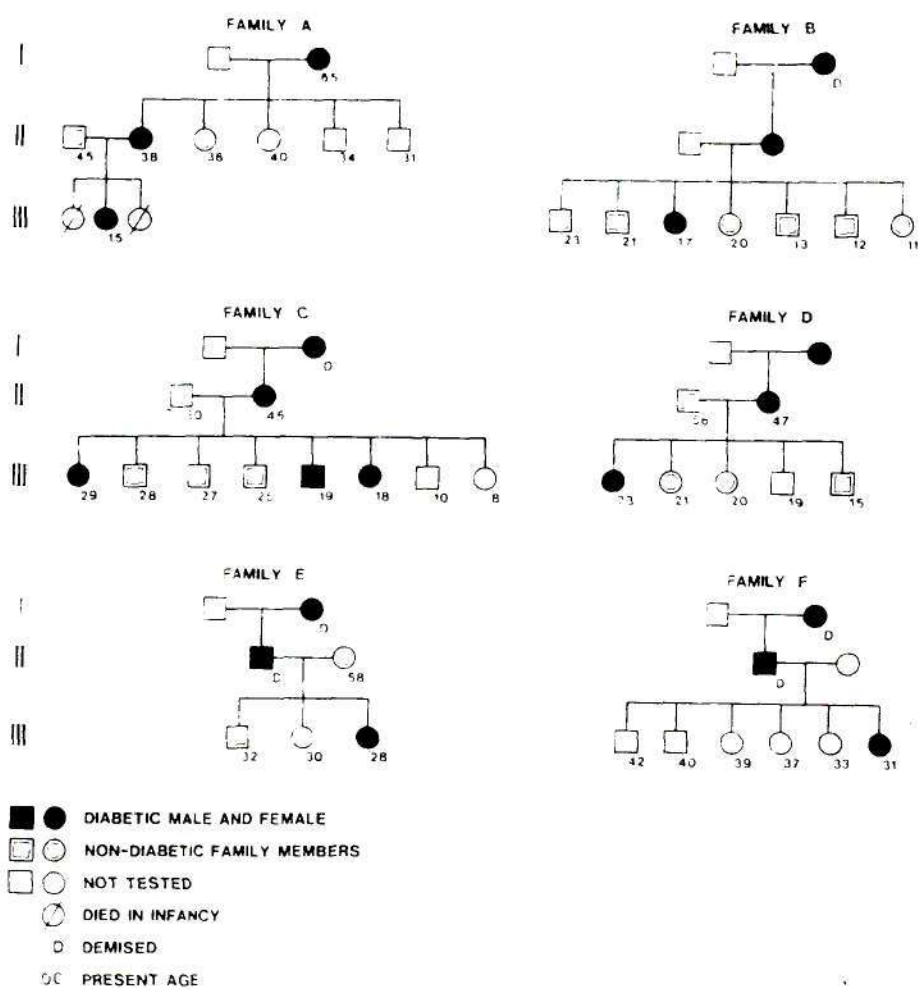


TABLE 2.2
THE CLINICAL AND BIOCHEMICAL FEATURES OF PATIENTS
WITH NIDDIY AND NON-DIABETIC FAMILY MEMBERS

| FAMILY | AGE (YEARS) | SEX | BMI ₋₂ (KG/M ²) | HbA ₁ | DURATION OF DIABETES (YEARS) | AGE AT ONSET OF DIABETES (YEARS) |
|--------------------------------|----------------|-----|---|------------------|------------------------------------|--|
| L.N. A ₁ | 65 | F | 24 | 11,9 | 40 | 25 |
| (A.N.) A ₂ | 38 | F | 27 | 12,5 | 20 | 18 |
| (N.N.) A ₃ | 15 | F | 39 | 9,5 | 2 | 13 |
| (A.S.) B ₁ | 48 | F | 22 | 8,0 | 20 | 28 |
| (C.S.) B ₂ | 17 | F | 21 | 6,3 | 5 | 12 |
| (M.C.) C ₁ | 45 | F | 23 | 10,0 | 18 | 27 |
| (S.C.) C ₂ | 19 | M | 19 | 16,0 | 2 | 17 |
| (H.C.) C ₃ | 18 | F | 22 | 5,4 | 2 | 16 |
| (J.M.) C ₄ | 29 | F | 25 | 8,2 | 8 | 21 |
| (S.D.) D ₁ | 49 | F | 29 | 7,9 | 20 | 29 |
| (K.D.) D ₂ | 23 | F | 29 | 6,5 | 3 | 20 |
| (A.P.) E | 28 | F | 24 | 8,0 | 8 | 20 |
| (T.E.) F | 31 | F | 23 | 8,9 | 9 | 22 |
| MEAN | 32,7 | | 25,2 | 9,2 | 12,1 | 20,6 |
| RANGE | (15-65) | | (19-39) | (6,3-16) | (2-40) | (12-29) |
| NON-DIABETIC FAMILY MEMBERS | | | | | | |
| MEAN | 29,7 | | 22,3 | 5,2 | - | - |
| RANGE | (11-58) | | (19-26) | (4,3-8,7) | - | - |

in the non-diabetic family members. These glucose levels were high as diabetics were off treatment for 72 hours prior to testing. The diabetics were all on a diet as well as oral hypoglycaemic agents. Most patients were on a combination of sulphonylureas and biguanides. None of the family members had any evidence of impaired glucose tolerance.

Renal function as assessed by 24 hour urinary protein, serum creatinine and GFR was abnormal in only one patient (Table 2.3)

Glycosylated haemoglobin levels were used as an index of glycaemic control. These were measured at six monthly intervals during the 3 years over which this study took place. Levels were higher in diabetics than in the non-diabetic family members [9,2 (6,3 - 16,0) : 5,2 (4,3 - 8,7%)]. The three patients with retinopathy had considerably elevated HbA_{1c} levels. (Table 2.2)

2.5.3 Diabetic Complications

To date, none of the patients in this series, have presented with diabetic ketoacidosis or hyperosmolar non-ketotic coma.

Only three of the thirteen patients (23%) had vascular complications. The duration of diabetes in these patients ~~were~~ 40 years, 20 years and 18 years, respectively. In all three microvascular complications presented as background and proliferative retinopathy while only one patient had evidence of nephropathy. This patient also had macrovascular disease which manifested as ischaemic heart disease (angina pectoris and ECG changes consistent with ischaemia). No patient had peripheral vascular disease or evidence of a cerebrovascular accident.

Furthermore, two of the patients with retinopathy were hypertensive (Table 2.3)

TABLE 2.3

COMPLICATIONS OF PATIENTS WITH NIDDDY

| PATIENTS | RETINOPATHY (PROLIFERATIVE AND BACKGROUND) | NEPHROPATHY | NEUROPATHY | HYPER TENSION | MACROVAS- CULAR DIS- EASE (I.H.D.) |
|----------------|--|-------------|------------|------------------|--|
| A ₁ | + | + | + | + | + |
| A ₂ | + | + | + | + | + |
| A ₃ | — | + | + | + | + |
| B ₁ | — | — | + | — | — |
| B ₂ | + | — | + | — | — |
| C ₁ | + | — | + | + | — |
| C ₂ | — | — | — | — | — |
| C ₃ | — | — | — | — | — |
| C ₄ | — | — | — | + | — |
| D ₁ | — | — | — | — | — |
| D ₂ | — | — | — | — | — |
| E | — | — | — | — | — |
| F | — | — | — | — | — |

Peripheral neuropathy as diagnosed by history and clinical examination was present in 4 patients (31%).

All patients were prone to infections and the commonest ones encountered were monilial vaginitis, urinary tract infections and infections of the skin. These infections were treated appropriately with anti-bacterial and anti-fungal agents. However, none of the patients were precipitated into ketosis by these infections.

2.5.4. The HLA Status of NIDDM.

HLA studies were performed in eleven of the patients as well as thirteen non-diabetic family members. The results are depicted in Table 2.4. It can clearly be seen that in the families studied, NIDDM did not segregate with any specific HLA type.

2.5.5. Discussion

In this study, all six Indian families had a three generation transmission of non-insulin-dependent diabetes and thus all eight probands had a diabetic parent. However, only 36% of siblings tested had diabetes. While the findings of diabetes in the parents of the probands (100% prevalence) accords with that of Tattersall and Fajans (85%) (1975), the occurrence of diabetes in only 36% of siblings tested is lower than the 53% prevalence of diabetes in the siblings studied by these workers. In a previous study (Panzram and Adolph, 1981) only 14% of the siblings of probands with non-insulin-dependent diabetes in the young were diabetic. These workers interpreted their data as evidence for genetic heterogeneity in patients with NIDDM. However, even though the sample size is small it seems probable that this syndrome is inherited as a Mendelian dominant mode: every affected person had an affected parent; there was direct transmission from parent to

TABLE 2.4
HLA-HAPLOTYPES IN FAMILIES WITH THREE GENERATIONS
OF NIDDY

FAMILY A

| BMI IN KG/M ² | AGE IN YEARS | | | | |
|-----------------------------|-----------------|-----|-------------|----------------------------|---|
| 24 | 65 | 1.1 | Grandmother | NIDDY (A ₁) | a A2 Cw-- B60 DR2 e A-- Cw-- B44 DR7 |
| 27 | 38 | 2.1 | Mother | NIDDY (A ₂) | a A2 Cw-- B60 DR2 b A1 Cw1 B55 DR1 |
| 22 | 36 | 2.2 | Aunt | Normal | a A2 Cw-- B60 DR2 f A-- Cw-- B44 DR7 |
| 26 | 45 | 2.3 | Father | Normal | c A24 Cw-- B35 DR4 d A-- Cw-- B58 DR-- |
| 39 | 15 | 3.1 | Child 1(F) | NIDDY (A ₃) | b A1 Cw1 B55 DR1 d A24 Cw-- B35 DR4 |
| FAMILY B | | | | | |
| - | - | 1.1 | Grandmother | NIDDY NT* | |
| 22 | 48 | 2.1 | Mother | NIDDY (B ₁) | a A2 C-- B51 DR-- b A28 C-- B8 DR3 |
| 25 | 52 | 2.2 | Father | Normal | c A2 Cw1 B37 DR10 d A1 Cw6 B57 DR7 |
| 22 | 23 | 3.1 | Child 1(M) | Normal | b A28 Cw-- B8 DR3 c A2 Cw1 B37 DR10 |
| 20 | 21 | 3.2 | Child 2(M) | Normal | a A28 Cw-- B8 DR3 d A1 Cw6 B57 DR7 |
| 21 | 17 | 3.3 | Child 3(F) | NIDDY (B ₂) | b A28 Cw-- B8 DR3 c A2 Cw1 B37 DR10 |
| 19 | 13 | 3.4 | Child 4(M) | Normal | b A28 Cw-- B8 DR3 c A2 Cw1 B37 DR10 |
| 19 | 11 | 3.5 | Child 5(M) | Normal | a A2 Cw-- B51 DR-- d A1 Cw6 B57 DR7 |
| 20 | 10 | 3.6 | Child 6(F) | Normal | b A28 Ce-- B8 DR3 d A1 Cw6 B57 DR7 |

FAMILY C

| BMI IN KG/M ² | AGE IN YEARS | | | | |
|-----------------------------|-----------------|-----|-------------|----------------------------|--|
| - | - | 1.1 | Grandmother | NIDDY NT* | |
| 23 | 45 | 2.1 | Mother | NIDDY (C ₁) | a A28 Cw-- B52 DR2 b A31 Cw-- B51 DR2 |
| 25 | 48 | 2.2 | Father | Normal | a A1 Cw-- B60 DR2 d A1 Cw-- B60 DR10 |
| 25 | 29 | 3.1 | Child 1(F) | NIDDY (C ₄) | a A28 Cw-- B52 DR2 c A1 Cw-- B60 DR2 |
| 19 | 19 | 3.2 | Child 2(M) | NIDDY (C ₂) | b A31 Cw-- B51 DR2 c A1 Cw-- B60 DR2 |
| 22 | 18 | 3.3 | Child 3(F) | NIDDY (C ₃) | b A31 Cw-- B51 DR2 d A1 Cw-- B60 DR10 |
| FAMILY D | | | | | |
| - | - | 1.1 | Grandmother | NIDDY NT* | |
| 29 | 49 | 2.1 | Mother | NIDDY (D ₁) | a A1 Cw-- B62 DR-- b A1 Cw-- B57 DR7 |
| 26 | 58 | 2.2 | Father | Normal | c A33 Cw-- B61 DR2 d A1 Cw-- B17 DR7 |
| 22 | 24 | 3.1 | Child 1(F) | Normal | b A1 Cw-- B57 DR7 c A33 Cw-- B61 DR2 |
| 29 | 23 | 3.2 | Child 2(F) | Niddy (D ₂) | a A1 Cw-- B62 DR-- c A33 Cw-- B61 DR2 |
| 22 | 21 | 3.3 | Child 3(F) | Normal | b A1 Cw-- B57 DR7 c A33 Cw-- B61 DR2 |
| 22 | 15 | 3.4 | Child 4(M) | Normal | b A1 Cw-- B57 DR7 c A33 Cw-- B61 DR2 |

*NT : Not Tested (Deceased)

M : Male

F : Female

child through three generations. The fact that only 36% of the siblings were affected as opposed to 53% reported by Tattersall and Fajans (1975) is most probably due to autosomal dominant inheritance with incomplete penetrance.

Rigid diagnostic criteria adopted in the selection of patients, confirms that NIDDM is a definite entity in the spectrum of diabetic types found in the local Indian community. The choice of 30 years as the upper limit of youth was necessary for broad comparative purposes (Fajans and Conn, 1960; Tattersall, 1974; Fajans et al 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978; Panzram and Adolph, 1981).

As common with most other reports on this condition, women were the dominant sex (Campbell 1960; Tattersall, 1974; Barbosa et al, 1978; Jialal et al, 1982a), suggesting sexual dimorphism in the prevalence of NIDDM.

An aspect which should be stressed is that diabetes was first diagnosed at a mean age of 20.6 years, the youngest patient being 12 years old. NIDDM in the Indian community is therefore essentially a disorder found in young adults and not in children, confirming the observation of Jialal et al (1982a).

In the present series of thirteen patients only four were obese by definition (31%). This finding accords with most previous reports on NIDDM, in which the percentage of obese patients was similar (Tattersall, 1974; Fajans et al, 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978). However, the incidence of obesity was 55% in the studies carried out by Jialal et al (1982a). Jackson (1978a) pointed out that in the local Indian population, increasing body mass is associated with an increasing frequency of diabetes. This finding is borne out in the present study, in that only two (10%) of the non-diabetic

family members were obese. It would thus appear that obesity is a definite risk factor in the development of diabetes.

A cardinal finding in this study was the fact that not a single non-diabetic family member had any evidence of impaired glucose tolerance. In other words, subjects were frankly diabetic or had no impairment of glucose tolerance at all. This aspect will be discussed in greater detail in the chapter to follow.

As a group, the diabetics had a mean HbA_{1c} level of 9.2% although this ranged from 6.3 - 16%. In this study, HbA_{1c} levels proved valuable as an index of the control achieved in the patients. The mechanism of protein glycosylation is a post translation, non-enzymatic reaction which yields HbA_{1c} as a major end product. This HbA_{1c} adequately reflects the exposure of HbA to the average blood glucose levels and therefore the control achieved. (Svensen et al, 1980; Goldstein et al, 1980) Perhaps of greater importance is that HbA_{1c} levels were related to the vascular complications in this group of patients. In the three patients with retinopathy (one of whom had nephropathy), HbA_{1c} levels were considerably elevated (mean 11.5%). There is good evidence that the basement membrane of the glomeruli is enriched by a glycoprotein (Brownlee et al, 1984) and it is possible that high blood glucose values affect the micro-vasculature adversely and relate aetiologically to microvascular complications. The only male patient in this series, who was subsequently put onto insulin therapy because of a secondary failure on oral hypoglycaemic agents, had a mean HbA_{1c} level of 16%. This has subsequently dropped to 7% on insulin therapy. While fasting plasma glucose measurements are subject to wide fluctuations dependent on the time of day, exercise and diet, HbA_{1c} levels afford an integrated measure of the diabetic state for the preceding two months and are independent of diet.

The overall prevalence of vascular complications was 23% in the patients with NIDDM, which is significant and supports the impression expressed by Jackson (1978b) that vascular complications are not uncommon in Indian patients with NIDDM. This percentage also compares favourably with the 19% reported by Jialal et al in a large series of 85 patients (1982b). Mean age and duration of diabetes in the patients with vascular complications were greater than that of most of the other patients. Nevertheless it should be noted that two other patients had a duration of diabetes of 20 years and yet had no evidence of vasculopathy. (Table 2.2) Microvascular involvement was manifestly the commonest complication. This finding contrasts with the experience of Fajans et al (1978) who found that 16 of their 69 patients had macrovascular disease, whereas only 9 had microvascular involvement.

On the basis of the criteria used, only one of the thirteen diabetics had evidence of nephropathy. In most series, the investigators failed to report renal involvement in any of their patients. (Tattersall, 1974; Tulloch and Adamson, 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978). In the series of Jialal et al (1982b) the incidence of nephropathy was 7%. Cognisance should be taken of the fact that Jialal utilized more refined techniques than the other authors in his search for nephropathy.

Macroangiopathy was found in only one patient in the present study, which is much lower than the 23% reported by Fajans et al (1978) and is similar to most other reports on NIDDM (Tattersall, 1974; Tulloch and Adamson, 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978; Koberling et al, 1980 and Jialal et al, 1982b).

The presence of hypertension in two patients (15%) appears to be no different from the 12% prevalence reported by Seedat et al (1978) in the Indian population of Durban. The only report on the incidence of

hypertension in NIDDM is that of Jialal (1982b) in which 18% of patients were hypertensive. A significant finding in the present study is that two of the three patients with vascular complications were hypertensive, suggesting that hypertension is an additional risk factor in the development of vascular complications in NIDDM.

The commonest chronic complication in NIDDM was neuropathy (31%) and this is in agreement with studies in Indian patients with NIDDM (Thandroyen et al, 1980; Asmai et al, 1981; Jialal et al, 1982b). A review of the literature reveals only a further 3 NIDDM patients with neuropathy (Soler et al, 1969; Tattersall, 1974; Bigler and Adler, 1981). It would appear that the prevalence of neuropathy in Indian NIDDM is far commoner than that reported in White patients with NIDDM. This could possibly be attributed to the more severe degree of hyperglycaemia in Indian patients.

Nelson and Pyke (1976) HLA-typed thirteen diabetics (MODY) as well as nine non-diabetic family members for A, B, C and D antigens. They found that there was no association between MODY and the HLA system. Others (Faber et al, 1978; Platz et al, 1982) confirmed these findings in two larger MODY pedigrees. Barbosa (1977) and Barbosa et al (1978a) carried out studies in patients with MODY that strongly suggested a linkage between the HLA haplotype A3, BW15 and the hyperglycaemic trait. However, after HLA typing ten large MODY families Barbosa (1983) refuted his two earlier findings and concluded that MODY was neither associated nor linked to the HLA system. In the present study, a further antigen HLA-DR was also measured and similar findings were observed in this group of Indian patients. Therefore we have shown that there is no relationship between NIDDM and the HLA A,B, C and DR antigens.

2.5.6. Conclusions

Using strict defined criteria, only patients belonging to families in which NIDDM was transmitted through three successive generations were investigated. This family history strongly suggests autosomal dominant inheritance with incomplete penetrance as only 50% of the siblings tested were diabetic. The presence of obesity in 71% of the diabetics would indicate that obesity is a definite risk factor in the development of diabetes.

While complications were rather mild, the commonest vasculopathy was retinopathy. Despite the proneness to intercurrent infections, not a single patient presented with ketonuria, which is an unusual feature as the group were severe diabetics. The commonest chronic complication was peripheral neuropathy.

The diabetics' responses to diet and oral hypoglycaemic agents were good as evidenced by their HbA_{1c} levels.

Finally, in the discrete genetic syndrome of NIDDM there was no association between the hyperglycaemia and the HLA system.

In conclusion we have demonstrated that this group of patients differ in no way from those described by Jialal et al (1982a and 1982b), in that their clinical and biochemical features as well as complications were similar. As the sample represents a genetically pure group, it would serve as a model to find a genetic marker for this syndrome.

CHAPTER 3

THE INSULIN AND C-PEPTIDE RESPONSES TO ORAL AND INTRAVENOUS
GLUCOSE IN PATIENTS WITH NIDDDY3.1 THE INSULIN AND GLUCOSE RESPONSES TO A 100 GRAM ORAL
GLUCOSE LOAD.

3.1.1. Purpose of Study

The clinical features of NIDDDY, as seen in the group of Indian patients selected, were considered in detail in the preceeding chapter. All patients belonged to families in which non-insulin-dependent diabetes was transmitted through three successive generations via one parent. One of the cardinal features of NIDDDY as described in the local Indian population concerns the apparent absence of antecedent periods of glucose intolerance progressing to frank diabetes as defined by WHO criteria. The families collected, presented an ideal opportunity to investigate whether the natural history of this form of diabetes progressed from glucose intolerance through to diabetes. Accordingly the insulin secretory status of the patients and 20 non-diabetic family members were studied in depth.

3.1.2. Patients and Non-Diabetic Family Members.

The 13 patients with NIDDDY described in the previous chapter are under consideration. Twenty non-diabetic family members consented to undergo glucose tolerance testing. Although the two groups were within similar age groups [32,7 (15-65); 29,7 (11-58)years; $p > 0,5$], the diabetics had a significantly higher BMI than the non-diabetics [25,2 (19-39); 22,3 (19-26) kg/m^2 ; $p < 0,05$]

3.1.3. Study Procedure

Since 12 of the 13 patients were on oral hypoglycaemic agents and

nominally on low carbohydrate diets, they were instructed as to what a mixed diet containing at least 300g carbohydrate was (30% protein, 20% fat, 50% carbohydrate) by issuing them with diet sheets. They were requested not to take their medication and to follow the diet routine for 72 hours prior to glucose tolerance testing. All 13 patients had an oral glucose tolerance test (OGTT). The non-diabetic family members were also requested to have mixed diets for 3-5 days prior to the OGTT. All participants in this study were ambulant and were not on any medication known to affect carbohydrate metabolism. After an overnight fast of 12 hours, a cannula was inserted into a deep antecubital vein. The patency of the cannula was ensured by a slow infusion of normal saline. Fasting blood samples were withdrawn after a rest period of 30 minutes. Thereafter 100 grams(g) of glucose, dissolved in 250 mls water was taken by the oral route over a period of 5 minutes. Further blood samples were withdrawn at 30 minute intervals for a period of 3 hours, via the indwelling catheter. At each sampling time, the first 5 mls of blood obtained was discarded to prevent the dilution effect of the slow saline infusion, following which the "true" sample was collected. Blood samples were collected in potassium-oxalate-fluoride tubes for glucose estimation and in tubes containing no preservative for insulin assays.

3.1.4. Methods

The methods detailed in Appendix A-1 and A-5 were used to measure glucose and insulin concentrations respectively. From the glucose and insulin values, a number of indices were calculated.

- a) Areas under the insulin and glucose curves (Chiles and Tzagournis, 1970).

$$\text{Areas under curve } [1/2 (\text{fasting level}) + (1/2 \text{ hour level}) + 1\frac{1}{2} (\text{1 hour level}) + 2 (2 \text{ hour level}) + (3 \text{ hour level}) \times 30/1000]$$

- b. Insulin-glucose ratios (Perley and Kipnis, 1966)

$$\frac{\text{Plasma insulin } (\mu\text{U/ml})}{\text{Plasma glucose } (\text{mg\%})}$$

- c. The modified Seltzer insulinogenic index (M.S.I.) (Gleuck et al, 1969).

$$\text{MSI} = \frac{\text{Area under insulin curve}}{\text{Area under glucose curve}}$$

3.1.5. Results

3.1.5.1. Glucose and Insulin Responses

The mean plasma glucose response to 100g glucose taken orally in both the patients and non-diabetic family members are presented in Figure 3.1. It can be seen that the NIDDDY group had a mean fasting plasma glucose of $15,1 \pm 1,0$ mmol/l and were unequivocally intolerant to glucose. On the other hand, the non-diabetics had no evidence of glucose intolerance.

Insulin responses are graphically presented in Figure 3.2. In the fasting state, insulin levels were significantly higher in the patients with NIDDDY ($19,2 \pm 5,3$ $\mu\text{U/ml}$; $13,4 \pm 2,0$ $\mu\text{U/ml}$; $p < 0,05$). Following the glucose challenge, the response in the NIDDDY group was delayed and attenuated. By contrast, the non-diabetic family members had a mean peak insulin response of $101,0 \pm 3,8$ $\mu\text{U/ml}$ at 60 minutes. With the exception of the fasting state, at all other times, NIDDDY displayed decreased insulin levels in comparison to the non-diabetics. Only one patient with NIDDDY had increments in insulin levels that exceeded the mean ± 1 S.D. of the responses of the non-diabetics ($401,2 \pm 20,1$; $310 \pm 30,2$ $\mu\text{U/ml}$; $p < 0,01$).

3.1.5.2. Glucose and Insulin Areas.

The glucose and insulin areas are shown in Figure 3.3. While the area

FIGURE 3.1

PLASMA GLUCOSE RESPONSE TO 100G ORAL GLUCOSE LOAD
IN PATIENTS WITH NIDDM AND NON DIABETIC FAMILY MEMBERS

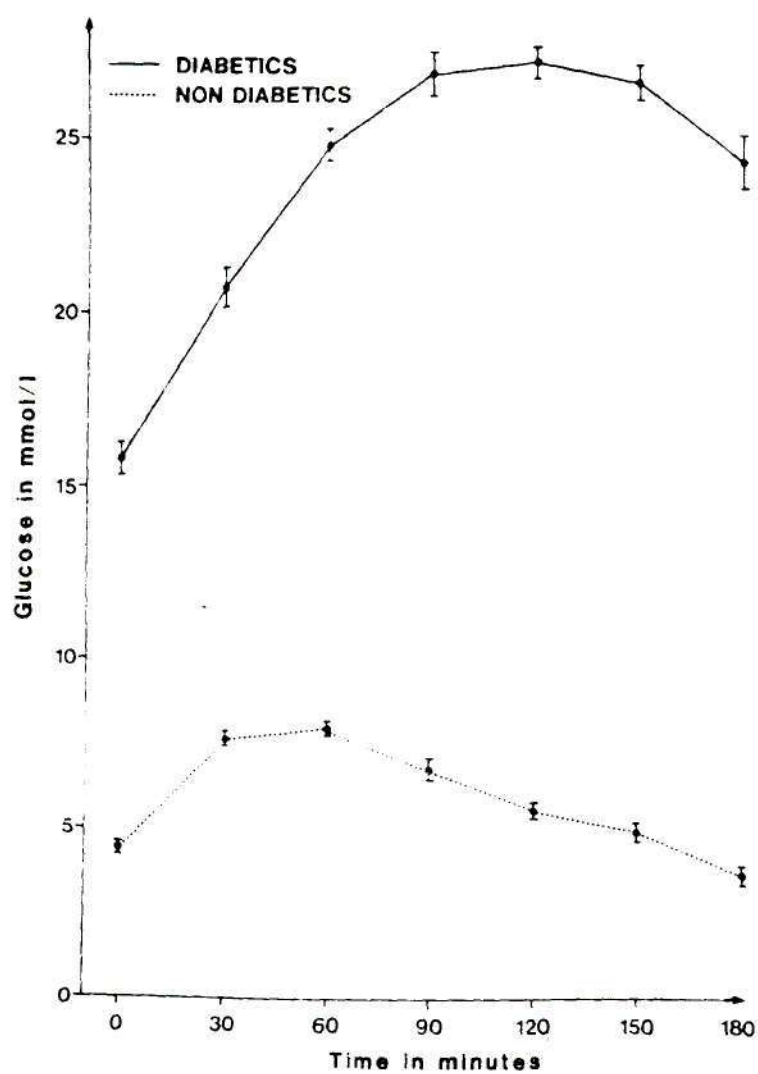


FIGURE 3.2

INSULIN RESPONSES TO 100G ORAL GLUCOSE IN NIDDY AND NON-DIABETIC
FAMILY MEMBERS

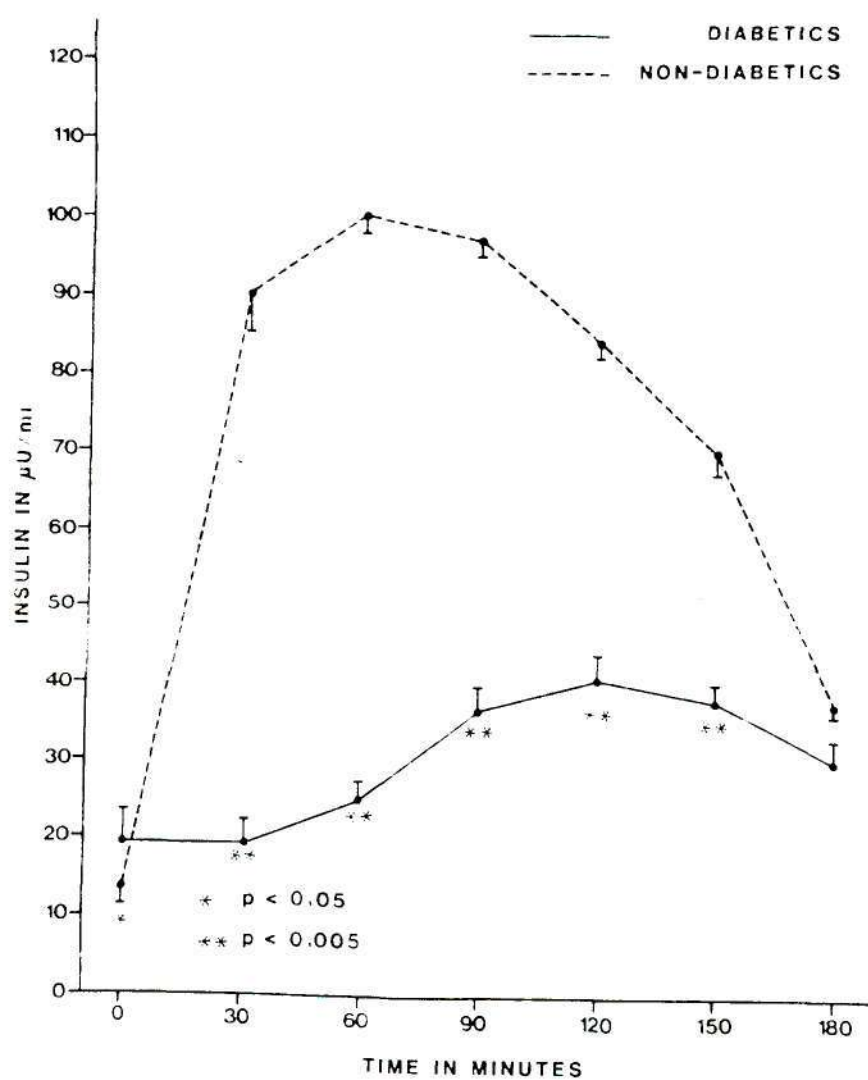
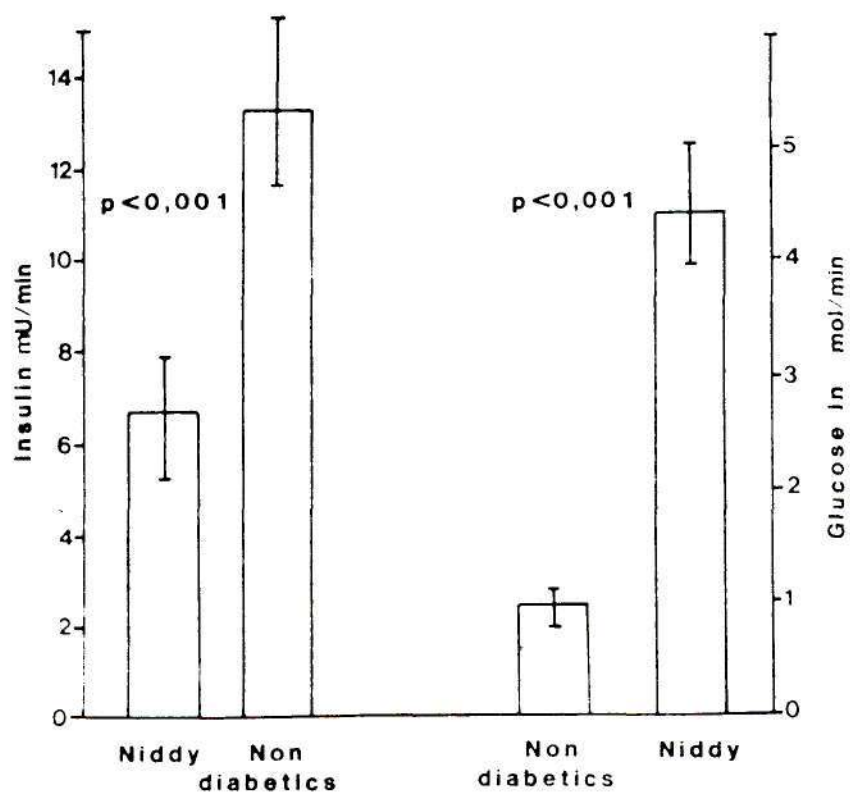


FIGURE 3.3

THE CALCULATED INSULIN AND GLUCOSE AREAS IN PATIENTS
WITH NIDDIY AND NON DIABETIC FAMILY MEMBERS



under the glucose curve was significantly higher in the patients as compared to the non-diabetic subjects ($4,5 \pm 0,5$; $1,0 \pm 0,3$ mol/min; $p < 0,001$); the area under the insulin curve in NIDDDY ($7,3 \pm 1,5$ mU/min) was significantly lower than that obtained in the non-diabetic family members ($13,0 \pm 2,0$ mU/min; $p < 0,001$).

3.1.5.3. Insulin-Glucose Ratios and modified Seltzer Insulinogenic Index.

At all time intervals during the OGTT, NIDDDY exhibited significantly lower insulin-glucose ratios (Table 3.1). The smallest disparity between the ratios was in the fasting state, whilst the most marked differences were apparent at 60-180 minutes. ($p < 0,001$). Comparison of the ratios of the insulin and glucose areas by the modified Seltzer insulinogenic index once again revealed much lower values in the patients (NIDDDY $1,6 \pm 0,4$; non-diabetics $13,0 \pm 2,5$; $p < 0,001$).

3.1.6. Discussion

Although the patients under study were selected on the basis of the WHO criteria for diabetes, which clearly defines a frank diabetic, the striking results in the present study are undoubtedly the severity of the diabetes in the patients with NIDDDY and the absence of any form of glucose intolerance in other members of the family. These findings should be contrasted with the earlier studies of Tattersall (1974), Fajans et al (1976) and Barbosa et al (1978). Quite apart from the fact that WHO criteria were of course, not used to identify diabetic members in those early studies, it is apparent that only a small percentage of their patients were indeed diabetic by present day criteria. Indeed, these patients presented a spectrum varying from impaired glucose tolerance (IGT) to the frankly diabetic, which contrasts sharply with the finding of perfectly normal glucose tolerance in all other

TABLE 3.1.
INSULIN-GLUCOSE RATIOS AND MODIFIED INSULINOGENIC INDEX
IN NON-DIABETIC FAMILY MEMBERS AND NIDDY

| | INSULIN-GLUCOSE RATIOS (MEAN \pm SEM) | |
|----------|---|------------------|
| | NON-DIABETICS | NIDDY |
| 0 min. | 0,20 \pm 0,08 | 0,11 \pm 0,1 * |
| 60 min. | 0,69 \pm 0,30 | 0,10 \pm 0,1 * |
| 120 min. | 0,72 \pm 0,39 | 0,14 \pm 0,1 * |
| 180 min. | 0,78 \pm 0,15 | 0,18 \pm 0,2 * |

*MSI 13,0 \pm 2,5 1,6 \pm 0,4

*MSI Modified Seltzer Insulinogenic Index

*p < 0,001

members of the families examined. However, the present results are similar to that obtained by Jialal et al (1982a) in randomly selected patients with NIDDM. They also remarked on the fact that in actively selecting NIDDM patients for study by WHO criteria for diabetes, glucose intolerant patients were obviously excluded from the study. Fajans et al (1976) argued that their patients represented one end of the spectrum of this disorder and that the probable natural history of the disorder progressed from glucose intolerance through to frank diabetes in much the same way as NIDDM (National Diabetes Data Group, 1979). The findings in the present study that the family members of the patients all have normal glucose tolerance suggests a rather different natural history of the disorder. If there were a natural progression from glucose intolerance through to frank diabetes one would have expected to have observed stigmata of glucose intolerance in some of the family members.

Looking at the insulin responses in the patients, it is clear that mean values are far lower than that obtained in the non-diabetic family members. Furthermore, there is no peak in the insulin values, only a gradual increase to a plateau value reached at 120 minutes. The serially lower insulin-glucose ratios, insulinogenic indices and areas under the insulin curve, are evidence of deficient insulin secretion in the diabetic patients. Only one patient in this series (A₃) was a "high" insulin responder. This high insulin responder is an unusual finding, in that the other two diabetic family members had hypoinsulinaemic responses. Fajans (1979) has demonstrated heterogeneity between families but not within families as regards insulin responses.

It is the view of Reaven (1980) that the basic lesson in NIDDM is insensitivity of the target tissues to insulin. As a result there is initially an increased insulin response to overcome the resistance

in the target organs, evoked by the increased hyperglycaemia. This stage corresponds to the stage of high insulin responses. However, the beta cells do not have an unlimited capacity for increased insulin secretion and ultimately a stage of beta cell exhaustion sets in, leading to a hypoinsulinaemic response. This spectrum of insulin responses conforms to a horseshoe pattern. In the non-diabetic family members, not a single one had a hyperinsulinaemic response. It could be argued again, that by the selection criteria used, high insulin responders would have been excluded from the study. But the fact that no family member not suffering from NIDDDY had evidence of hyperinsulinaemia, suggests that the aetiology of NIDDDY is somewhat different from that of NIDDM.

One other factor which can significantly modulate insulin secretion requires to be considered, namely, obesity. As mentioned in the previous chapter, the patients tended to obesity. However, when Jialal et al (1984c) divided NIDDDY into obese and non-obese subgroups and compared their insulin and glucose responses, no differences could be found between the two groups. Reaven et al (1982) also confirmed this finding in patients with NIDDM. Thus obesity was not regarded as a major factor affecting insulin secretion in diabetes.

Specific comment is necessary on 3 patients at this stage. Patient A₁ (Table 2.2) had evidence of microvascular (retinopathy and nephropathy) and macrovascular complications. The second patient (A₃) had fasting insulin levels and incremental insulin responses to oral glucose that were consistently $> \text{mean} \pm 1 \text{ S.D.}$ of the non-diabetic family members. She also had evidence of acanthosis nigricans. Patient C₂ had a secondary failure on sulphonylureas and biguanides, as he persistently presented with plasma glucose levels $> 17 \text{ mmol/l}$. He was subsequently put onto insulin therapy and is presently well controlled

(plasma glucose < 8 mmol/l). For these reasons, the above 3 patients will be specifically excluded from further studies.

3.1.7. Conclusion

The evidence is clear that in NIDDM, inappropriate and inadequate insulin responses to oral glucose underlie the glucose intolerance. No evidence could be found that this stage is the end result of an initial hyperinsulinaemic response to glucose in the natural development of the disorder. Hence, further studies on insulin secretion in NIDDM seem indicated.

3.2. THE INSULIN AND GLUCOSE RESPONSE TO AN INTRAVENOUS GLUCOSE STIMULUS.

3.2.1. Purpose of the Study.

The phasic release of insulin can be determined using a bolus administration of glucose IV : the first phase of insulin secretion represents the stored pool of insulin which is immediately released in response to an acute rise in the plasma glucose levels (Lerner and Porte, 1971); the second phase insulin response occurs more gradually and persists for the duration of the hyperglycaemia. This is said to represent mainly de novo synthesis of insulin (Lerner and Porte, 1971). There is some evidence that the intravenous glucose tolerance test (IVGTT) is a superior method of quantifying pancreatic insulin secretion than the OGTT (Ward et al, 1984). Firstly, insulin responses to oral glucose are influenced by gut factors and neural responses to various stimuli, which may differ in magnitude among individuals. Secondly, gastro intestinal motility and gastric emptying which vary from person to person influence the rate of rise in plasma glucose and thus the degree of β cell stimulation. Thirdly, the variable increases in plasma glucose levels after an oral glucose load make comparisons of

insulin secretion among different subjects difficult. As a first approach to insulin secretion in NIDDM patients, the IVGTT was used.

3.2.2. Patients, non-diabetic Family Members and Reference

Subjects.

The 10 patients that remained after the exclusions (Paragraph 3.1.6) were studied. In addition 10 reference subjects and 10 non-diabetic family members were also included in this study. Ages in the diabetics and reference subjects were similar ($32,6 \pm 3,8$; $30,3 \pm 2,8$ years; $p > 0,5$). The body mass indices of the diabetics and reference subjects were alike ($24,5 \pm 0,9$; $24,4 \pm 0,7$ kg/m²; $p > 0,5$); the non-diabetic family members had a mean age of $28 \pm 4,7$ years and a mean BMI of $21,8 \pm 0,8$ kg/m², which was not significantly different from the other two groups.

3.2.3. Study Procedure.

The preparation in respect of diet and oral hypoglycaemic agents in all participants were as detailed in Section 3.1.3. None of the patients, reference subjects or non-diabetic family members had any evidence of renal or hepatic dysfunction. The IVGTT was carried out according to the method of Felig et al (1981). After an overnight fast 0,5 g glucose /kg body weight was administered as 50% dextrose water intravenously over a three minute period. Venous samples for glucose and insulin assays were taken in the fasting state and then at 1, 3, 5, 10, 20, 30, 40, 50, 60, 90 and 120 minutes after the glucose infusion, via an indwelling catheter in an antecubital vein. Intravenous glucose was administered into one arm, while samples for glucose and insulin estimation were taken from the opposite arm. Blood was taken into tubes containing fluoride-oxalate for glucose measurements and into tubes containing no preservative for insulin assays.

3.2.4. Analytical Methods

The methods detailed in Appendix A-1 and A-5 were used to measure glucose and insulin concentrations.

The glucose disposal constant (kgtt) was calculated according to Lundbaek (1962):

$$K_{gtt} = 0,693 / t_{\frac{1}{2}}$$

where $t_{\frac{1}{2}}$ is the time during which the blood sugar has fallen from fasting value to half it's value (half-time).

Early peak insulin responses to intravenous glucose were calculated by adding the sum of the one and three minute insulin levels (Srikanta et al, 1983).

3.2.5. Results

3.2.5.1. Glucose Response

Fasting glucose levels were significantly higher in the diabetics ($15,0 \pm 1,4$ mmol/l), as compared to mean levels of $4,7 \pm 0,1$ in the reference group and $5,0 \pm 0,1$ mmol/l in the non-diabetic family members, respectively. However, all three groups attained peak glucose levels at one minute. Thereafter glucose values fell steadily, reaching fasting levels at 120 minutes in the diabetics and at 60 minutes in the reference subjects and non-diabetic family members. These results are graphically presented in Figure 3.4.

The glucose disposal constant, kgtt, represents the rate of glucose utilization per unit time. Whereas the kgtt values were low in the diabetics (0,2 - 0,9), the reference subjects and non-diabetic family members had values ranging from 1,2 - 5,0. (Figure 3.5).

FIGURE 3.4

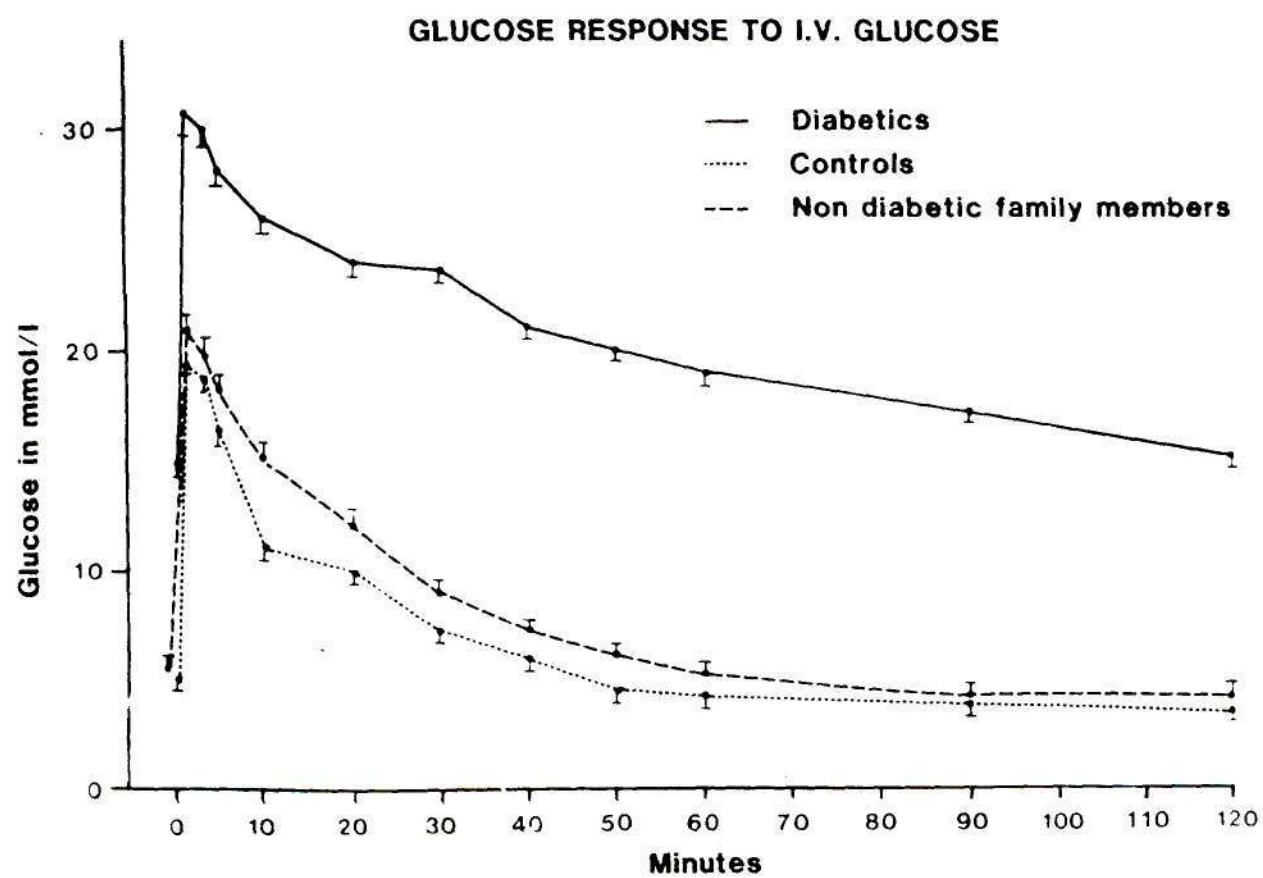
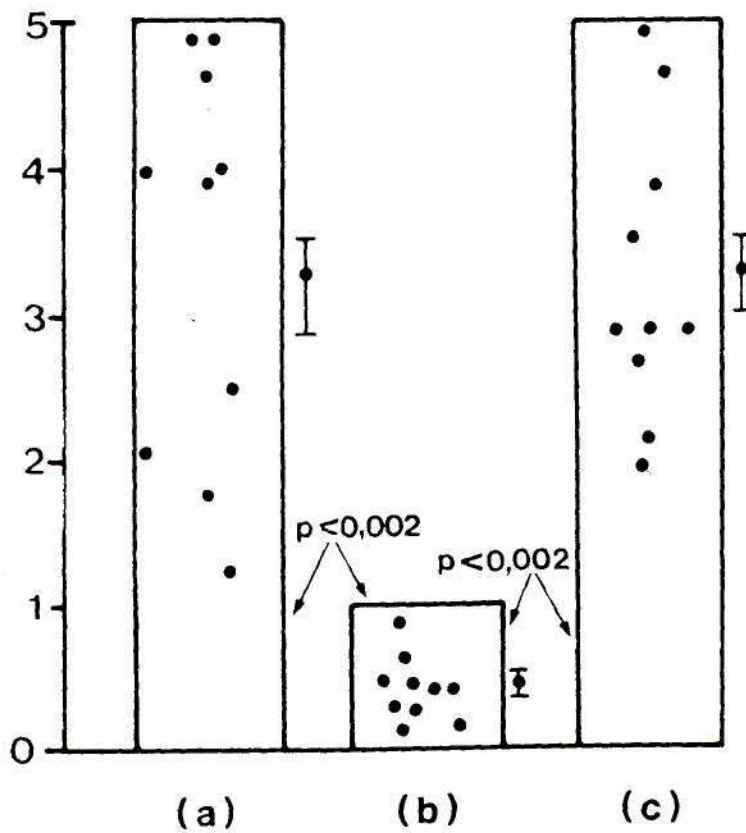


FIGURE 3.5

K values (Glucose disposal)

Non Diabetic
Family

Members Diabetics Controls



(a) vs (c) Not significant

3.2.5.2. Insulin Responses

Mean fasting insulin levels were apparently higher in the diabetics ($18,4 \pm 1,0 \mu\text{U/ml}$) than in the reference subjects and non-diabetic family members ($14,1 \pm 1,0$; $13,0 \pm 2,1 \mu\text{U/ml}$ respectively). Whereas the diabetics had no first phase insulin release in responses to IV glucose, the non-diabetic family members had a rapid release of insulin which peaked at one minute ($175 \pm 25 \mu\text{U/ml}$); the reference subjects also attained a maximum insulin response at one minute ($128 \pm 20 \mu\text{U/ml}$). This latter result was not significantly different from that of the non-diabetic family members ($p > 0,5$). These results are presented in Figure 3.6.

Mean early peak insulin responses ($38,1 \pm 5,0 \mu\text{U/ml}$) in the diabetics were significantly lower than that obtained in the reference subjects ($260,7 \pm 20 \mu\text{U/ml}$) and in the non-diabetic family members ($268,8 \pm 40 \mu\text{U/ml}$). The latter two groups had considerably higher insulin responses than the diabetics ($p < 0,002$) at certain time intervals (Figure 3.7). There was a positive correlation between k values and mean peak insulin responses ($r = 0,642$; $p < 0,001$).

3.2.6. Discussion

The value of the IVGTT has already been elaborated in the introduction to this study (section 3.2.1.) and to these may be added the simplicity of calculating the glucose disposal constants (kgtt).

As expected, in the light of the OGTT results, glucose disposal was substantially decreased in the NIDDM patients, whether measured by the time taken to return to fasting values or by kgtt values. Significantly, there were no differences in glucose disposal between the reference group and non-diabetic family members, but equally significant was the fact that the fasting glucose concentrations in the NIDDM

INSULIN RESPONSE TO IV GLUCOSE

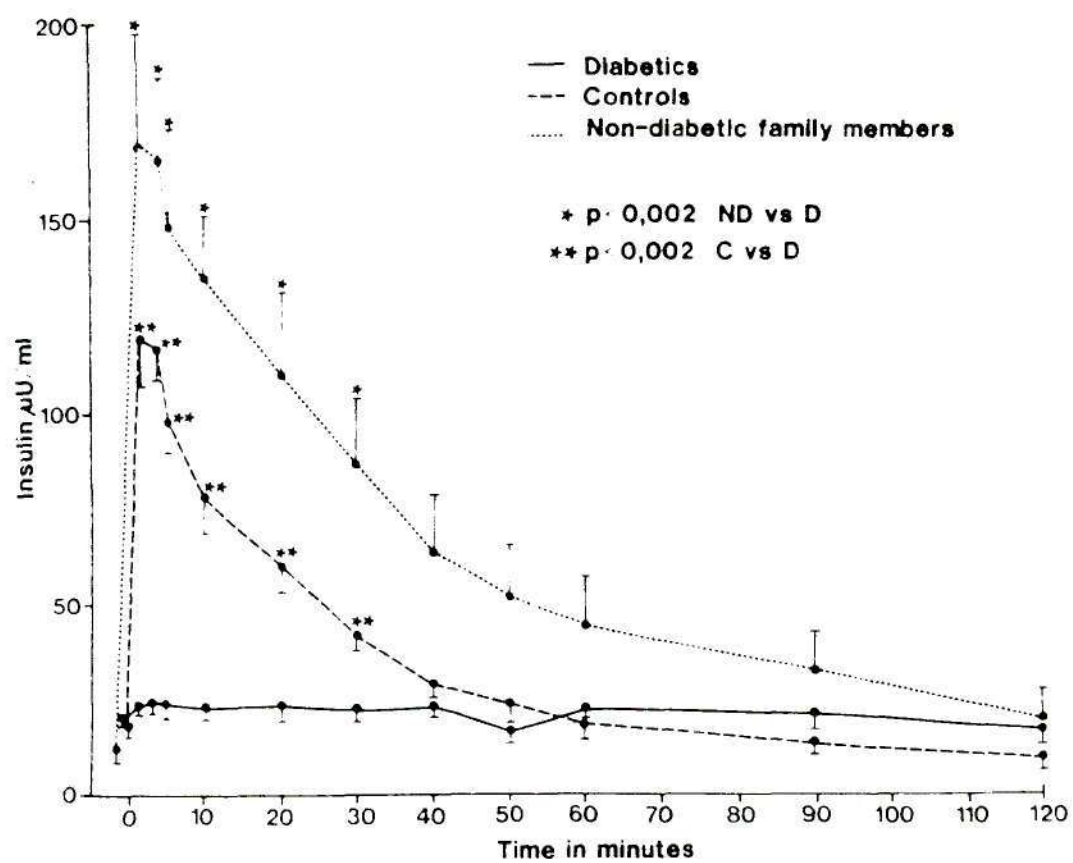
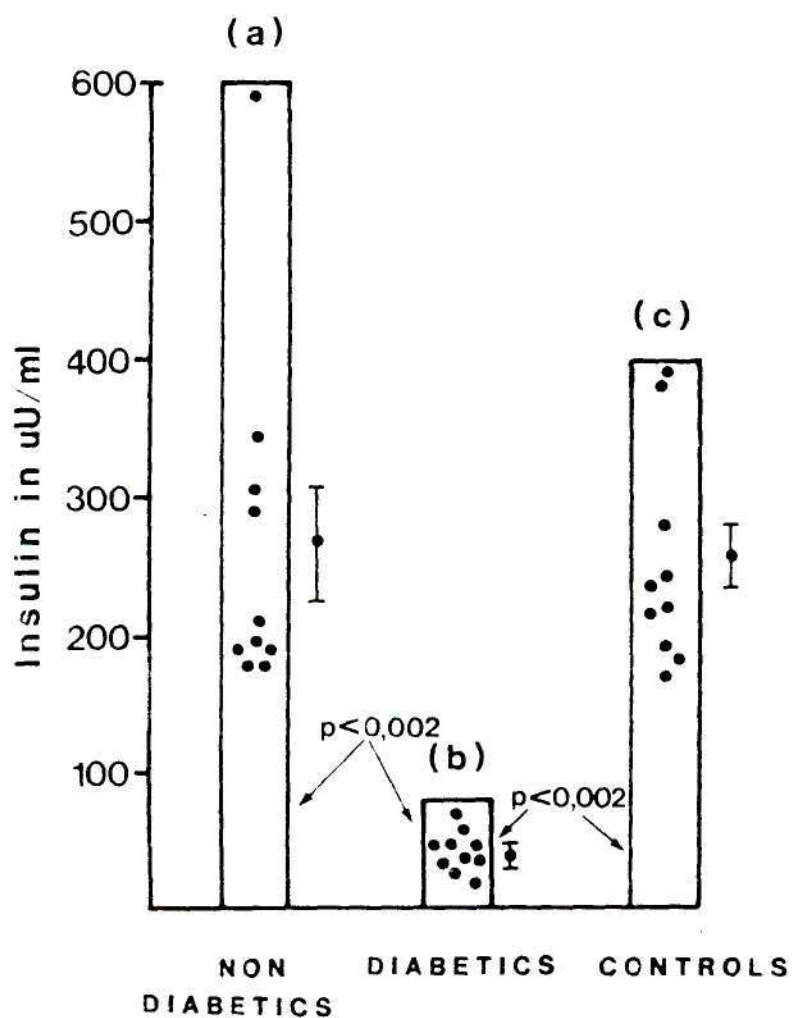


FIGURE 3.7

EARLY PEAK INSULIN RESPONSES
(mean of 1min and 3min levels)

(a) vs (c) NOT SIGNIFICANT



patients were about three times greater than the values in the other two groups. It has been shown that k values correlate with early insulin release (Lerner and Porte, 1971) as was the case, in this study ($r=0,642$; $p < 0,001$) Cerasi and Luft (1963) found a decreased early phase of insulin release in potential diabetics that had normal kg_{tt} values. However, the findings in this study are in accordance with those of Soeldner et al (1968) in that the non-diabetic family members had similar early insulin responses and kg_{tt} values to the reference subjects.

The major interest in this study centres naturally on insulin secretion itself. There was no early phase insulin to IV glucose in patients with NIDDM. Inasmuch as the early phase release represents stored insulin (Lerner and Porte, 1971) it must be concluded that such stores were non-existent or that the release mechanism was defective. Bearing in mind that the fasting glucose concentrations in patients were about 15 mmol/l, it may be argued that these high levels are stimuli enough to release stored insulin from the β cells of the pancreas and thus an early phase insulin response to IV glucose can hardly be expected. Nevertheless, no definitive conclusion can be drawn from this finding alone, as it does not answer any question in respect of the adequacy of the release mechanism.

As far as the overall insulin response to IV glucose is concerned, the evidence is quite clear that it was inadequate; as was the finding with oral glucose but with this difference: IV glucose evoked even less insulin secretion than oral glucose, an observation previously made by others (Simpson et al, 1966; Robertson et al, 1973). The hypoinsulinaemic responses in the patients with NIDDM are similar to the responses recorded by previous workers (Cerasi and Luft, 1967; Brunzell et al, 1976) in patients with NIDDM. Although in the mechanistic sense

it is not possible to pinpoint any specific lesion in the β -cell, the evidence clearly implicates the inability of the β -cells to respond adequately by release of insulin to the appropriate glucose stimulus.

But, having said as much, the true dilemma facing the investigator must also be recognised. Under normal physiological conditions, responses to specific stimuli can be defined within limits, between a number of interdependent variables. Given a condition in which one variable is disturbed, such as diabetes with high fasting plasma glucose levels: Is it still valid to expect the same response to the same stimulus? Or, put more pertinently : Can an abnormal response be interpreted as inappropriate or is it appropriate to the perturbation in the basic condition? One approach to this problem is to study the effect of secretagogues acting mechanistically different from glucose and such an approach will be dealt with in subsequent chapters. For the present, it seems reasonable to conclude that insulin secretion is impaired in patients with NIDDM, although the mechanism cannot be deduced from the present study. Nevertheless it should still be demonstrated that no abnormal metabolism of insulin occurs in the liver. This study will be reported in the following section.

Very significantly again, both phases of insulin secretion in all family members were perfectly normal and if Cerasi and Luft's (1963) observation that the first phase insulin release is depressed in potential diabetics has any substance, it only reinforces the earlier observations that non-diabetic family members have no stigmata that they are potential diabetics. As the non-diabetic family members were within a similar age group as their diabetic counterparts, this observation argues strongly for autosomal dominant inheritance of NIDDM in these families.

3.2.7 Conclusions

Using another route of glucose administration, namely intravenously, it has been demonstrated that NIDDM have hypoinsulinaemic responses. Whether this is due to a "true hypoinsulinaemia" or due to a defect in hepatic extraction of insulin is still to be determined.

3.3. THE GLUCOSE, INSULIN AND C-PEPTIDE RESPONSE TO A 100 GRAM ORAL GLUCOSE LOAD.

3.3.1. Purpose of the Study

In section 3.1 and 3.2 it has been demonstrated that patients with NIDDM have an attenuated insulinaemic response to glucose administered orally and intravenously. However, it has not been ascertained whether the hypoinsulinism is due to an increase in hepatic extraction of insulin.

Although equimolar quantities of insulin and C-peptide (CPR) are secreted by the pancreatic β -cell (Ashby et al, 1981), the liver extracts approximately 50% of the insulin delivered to it on the first pass (Field, 1973). On the other hand CPR is metabolised principally by the kidney, renal extraction accounting for about 69% of total clearance (Katz et al, 1973). Peripheral plasma ratios of CPR to insulin may therefore be expected to reflect whether endogenous insulin is extracted normally.

Accordingly, the CPR response to a 100g oral glucose load was studied in NIDDM and reference subjects.

3.3.2. Patients and Reference Subjects

The same 10 patients with NIDDM and reference subjects described in Section 3.2.2. were studied.

3.3.3. Study Procedure

The preparation of patients and reference subjects was as described in Section 3.1.3.

Venous samples of blood for glucose, insulin and CPR were taken in the fasting state and then at 30, 60, 90, 120 and 180 minutes after the oral glucose load. Blood for CPR was taken into tubes containing no preservative.

3.3.4 Analytical Methods

The method for the measurement of CPR is described in Appendix A-6.

Mean incremental CPR responses were calculated by subtracting the fasting concentrations from the concentrations at each time interval.

CPR: insulin molar ratios were worked out using the following formula:

$$\frac{\text{CPR (ng/ml)}}{\text{Insulin (\mu U/ml)}} \times 48,04.$$

3.3.5 Results

3.3.5.1. Glucose Responses

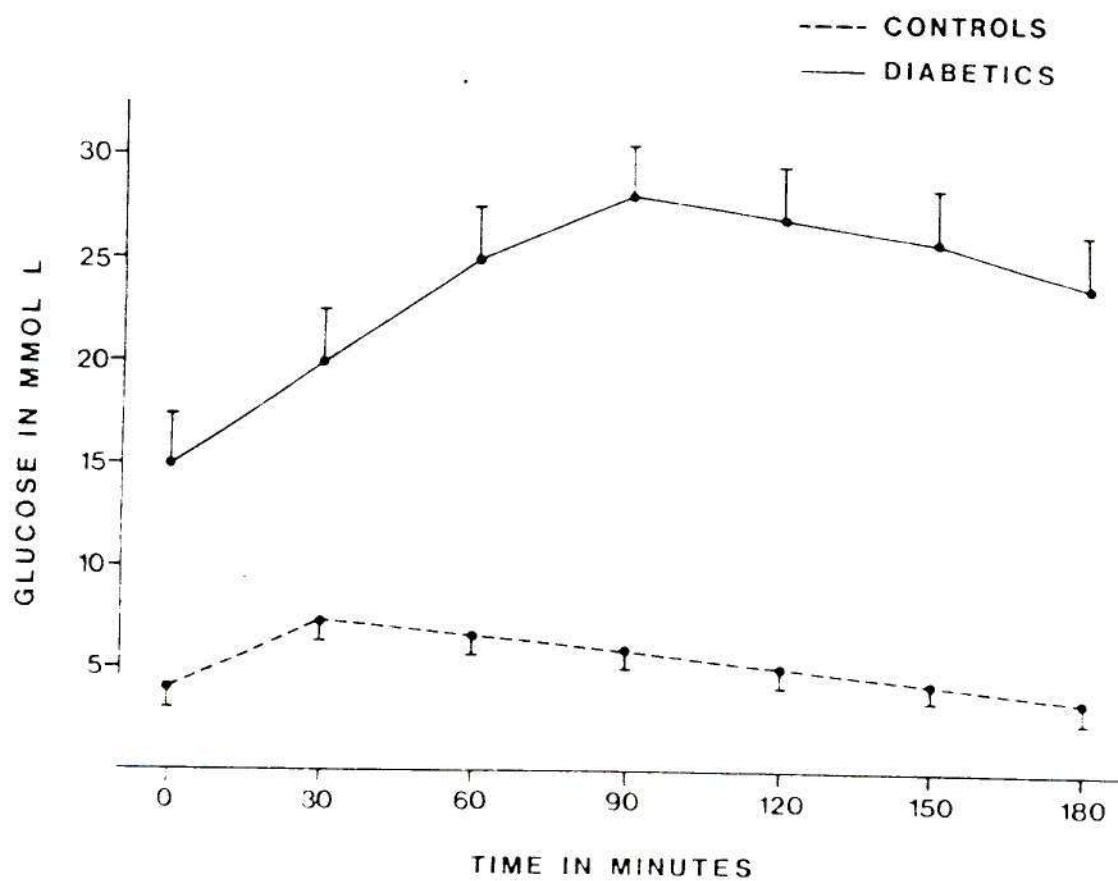
Fasting plasma glucose levels were significantly higher in the diabetics than in the reference subjects ($14,8 \pm 1,6$; $4,3 \pm 0,9$ mmol/l; $p < 0,002$). At 90 minutes, glucose values in the diabetics were $27,5$ mmol/l and levels fell thereafter. In the reference subjects, the peak glucose value of $7,5$ mmol/l was attained at 30 minutes. Fasting levels were resumed at 180 minutes (Figure 3.8)

3.3.5.2. Insulin Responses

In the fasting state, plasma insulin levels were higher in the diabetics ($18,2 \pm 2,4$; $10,5 \pm 0,7$ μ U/ml; $p < 0,002$). Maximum levels were attained between 60 - 120 minutes in the diabetics and 30 - 60 minutes in the controls. The diabetics had a decrease in the mean maximal in-

FIGURE 3.8

GLUCOSE RESPONSES TO 100G ORAL GLUCOSE IN NIDDDY AND CONTROLS



ulin response when compared with the reference subjects ($45,8 \pm 10,2$; $91,0 \pm 8,8$ $\mu\text{U/ml}$; $p < 0,002$). These results are depicted in Figure 3.9. Mean incremental insulin responses were lower in diabetics ($100,4 \pm 11,6$; $314,5 \pm 18,5$ $\mu\text{U/ml}$).

3.3.5.3.C-peptide Responses

Prestimulation levels of CPR were significantly greater in the diabetics than their matched reference subjects ($1,8 \pm 0,1$; $1,3 \pm 0,1$ ng/ml ; $p < 0,02$). Whereas the reference subjects had a peak response of $6,6 \pm 0,8$ ng/ml at 90 minutes, the diabetics had significantly lower peak CPR concentrations ($3,2 \pm 0,3$ ng/ml ; $p < 0,002$). These results are seen in Figure 3.10.

Mean incremental CPR responses were lower in the diabetics ($5,1 \pm 0,9$; $23,9 \pm 3,5$ ng/ml ; $p < 0,002$). The CPR : insulin molar ratios are given in Table 3.2. At all time intervals the ratios were similar in both groups ($p > 0,5$).

3.3.6 Discussion

It has already been demonstrated in the two antecedent studies that patients with NIDDM have an attenuated insulin response to an oral glucose load, an absent first phase response to IV glucose and a decreased overall insulin response to IV glucose.

This study has shown that NIDDM have a delayed and diminished CPR response to oral glucose. The fact that the CPR peak occurred later than that of insulin, could be explained on the basis of the longer half life of CPR as compared to that of insulin (Faber and Binder, 1977). Decreased responses of insulin and CPR are confirmed by the lower mean incremental responses in the diabetic subjects. These findings support those reported by Mohan et al (1985) who also demonstrated this ab-

TABLE 3.2

CPR/INSULIN MOLAR RATIOS IN NIDDIY AND REFERENCE SUBJECTS

| | NIDDIY | REFERENCE SUBJECTS |
|---------|-----------|--------------------|
| 0 min. | 6,0 + 0,8 | 7,2 + 0,6 * |
| 30 min. | 5,2 + 0,6 | 3,4 + 0,4 * |
| 60 min | 3,8 + 0,7 | 3,9 + 0,4 * |
| 90 min | 4,1 + 0,8 | 4,3 + 0,5 * |
| 120 min | 4,4 + 0,8 | 4,7 + 0,6 * |
| 180 min | 5,3 + 0,8 | 4,8 + 0,7 * |

* $p > 0,5$

FIGURE 3.9

INSULIN RESPONSES TO 100G ORAL GLUCOSE IN DIABETICS AND CONTROLS

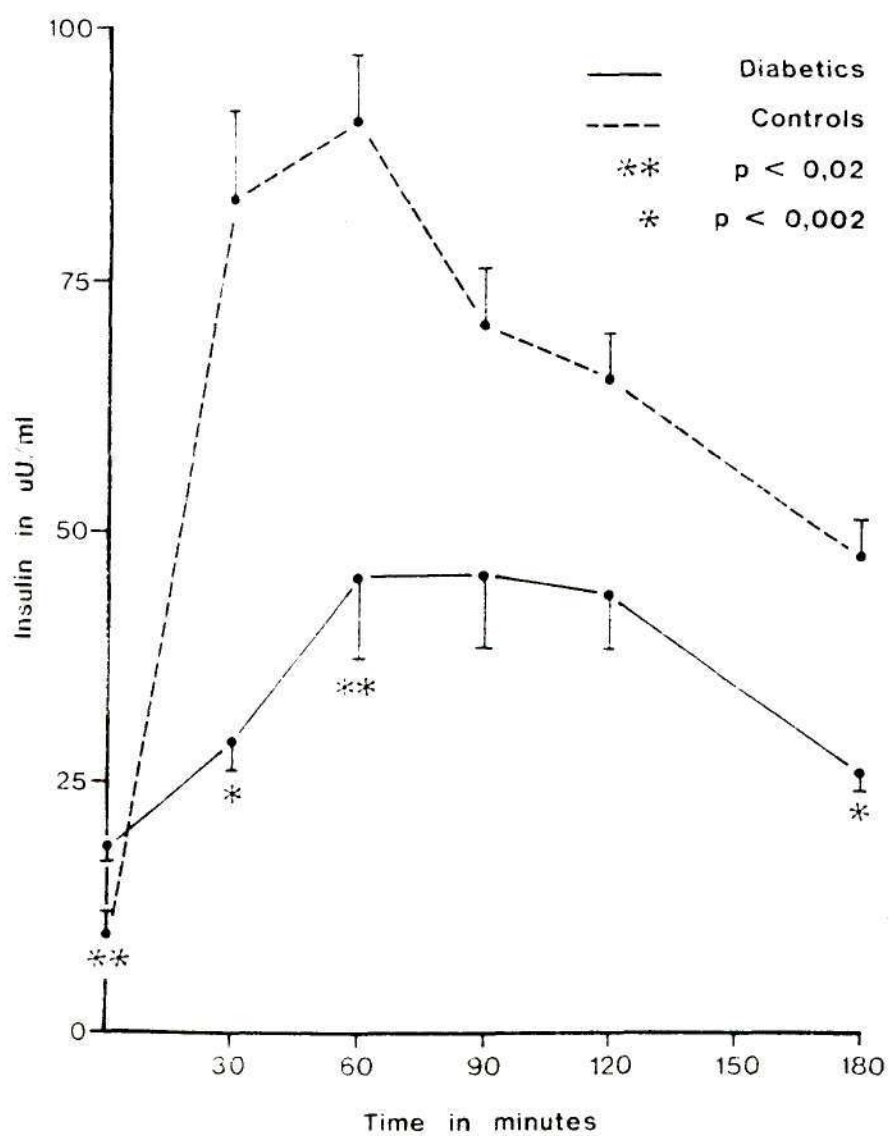
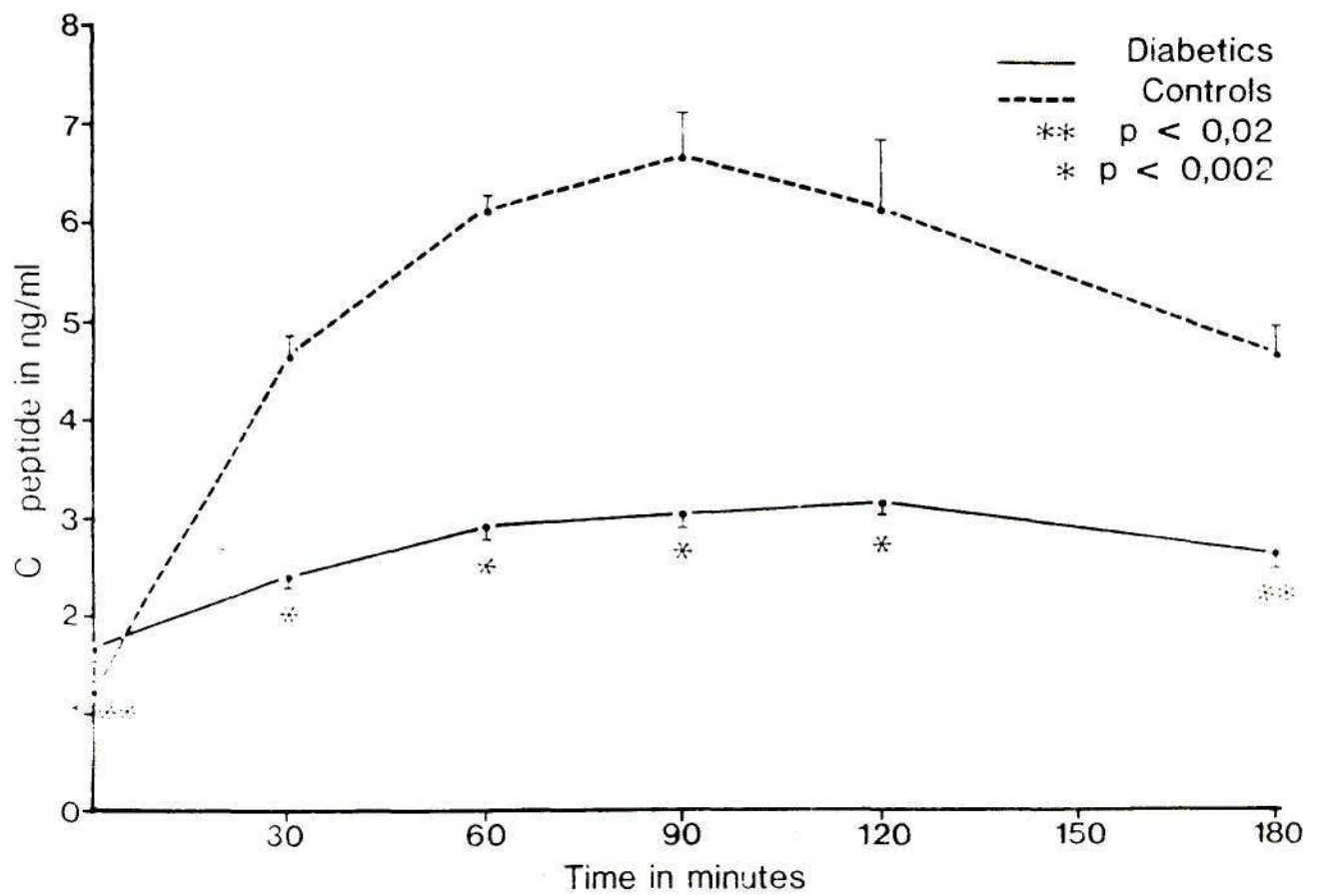


FIGURE 3.10

C-PEPTIDE RESPONSES TO 100G ORAL GLUCOSE IN DIABETICS AND CONTROLS



erration in a group of Indian patients in India with NIDDM.

The CPR: insulin molar ratios were used as an indicator of hepatic insulin extraction in this study. The molar ratios were greatest in the fasting state, but fell after the ingestion of glucose. At all times, these ratios were similar in the diabetics and reference subjects. Although these molar ratios are dependent upon many factors such as secretion, metabolism, distribution and half-lives of CPR and insulin (Polonsky et al, 1984), the ratios were nevertheless utilized as a semi-quantitative index of hepatic insulin extraction on the grounds that all patients had normal hepatic and renal functions. Other workers have used the ratios as indices of hepatic extraction of insulin (Sando et al, 1980; Johnston et al, 1977; Faber et al, 1981).

It may be concluded on the basis of the results presented that NIDDM have a decreased pancreatic β -cell response to oral and IV glucose and that the attending hypoinsulinaemia is unlikely to be due to a defect in the extraction of insulin by the liver.

3.3.7 Conclusion

From the studies reported in Section 3.1, 3.2 and 3.3, it may be concluded that overtly in NIDDM the first phase insulin release is absent and the second phase secretion of insulin is decreased in response to appropriate glucose stimuli. It cannot be determined from these studies whether the mechanisms of insulin secretion in these two phases are defective or whether it merely reflects appropriate responses in the face of sustained high levels of glucose in the patients with NIDDM.

An observation of significance in these studies is the absence of any stigmata of glucose intolerance in non-diabetic family members of the patients. Not only does it have a bearing on the natural history of the disorder, but it argues cogently for a dominant inheritance in this

disorder.

The question now arises as to what defect is inherited. Before suggesting that there is a β -cell anomaly, the insulin responses to non-glucose stimuli will have to be studied.

CHAPTER 4

THE INSULIN AND GLUCOSE RESPONSES TO NON-GLUCOSE
SECRETOGOGUES : GLUCAGON, TOLBUTAMIDE AND ARGININE IN
NON-INSULIN-DEPENDENT DIABETES IN THE YOUNG

4.1 THE INSULIN AND GLUCOSE RESPONSE TO INTRAVENOUS GLUCAGON
AND TOLBUTAMIDE

4.1.1. PURPOSE OF STUDY

In the previous chapter it has been shown that patients with NIDDM display delayed and attenuated insulinaemic responses to oral glucose, as well as an absent early phase release of insulin following intravenous glucose administration. These findings could be interpreted as indicating the absence of stored insulin and defective second phase insulin secretion in the patients. However, it should be borne in mind that the patients have fasting hyperglycaemia which would be a constant stimulus for insulin secretion. Hence it may be argued with equal validity that, in the face of an existing hyperglycaemia, the additional hyperglycaemic effect of glucose administration can hardly be expected to release more stored insulin, when that pool has already been released by the endogenous hyperglycaemia. Equally the β -cell response to increments in hyperglycaemia occasioned by glucose administration in the face of pre-existing hyperglycaemia must surely reflect an adapted response rather than a comparable physiological response.

A possible way out of this interpretational impasse would be to study insulin secretagogues, which achieve insulin release or secretion by different mechanisms. As non-glucose stimuli of insulin secretion, glucagon and tolbutamide were chosen. It has been suggested that glucagon stimulates insulin release by the activation of membrane bound adenylase and gen-

eration of intracellular cyclic AMP (Berson and Yalow, 1970); while tolbutamide increases the sensitivity of the β cell to β adrenergic input (Lundquist et al, 1984). In this way it would also be possible to gain further insight into β cell function in NIDDM.

In the following sections the actions of administered glucagon and tolbutamide are reported in patients with NIDDM.

4.1.2. PATIENTS AND REFERENCE SUBJECTS

The same ten diabetics and reference subjects described in Section 3.2.2. were studied.

4.1.3. STUDY PROCEDURE

The preparation of patients was identical to that set out in Section 3.1.3. After an overnight fast, 1mg glucagon (Lilly) was administered as an IV bolus. Blood samples for glucose and insulin were taken at 0, 3, 6, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 90 and 120 minutes via an indwelling catheter in an ante-cubital vein (Simpson et al, 1966)

One week later (also following an overnight fast) 1g sodium tolbutamide (Hoechst) was given IV over a period of two minutes. Venous samples for insulin and glucose were taken in the fasted state and then at 1, 3, 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 minutes following tolbutamide administration (Varsano-Aharon et al, 1970)

The doses of glucagon and tolbutamide used were MAXIMAL dosages (Marks and Samols, 1968; Marks, 1930)

Blood samples were collected in potassium-oxalate-fluoride tubes for glucose estimation and in tubes containing no preservative for insulin assays.

4.1.4. METHODS

The methods detailed in Appendix A-1 and A-5 were used to measure glucose and insulin concentrations respectively.

4.1.5. RESULTS

4.1.5.1. Glucose responses to glucagon

Mean fasting glucose levels in the patients with NIDDM were 14,5 mmol/l as compared with mean levels of 4,2 mmol/l in the controls. Blood glucose concentrations rose to a level of 16,5 mmol/l at 20 minutes in the diabetics; fasting levels were approximated at 90 minutes (Figure 4.1). In the reference group glucose levels reached a peak of 6,5 mmol/l at 20 minutes and fell thereafter to reach prestimulation values at 40 minutes.

4.1.5.2. Glucose responses to tolbutamide

At the outset, mean fasting blood glucose values were considerably higher in the diabetics than the control subjects ($12,5 \pm 1,5$; $4,5 \pm 0,4$ mmol/l). In response to IV tolbutamide administration, diabetics had a fall in plasma glucose (8,7 mmol/l at 120 minutes). The reference subjects also had a decrease in plasma glucose levels (2 mmol/l at 30 minutes). These responses are presented in Figure 4.2

4.1.5.3. Insulin responses to glucagon

Fasting insulin levels were higher in the diabetics than the reference group ($20,4 \pm 1,4$; $12,1 \pm 4,4$ μ U/ml; $p < 0,02$). A peak insulin response of 60 μ U/ml was attained at 3 minutes in the diabetics. The highest insulin level reached in the reference subjects was 106,0 μ U/ml within the first 10 minutes (Figure 4.3). Mean peak insulin responses attained in the patients with NIDDM were significantly lower than that achieved in the reference subjects ($p < 0,05$). Prestimulation insulin levels were

FIGURE 4.1

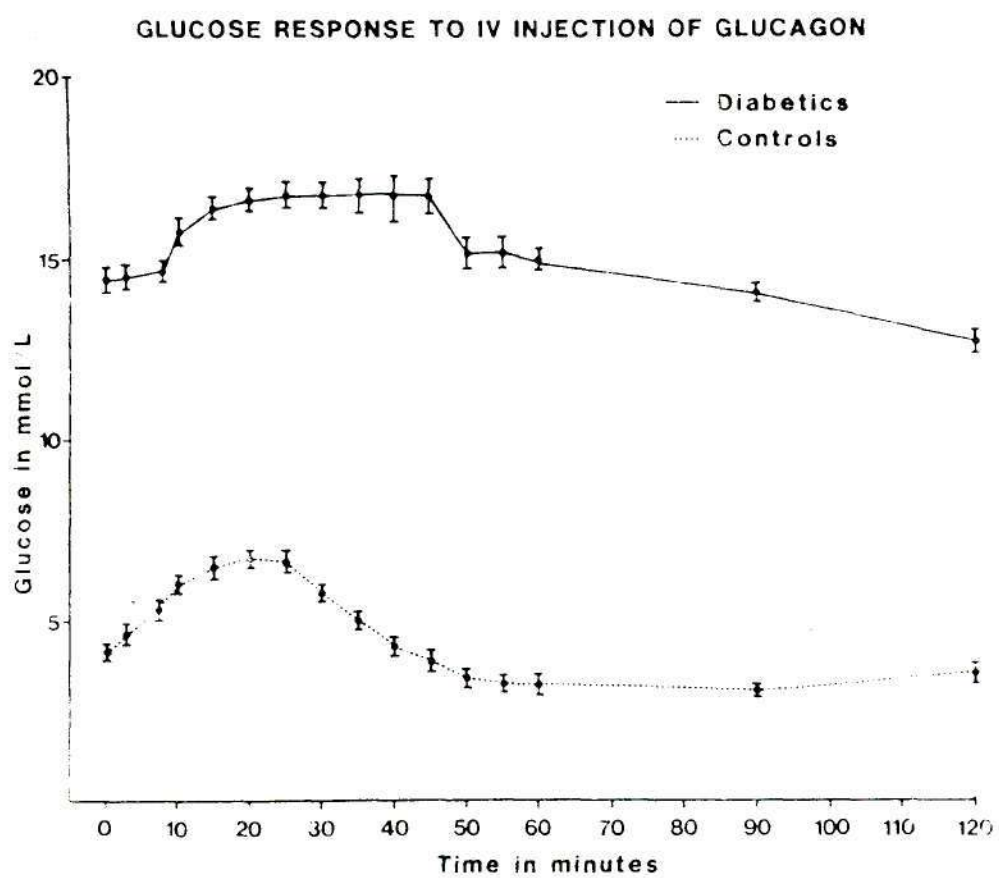


FIGURE 4.2

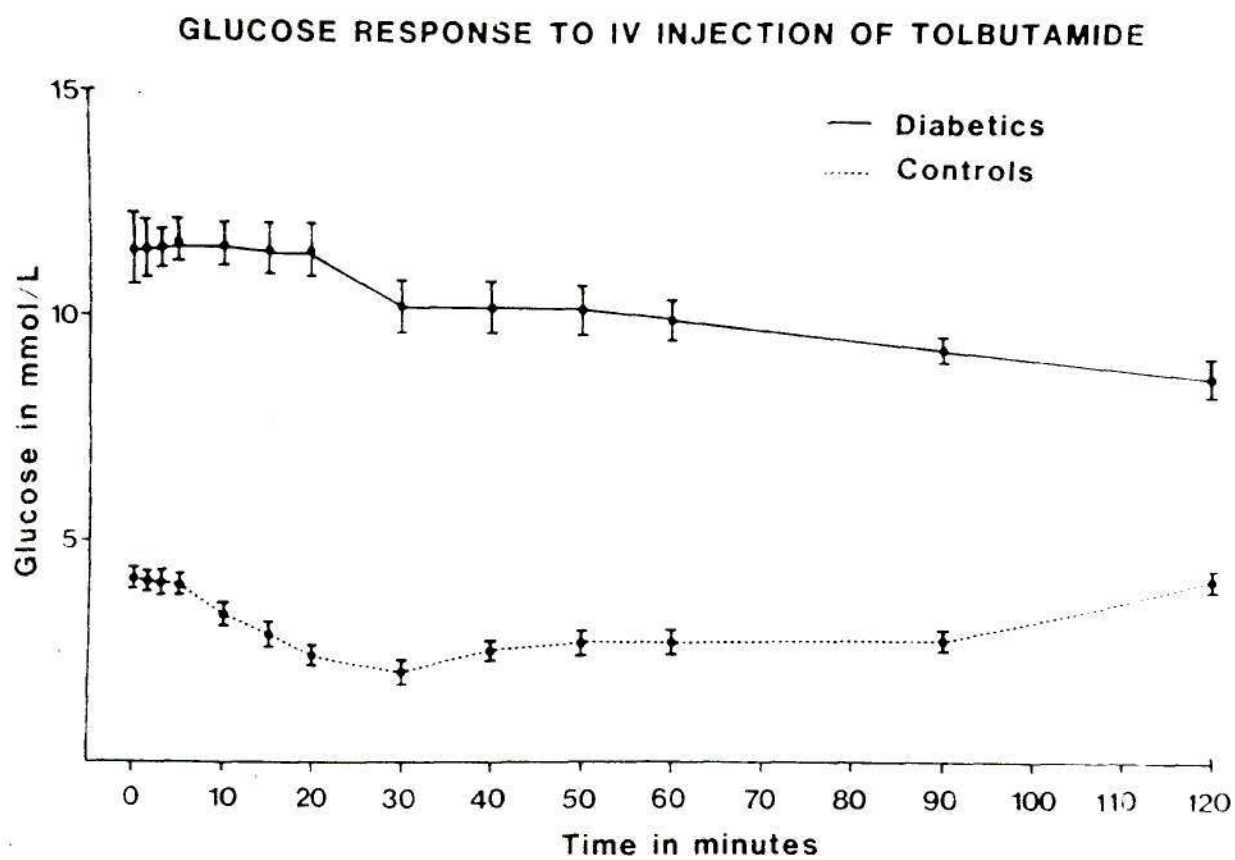
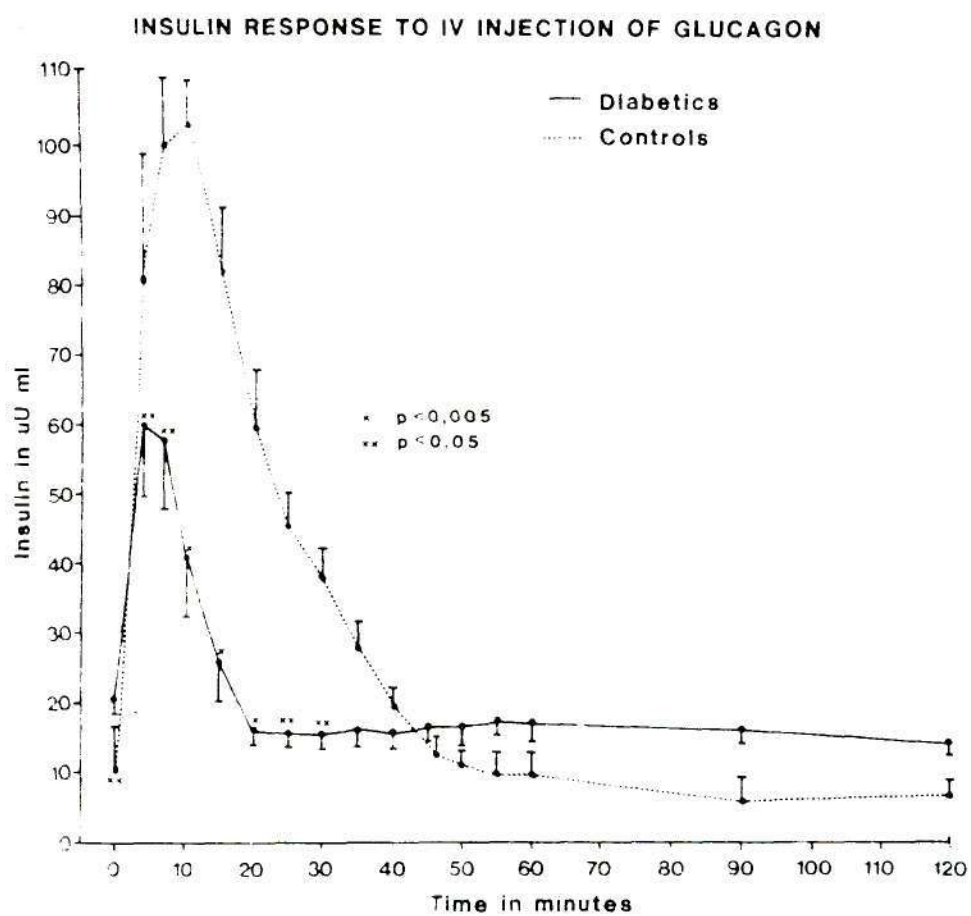


FIGURE 4.3



resumed at 20 minutes in the diabetics and 45 minutes in the controls.

4.1.5.4. Insulin response to tolbutamide

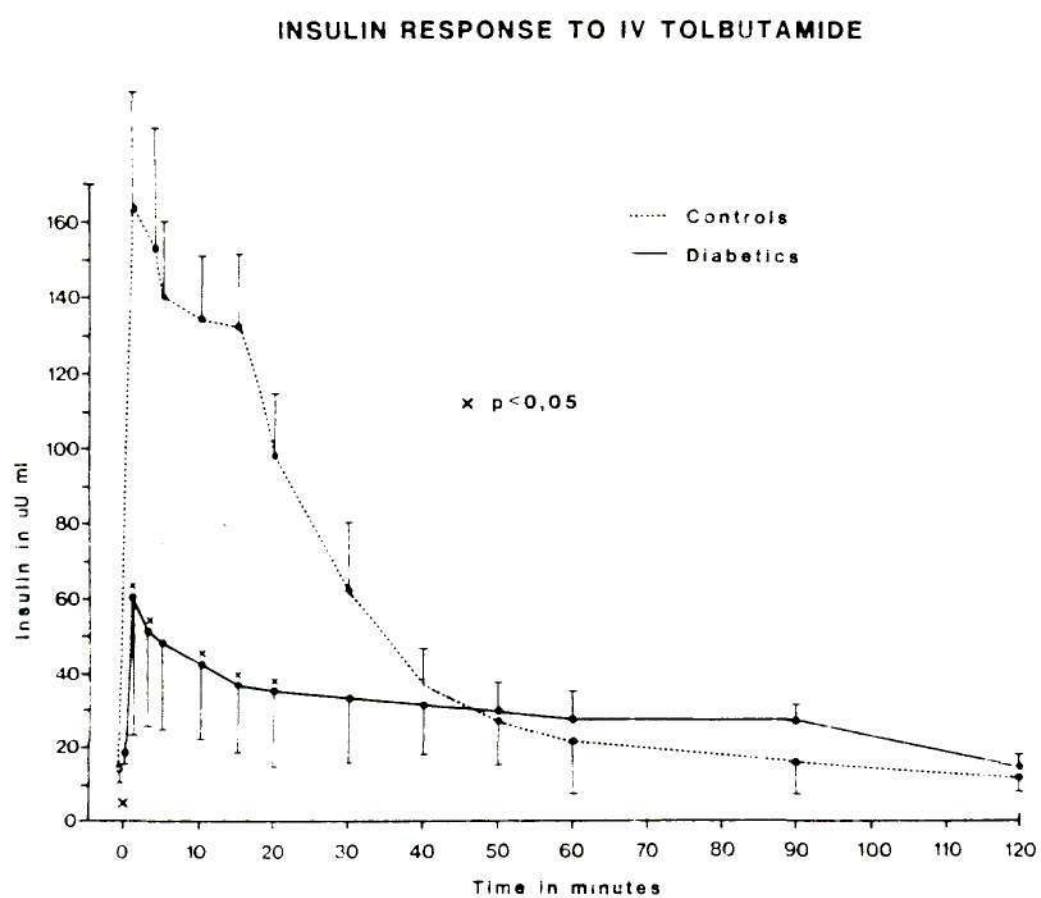
In the fasting state insulin levels were higher in the diabetics as compared with the reference subjects ($18,1 \pm 2,1$; $12,2 \pm 3,4$ $\mu\text{U/ml}$; $p < 0,05$). In the reference subjects, insulin levels peaked at one minute, a mean value of 163 $\mu\text{U/ml}$ being attained (Figure 4.4). The diabetics also reached a peak insulin level at one minute. However, the mean value obtained (63 $\mu\text{U/ml}$) was significantly lower than that attained in the controls ($p < 0,05$).

4.1.6. DISCUSSION

In this study two non-glucose secretagogues that act mechanistically different to glucose were used, in an attempt to determine the basis of the decreased first phase insulin release in NIDDM.

When glucagon was administered, NIDDM displayed a prompt release of insulin during the first three minutes (although the response was only 56,7% of that reached in the reference subjects). This is similar to the findings of Simpson et al (1968) who studied patients with NIDDM. The fact that glucagon stimulated a quicker and greater insulin response than glucose, indicates that the former stimulates insulin secretion via a different mechanism from the latter. Glucagon stimulated release of insulin also occurs independently of its hyperglycaemic action, since it also releases insulin in patients with Type I glycogen storage disease (Crockford et al, 1966) Pipeleers et al (1982) have reported that adjacent islet cells appear to influence each other by direct (paracrine) interactions. In this way paracrine signals from non- β cells (eg α cells) may enhance the insulin secretory capacity of the β cells.

FIGURE 4.4



Patients with NIDDM had a 3.5 fold increase in insulin levels in response to IV tolbutamide and this occurred at one minute after administration. However, there have been conflicting reports during the tolbutamide test. Yalow et al (1960) have demonstrated a less marked insulin rise in response to tolbutamide administration as compared with glucose administration in NIDDM. On the other hand Varsano-Aharon et al (1970) found a higher insulin secretory response to tolbutamide than to IV glucose. This response was greater in NIDDM than in the reference subjects. It should be noted though, that the diabetics in both the above cited studies had mean fasting plasma glucose levels of approximately 7 mmol/l which was considerably lower than the levels in the NIDDM patients of this study. Hence comparisons between these various groups might not be valid.

Although patients with NIDDM in this study responded to IV tolbutamide by increasing circulating levels of insulin, the mean peak insulin levels were far less than that achieved in the reference subjects. Perley and Kipnis (1966) studied patients with NIDDM and reported that their obese diabetics had higher tolbutamide-stimulated insulin levels than normal individuals, whereas the normal weight diabetics had lower levels than normal subjects. In the present study both obese and non-obese diabetics had lower insulin responses than the reference subjects. The severity of the fasting hyperglycaemia in NIDDM could be one factor accounting for this discrepancy.

Although the insulin responses to glucagon and tolbutamide were lower than that in the control subjects, one has to take cognisance of the fact that the dilemma facing the investigator is the hyperglycaemia which is a constant feature in the diabetics. This chronic hyperglycaemia must surely affect the quantitative response to glucose stimuli.

One could postulate that the persistent hyperglycaemia, stimulates the β cell to maximal capacity and thus further increments in hyperglycaemia fail to evoke an adequate insulin response. Likewise with non-glucose stimulation, although insulin responses were far less than in the reference group, this could be regarded as appropriate to the prevailing fasting glucose and insulin levels.

In comparison to the insulin responses achieved with IV glucose (section 3.2.5) it is quite apparent that the diabetics have higher insulin responses to a single maximal dose of IV glucagon and tolbutamide than to a single dose of IV glucose. These findings concur with those of Lundquist et al (1984) who demonstrated that patients with NIDDM have a selective inability to release insulin in response to a glucose load, whereas the early insulin release is relatively preserved for non-glucose stimuli. Another factor which seems to play an important role in insulin secretion in diabetes, is the effect of hyperglycaemia to augment the acute insulin response to a non-glucose stimulus - "glucose potentiation" (Ward et al, 1984). This aspect will be dealt with in great depth in section 4.2.

4.1.7. CONCLUSION

Although the mechanisms whereby glucagon and tolbutamide evoke insulin release are not exactly known, this study has confirmed that they do act mechanistically different from glucose, in that these two secretagogues elicited a greater insulin response than glucose stimulation alone.

Since tolbutamide and glucagon evoked greater insulinaemic responses than glucose, it would appear that the storage of insulin is not affected to a great extent. Before any firm conclusions can be reached, the effect of a third non-glucose stimulus, arginine monohydrochloride

will be studied.

4.2. THE INSULIN, C-PEPTIDE AND GLUCOSE RESPONSE TO A 5 GRAM ARGININE MONOHYDROCHLORIDE PULSE IN NIDDY

4.2.1. PURPOSE OF STUDY

In the previous chapter it has unequivocally been demonstrated that patients with NIDDY have inappropriate insulin responses to oral and intravenous glucose stimuli. On the other hand, these patients displayed a greater insulinaemic response to two non-glucose stimuli : glucagon and tolbutamide, which act mechanistically different to glucose.

Arginine and leucine have been shown to be the two most potent amino acids to stimulate insulin release in man (Floyd et al, 1968; Palmer et al, 1975). Furthermore it has been suggested that for arginine to adequately release insulin, a pre-existing hyperglycaemia should prevail (Halter et al, 1979).

Thus first phase insulin release in response to a third non-glucose secretagogue was studied; the hypothesis that glucose potentiates the insulin response to non-glucose stimuli was also investigated.

4.2.2. PATIENTS AND REFERENCE SUBJECTS

The body mass indices and ages of the ten patients and controls used in this study have been described in section 3.2.2.

4.2.3. STUDY PROCEDURE

The procedure was essentially similar to that set out in Section 3.1.3. After an overnight fast, 5g arginine monohydrochloride (Merck) as a 10% solution was administered intravenously over 30 seconds. Blood

samples for glucose, insulin and C-peptide were taken in the fasted state and then at 2-minute intervals for the first 10 minutes and at 15, 20 and 30 minutes after arginine administration, via an indwelling catheter in an ante cubital vein (Palmer et al, 1976).

On a subsequent occasion (one week later) also following an overnight fast, 5 of the diabetics and reference subjects underwent an intravenous insulin tolerance test (ITT) in which 0,1 U soluble insulin/kg body weight (Novo, Denmark) was administered in the fasting state. Sixty minutes after the insulin injection, when there was a considerable fall in the plasma glucose levels, 5g arginine (10% solution) was administered IV and β cell secretory responses (C-peptide levels) were quantitated over a 30 minute period as described above. This was done to confirm the hypothesis that hypoglycaemia attenuates the insulin-releasing action of arginine (Effendic et al, 1972).

4.2.4. METHODS

The methods outlined in Appendix A-1, A-5 and A-C were used to measure glucose, insulin and C-peptide concentrations respectively.

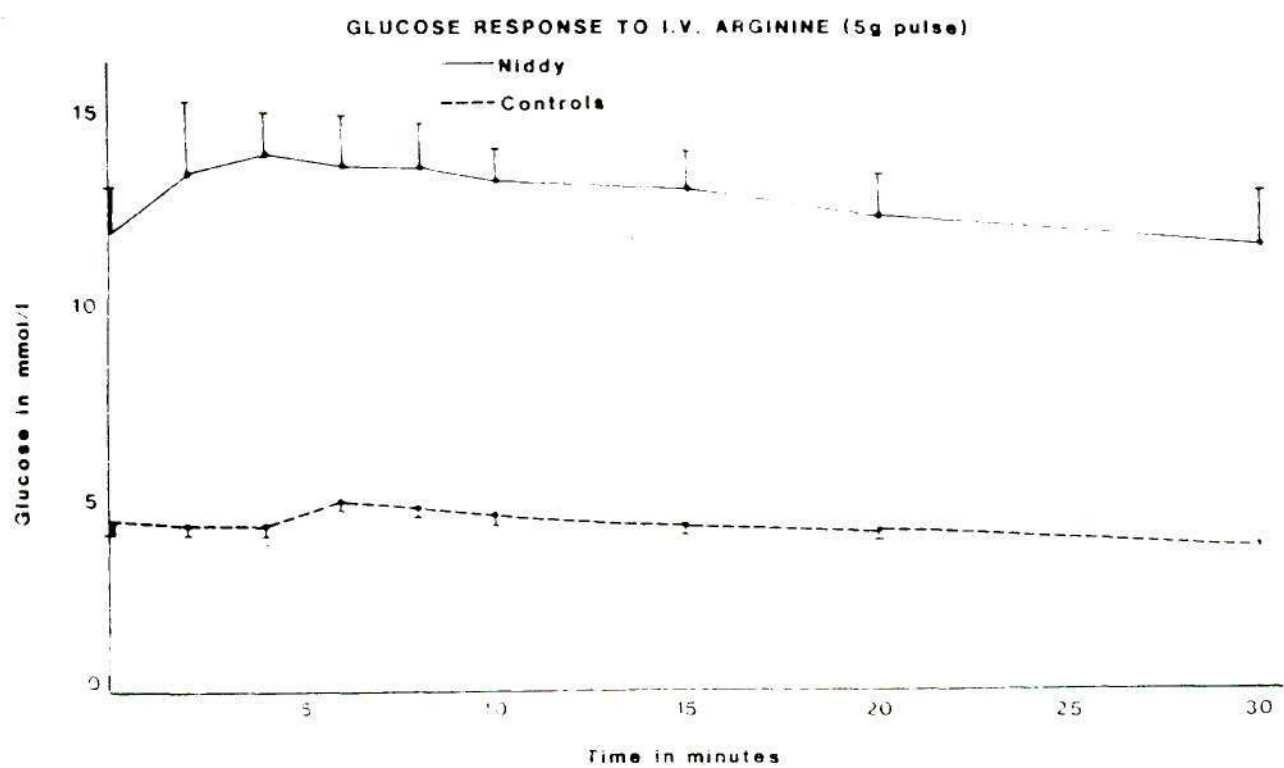
4.2.5. RESULTS

4.2.5.1. Arginine pulse only

At the outset, plasma glucose levels were considerably higher in the diabetics than the reference subjects ($11,8 \pm 1,0$; $4,6 \pm 0,1$; $p < 0,02$). Throughout the period of testing, glucose levels remained around the fasting values in both groups (Figure 4.5)

In the fasting state, diabetics displayed significant hyperinsulinaemia ($21,2 \pm 2,0$; $11,1 \pm 1,0$ $\mu\text{U/ml}$; $p < 0,02$). Peak insulin responses were attained in both groups at two minutes ($84,1 \pm 10,6$; $68,2 \pm 8,0$ $\mu\text{U/ml}$;

FIGURE 4.5



$p > 0,5$). Throughout the period of testing (with the exception of the fasting state) there was no significant difference between the insulin responses in the diabetics and reference subjects (Figure 4.6)

C-peptide (CPR) values were higher in the diabetics than the reference subjects in the fasting state, but this did not attain statistical significance ($2,6 \pm 0,3$; $2,1 \pm 0,4$ ng/ml; $p > 0,5$). During the remainder of the test, levels were similar in diabetics and control subjects (Figure 4.7).

4.2.5.2. Administration of arginine pulse after insulin-induced hypoglycaemia

In the five diabetics that received a bolus of soluble insulin, plasma glucose levels fell from a mean level of $13,2 \pm 2,2$ to $5,0 \pm 1,2$ mmol/l at sixty minutes ($p < 0,001$). When the glucose level in the diabetics had fallen to a value of 5 mmol/l, the arginine pulse was commenced and CPR responses were recorded. The CPR responses in the patients with NIDDM were considerably lower (Figure 4.8) than the previous CPR responses to arginine injection only (Figure 4.7). When comparing the CPR responses with those of the reference subjects, the diabetics now had significantly lower responses following the insulin primed arginine pulse (Figure 4.9).

4.2.6. DISCUSSION

The early phase of insulin release in response to glucose, glucagon and tolbutamide is diminished in patients with NIDDM (Lundquist et al. 1984) and is also the case in patients with NIDDM (Chapter 3 and 4). However, in response to a 5 gram arginine pulse, NIDDM displayed similar mean peak insulin responses to those of their age, weight and sex matched controls. The timing of the response was also identical,

FIGURE 4.6

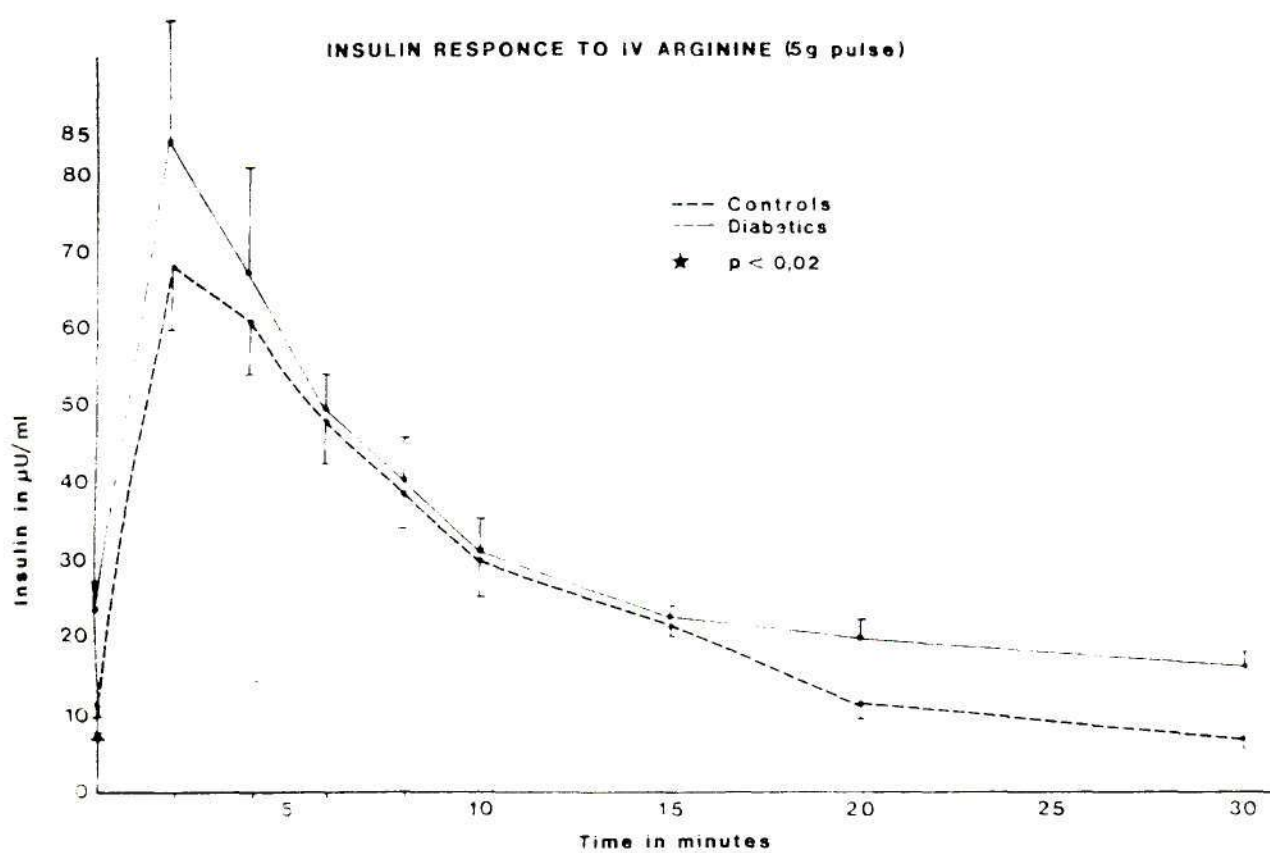


FIGURE 4.7

C-peptide responses to intravenous arginine only.

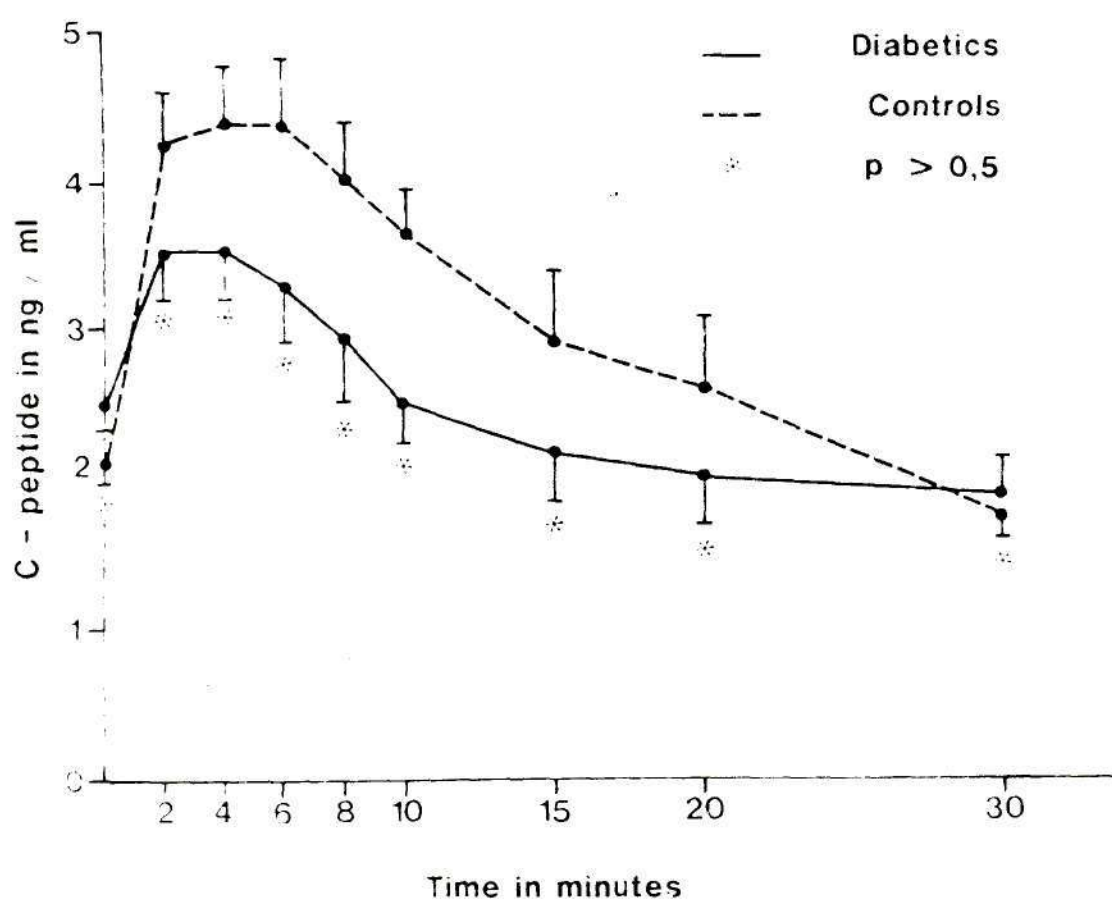


FIGURE 4.8

C-peptide responses to arginine in patients with NIDDM - pre and post insulin infusions.

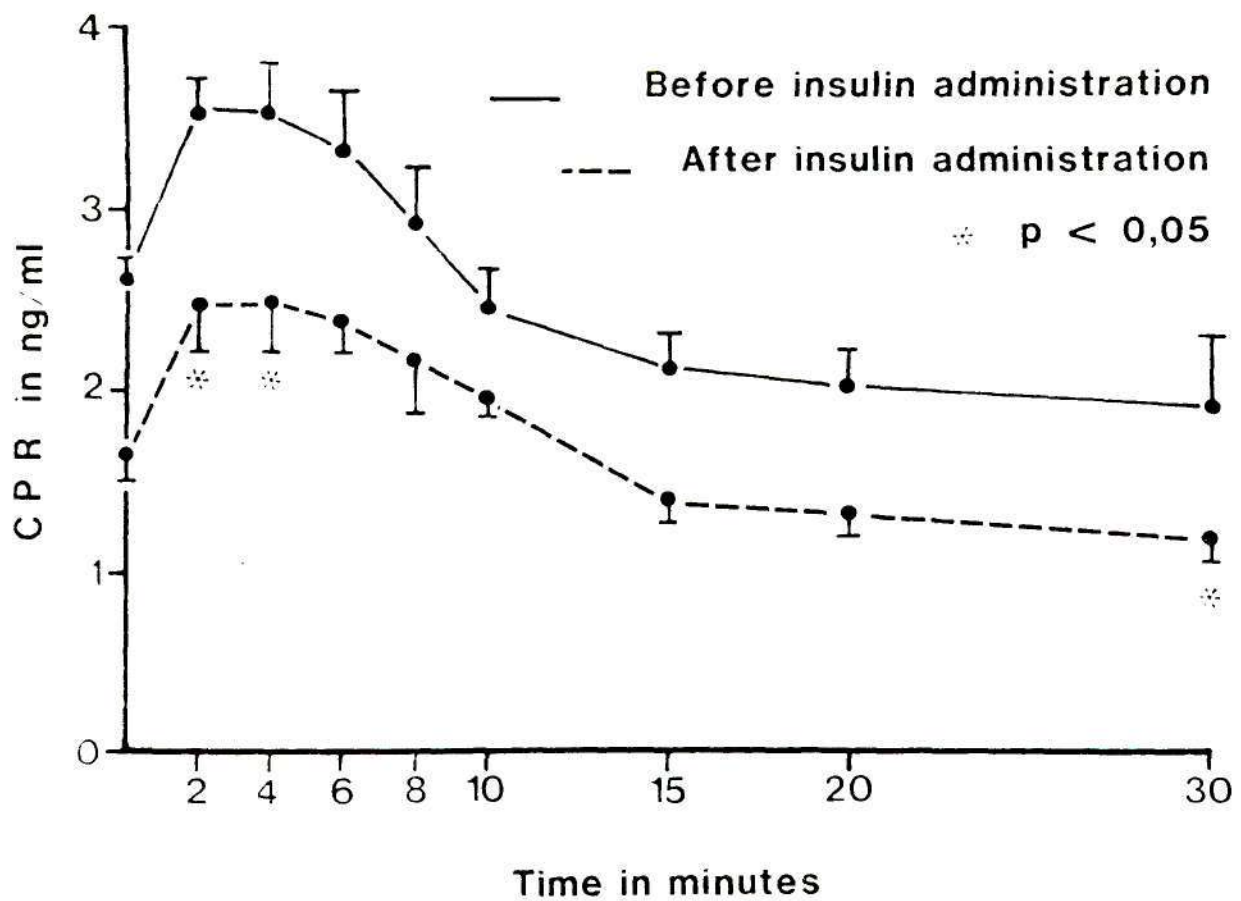
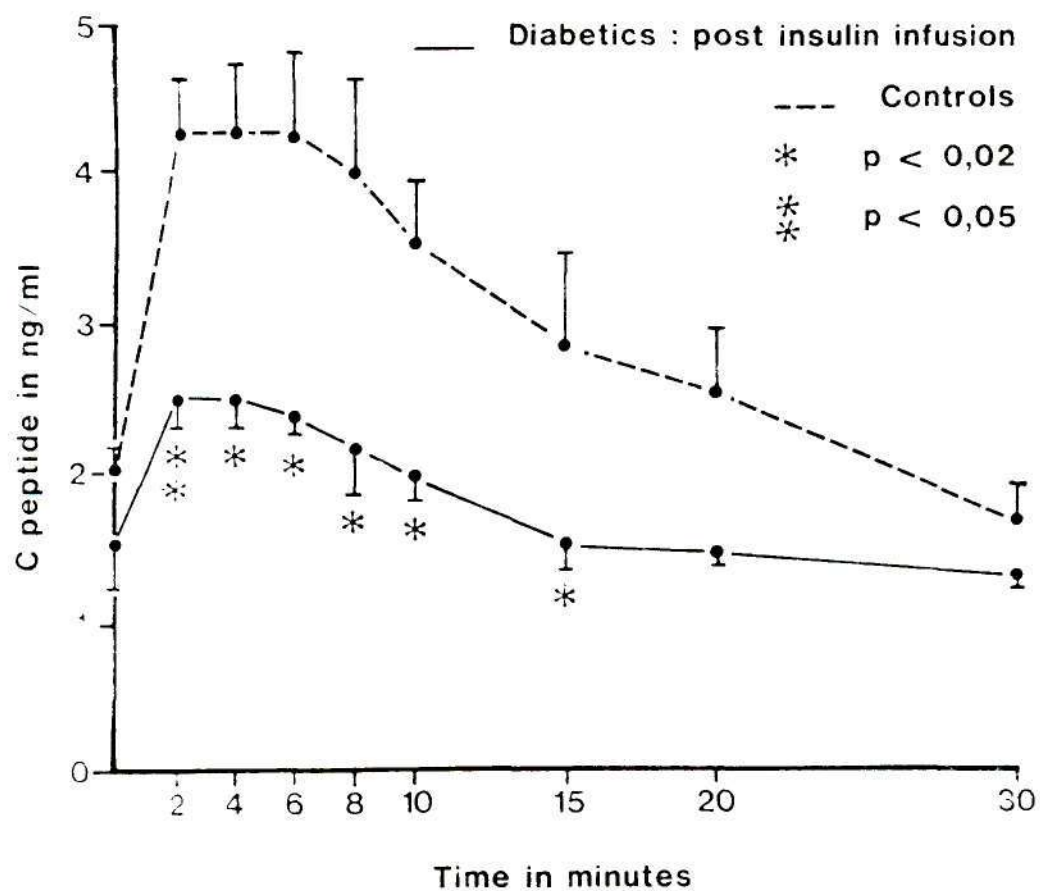


FIGURE 4.9

C-peptide responses in controls and diabetics (post-insulin infusion)



in that insulin levels peaked at two minutes in both groups. These findings are similar to those reported by others in patients with NIDDM (Palmer et al, 1976; Savage et al, 1979; Pfeifer et al, 1981), but are at variance with the findings of Fajans et al (1972) who found greater insulin responses in healthy controls as compared to mildly non-insulin-dependent diabetic patients.

Pancreozymin, a potent insulin secretagogue (Meade et al, 1967) is released following amino acid ingestion (Wang et al, 1951). However, with IV administration of arginine. Effendic et al (1972) have suggested that here the mechanism of its' insulin releasing action is by potentiation of the glucose signal at the β cell. The mediation of the glucose signal in the β cell involves the cyclic AMP system, since agents influencing the intracellular levels of cyclic AMP also modify insulin secretion induced by glucose (Cerasi and Luft, 1969; Cerasi et al, 1969). Thus for arginine to adequately release insulin, a pre-existing hyperglycaemia must prevail (Halter et al, 1979). Knowing that hyperglycaemia augments the insulin response to arginine, the researcher is faced with two problems : to study this effect, one can either administer glucose to the reference subjects and then compute the insulin responses to an arginine pulse or alternately, by rendering the diabetics "hypoglycaemic" with a bolus dose of insulin, the β cell secretory responses could be measured. However, for ethical reasons, it was decided to render the diabetics "hypoglycaemic" with a bolus of intravenous insulin in an attempt to "normalize" the diabetic state rather than to administer glucose infusions to normal volunteers. It could be argued that a dose of exogenous insulin might theoretically suppress endogenous insulin production; also the effect of hypoglycaemia with it's consequent counter-regulatory response can be uncomfortable. For the purpose of this study only a small, single dose of insulin was

administered and there were no untoward side effects of hypoglycaemia as the diabetics started out with a plasma glucose level of 13,2 mmol/l. Furthermore the arginine infusion was only commenced one hour after the insulin injection.

The administration of insulin to the diabetics introduced a variable in this study and one wonders whether it is justifiable to compare the diabetics (at this euglycaemic level) with the reference subjects. Unfortunately, there are currently no other methods available whereby one can test the hypothesis that glucose augments the insulin response to non-glucose secretagogues. At this decreased glucose level, diabetics now had substantially lower β cell secretory responses and the only conclusion that can be reached is that hyperglycaemia definitely potentiates the insulin response to arginine stimulation.

Levin et al (1971) and Palmer et al (1975) have shown an enhancement of arginine-induced insulin secretion by prior administration of glucose in normal man. Due to ethical limitations the reference subjects were not given IV glucose infusions, prior to arginine injections, rather the diabetics were administered with IV insulin.

4.2.7. CONCLUSION

The quantitative insulin response to an arginine pulse in NIDDM was essentially similar to that in the reference subjects. Although this was shown to be due to the effect of glucose potentiation on the β cell secretory capacity, it must still be concluded that the mechanism of insulin release in NIDDM is intact and that the storage of insulin is in no way defective.

4.2.8. SUMMARY AND CONCLUSIONS ON INSULIN SECRETION IN NIDDM

In comparison to their reference subjects patients with NIDDM display a marked fasting hyperinsulinism. However when taken in conjunction with their basal hyperglycaemia, it is evident that NIDDM in fact have a deficient basal insulin output.

In response to glucose stimuli (oral and intravenous) the diabetics displayed considerably lower insulin levels than their reference subjects and non-diabetic family members. This response was far lower with intravenous glucose than oral glucose suggesting that gut factors (probably gastro-intestinal peptides) are important in enhancing insulin secretion after oral glucose. The fact that insulin levels were higher in response to non-glucose stimuli would suggest that the β cell displays a relative "blindness" to purely glucose stimuli.

On the other hand, not a single non-diabetic family member had any evidence of glucose intolerance. In fact, all displayed normal insulinaemic responses to oral and intravenous glucose. But, it is important to note that the sample size was rather small and perhaps if larger numbers were studied, this phenomenon would manifest itself.

In response to glucagon and tolbutamide diabetics displayed higher insulin responses than that attained with oral and intravenous glucose. Although these responses were greater with non-glucose stimuli, they were still far less than the insulinaemic responses of the matched reference subjects. Cognisance should be taken of the fact that diabetics had significantly higher fasting insulin levels which would tend to deplete the insulin storage pool. After the administration of arginine, diabetics had a similar insulinaemic response to that of their control subjects, suggesting that NIDDM have no defect in releasing stored insulin. This "normal" insulin response was later con-

firmed to be due to the effect of hyperglycaemia to augment the acute insulin response to arginine. When the diabetics were given an insulin bolus to decrease their fasting plasma glucose levels, their β cell secretory responses to the arginine pulse also fell.

Thus the diabetic's displayed a differential response to varying stimuli, in that the most potent stimulus was arginine and the weakest IV glucose. The responses to the various secretagogues are set out in Table 4.2. It may be that the suggestion of Robertson and Porte (1973) that two sets of receptors reside within the β cell namely : glucose and non-glucose receptors, is a tenable one.

In conclusion it has been shown that NIDDM have attenuated insulin responses to glucose (oral and intravenous) glucagon, tolbutamide and arginine. In this regard NIDDM differs in no way from NIDDM of late onset. Although insulinaemic responses were decreased in diabetics, it would appear that these levels are in keeping with the fasting hyperinsulinism, which would tend to deplete the acute storage pool of insulin. Using a variety of different non-glucose secretagogues, different insulinaemic responses were provoked, confirming that glucagon, tolbutamide and arginine act mechanistically different from glucose. Thus, it can be concluded that the storage of insulin in NIDDM does not seem to be affected to a great extent and that the mechanisms of insulin release are intact, though somewhat overloaded. The constant fasting hyperglycaemia in the diabetics is a constant stimulus to the pancreatic β cell which ultimately leads to β cell "exhaustion" and its consequence of insulin deficiency. This theory of β cell "exhaustion" concurs with the findings of DeFronzo and Ferrannini (1982) and Reaven (1984). Besides the fact that the β cells are working at maximal capacity in an attempt to produce adequate amounts of insulin, it has also been reported that chronic hyperglycaemia can permanently damage

TABLE 4.1. THE MEAN PEAK INCREMENTAL INSULIN RESPONSE (μ U/ML)
TO VARYING STIMULI IN NIDDY AND CONTROLS

| STIMULUS | NIDDY | CONTROL |
|--------------------------|----------------|-----------------|
| ORAL GLUCOSE 100 GRAM | 24,8 \pm 10 | 89,8 \pm 4,0 |
| IV GLUCOSE 0,5g/Kg | 8,8 \pm 1,5 | 107,5 \pm 5,9 |
| IV GLUCAGON 1 mg | 35,0 \pm 6,3 | 84,2 \pm 5,4 |
| IV TOLBUTAMIDE 1 Gram | 42,5 \pm 11 | 134,1 \pm 7,3 |
| IV ARGININE 5 Grams | 61,6 \pm 18 | 58,0 \pm 8,5 |

TABLE 4,2 DIFFERENCES IN THE INSULIN RESPONSES TO VARIOUS
STIMULI IN NIDDDY

| | | | | |
|-----|----------------|----|----------------|-----------|
| (a) | Oral glucose | vs | iv glucose | p < 0,02 |
| (b) | Oral glucose | vs | iv tolbutamide | N.S. |
| (c) | Oral glucose | vs | iv glucagon | N.S. |
| (d) | Oral glucose | vs | iv arginine | p < 0,05 |
| (e) | IV glucose | vs | iv glucagon | p < 0,002 |
| (f) | IV glucose | vs | iv tolbutamide | p < 0,02 |
| (g) | IV glucose | vs | iv arginine | p < 0,002 |
| (h) | IV tolbutamide | vs | iv glucagon | N.S. |
| (i) | IV glucagon | vs | iv arginine | N.S. |
| (j) | IV tolbutamide | vs | iv arginine | N.S. |

vs : versus

NS : not significant

these pancreatic cells (Unger and Grundy, 1985). This would further compound the insulin deficiency.

The fact that the fasting hyperinsulinism does not alleviate the hyperglycaemia would suggest, that perhaps insulin resistance is also another factor contributing to the diabetic milieu. Olefsky (1981) has reported that insulin resistance is a cardinal feature of NIDDM. Since published information on insulin resistance in NIDDM is scanty, it will be interesting to study this phenomenon in these patients.

CHAPTER 5

INSULIN RESISTANCE IN NON-INSULIN-DEPENDENT DIABETES IN THE YOUNG

5.1 THE GLUCOSE AND FFA RESPONSE TO INTRAVENOUS INSULIN ADMINISTRATION.

5.1.1. Purpose of Study

In the preceeding two chapters, evidence was presented which indicates that in patients with NIDDDY, insulin secretion in response to a variety of stimuli is diminished when compared to age, weight and sex-matched non-diabetics. The interpretation placed on these observations is, as in the case of NIDDM, that insulin secretion is defective, or in quantitative terms inadequate to the stimulus. The validity of such a conclusion in the face of the constant hyperglycaemic stimulus in the patients with NIDDDY has already been argued in Section 4.1.6. Be this as it may, if it is held that insulin secretion is defective, it follows that hyperglycaemia is a consequence of the quantitative deficiency in insulin secretion. On the other hand, if there is peripheral resistance to the action of insulin, it may be argued that the overload of a sustained hyperglycaemic stimulus may modify insulin secretion in the manner observed. Hence the insulin secretory defect may be a consequence of the hyperglycaemia. In either event, once hyperglycaemia is established, a self-sustaining situation develops.

The evidence for peripheral insulin resistance in NIDDDY was critically examined in Section 1.3.4., but in a sense the investigator faces the same problem as in the case of insulin secretion: the NIDDDY group are not normoglycaemic and therefore not strictly comparable to non-diabetics; alternatively, if patients or non-diabetics are rendered comparable in glycaemic or insulin status, the counter-regulatory responses will render the groups non-comparable on other grounds.

However, Jialal and Joubert (1985) reported an interesting observation on FFA responses in NIDDM following oral glucose. Their evidence suggested that adipose tissue is at least normally sensitive to endogenous insulin. If this is correct, it would be of manifest interest to monitor the responses of glucose and FFA's simultaneously after insulin administration, inasmuch as the responses in different tissues can be observed when the same counter-regulatory milieu exists. The purpose of the study was to examine glucose and FFA responses to administered insulin in patients with NIDDM.

5.1.2. Patients and Reference Subjects

Ten patients with NIDDM were studied as well as 10 age, weight and sex-matched reference subjects. The BMI and ages of both groups were similar (Section 3.2.2.).

5.1.3. Study Procedure

Patient and reference subject preparation was described in Section 3.1.3. After an overnight fast, 0.1U soluble insulin (Novo, Denmark/kg body weight was administered intravenously as a bolus. Blood samples for FFA and glucose estimation were taken in the fasting state and then at half-hourly intervals for three hours from an indwelling catheter in an ante-cubital vein. Fasting blood samples for the assay of the counter-regulatory hormones cortisol, glucagon and growth hormone were also taken.

Blood for glucose assay was taken into potassium-oxalate fluoride tubes; for FFA assay, blood was taken into tri-potassium-EDTA tubes on ice. The latter tubes were centrifuged at 4°C and the separated plasma stored at -20°C. Blood for glucagon assay was taken in tubes containing 1000IU trasylol and 2mg EDTA per ml of blood, on ice; bloods

for cortisol and growth hormone (HGH) assay were taken in tubes containing no preservative or anticoagulant.

5.1.4. Methods

The methods used for the measurement of glucose, FFA, cortisol, glucagon and HGH are set out in Appendix A-1, A-7, A-8, A-9 and A-10 respectively.

The glucose disposal constant (k_1) was calculated from the slope of the initial linear portion of the regression line obtained by plotting the natural logarithm of plasma glucose concentration against time (Franckson et al, 1966): k_1 was used as a measure of insulin resistance.

insulin/glucagon molar ratio's were calculated using the following formula:

$$\frac{*25,3 \text{ (insulin } \mu\text{U/ml)}}{\text{glucagon (pg/ml)}}$$

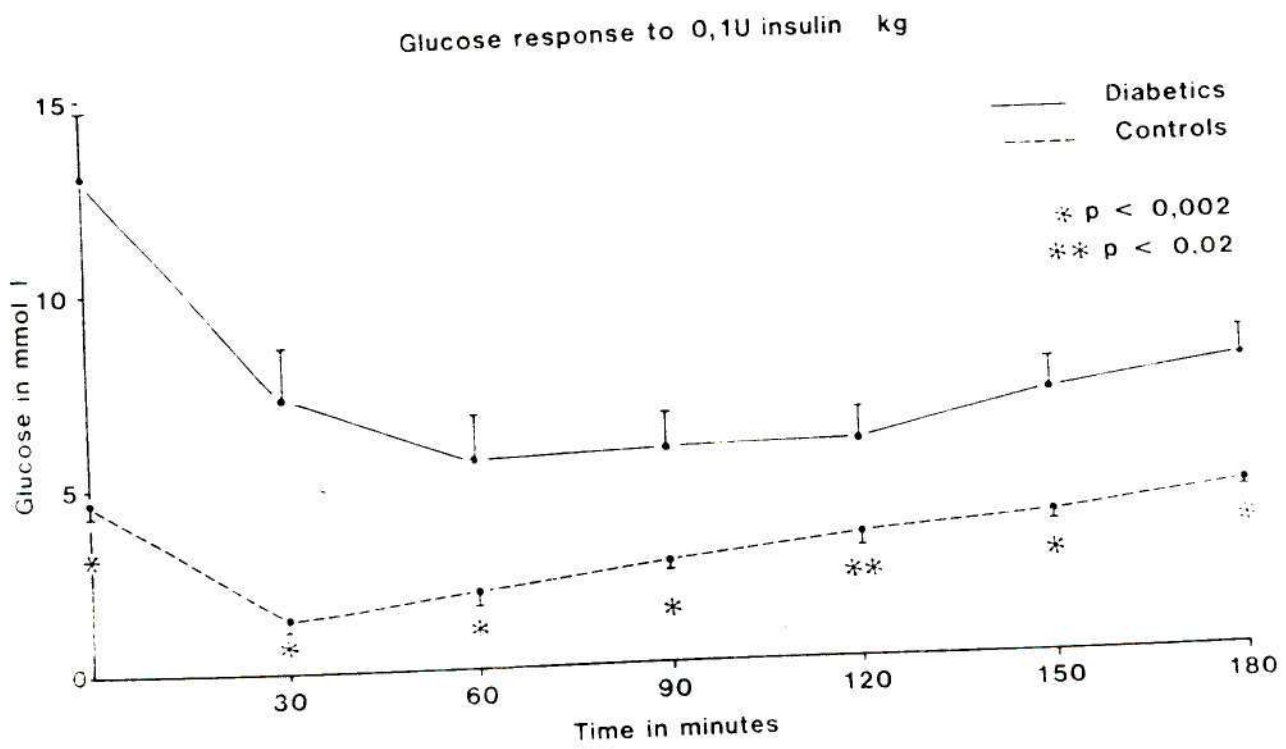
* constant to convert the ratio to a molar ratio.

5.1.5. Results

Fasting plasma glucose levels were significantly higher in the diabetics than in the reference subjects ($13,0 \pm 1,3$; $4,6 \pm 0,1$ mmol/l; $p < 0,002$). Glucose levels fell rapidly in the controls ($1,4 \pm 0,4$ mmol/l) at 30 minutes, whereas the lowest mean glucose value attained in the diabetics was $5,2$ mmol/l at 120 minutes (Figure 5.1)

The glucose disposal constant (k_1) was significantly lower in the diabetics: $2,36$ ($1,3 - 3,0\%/min$) as opposed to $6,39$ ($2,5 - 12,5\%/min$) in the reference group; K_1 correlated inversely with fasting plasma glucose levels ($r = -0,61$; $p < 0,05$)

FIGURE 5.1



Glucagon levels were considerably higher in the diabetics than the reference subjects ($205,5 \pm 15,7$; $125,3 \pm 12,6$ pg/ml; $p < 0,002$).

Cortisol levels were also higher in the diabetics ($319,2 \pm 44,3$; $240 \pm 14,8$ nmol/l; $p < 0,5$)

The mean fasting insulin/glucagon molar ratios were similar in diabetics and controls ($2,5 \pm 0,6$; $1,9 \pm 0,2$; $p > 0,5$).

HGH levels were lower in the diabetics ($0,62 \pm 0,01$; $1,75 \pm 0,06$ ng/ml; $p < 0,001$).

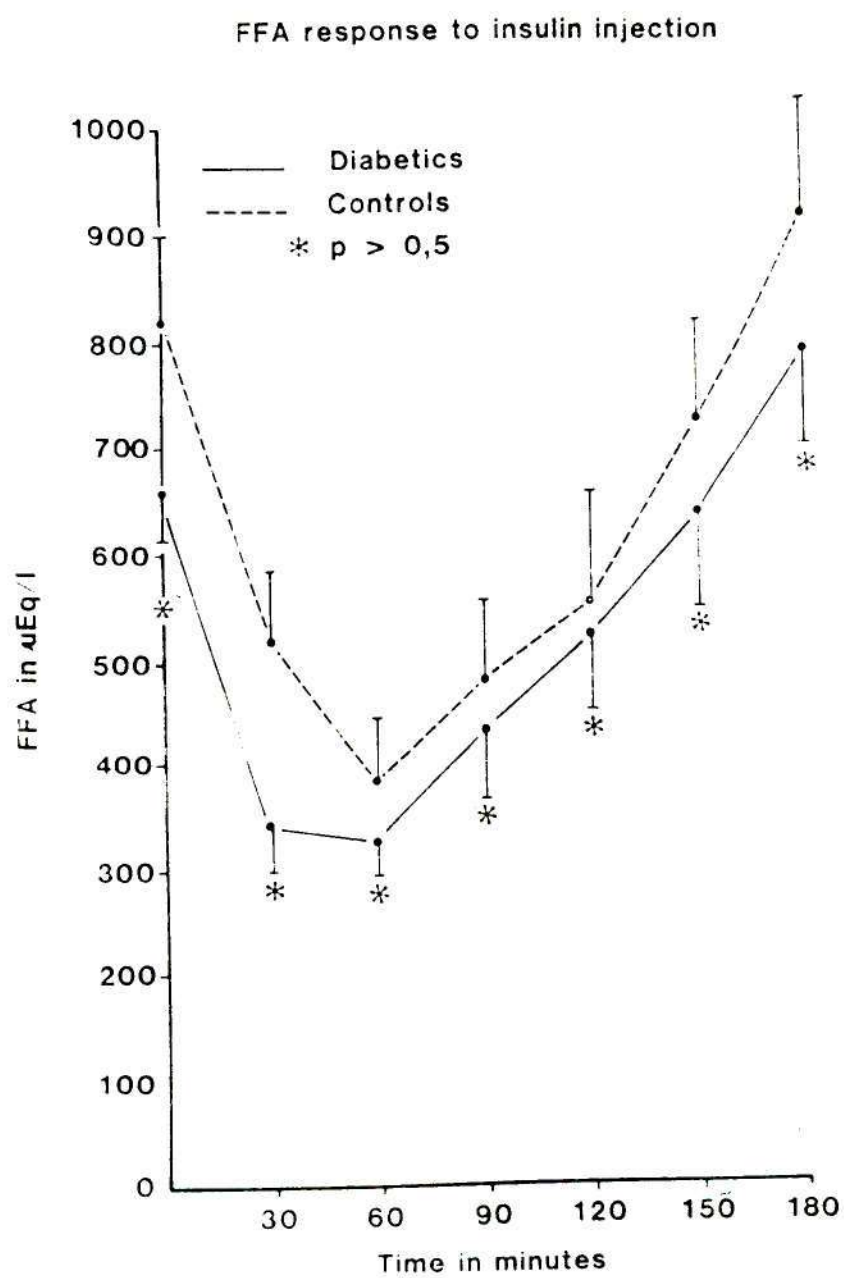
Plasma FFA's were similar in both groups in the fasting state ($650 \pm 44,7$; $802,5 \pm 82,9$ μ Eq/l; $p > 0,05$). In the diabetics and control subjects maximum decrements in FFA levels occurred at 60 minutes (Figure 5.2). The maximum percentage fall of plasma FFA's in the patients with NIDDM (58%) was similar to that attained in the reference group (54%). Nadir values in the diabetics and reference subjects were statistically similar ($325 \pm 30,5$; $375 \pm 50,4$ μ Eq/l; $p > 0,5$).

5.1.6. DISCUSSION

This rather simple study has yielded quite remarkable information: on the one hand it seems quite obvious that administered insulin was incapable of accelerating glucose disposal to the extent observed in non-diabetics; on the other hand, the ability of administered insulin to control fat metabolism seems to be unimpaired. Since there was a significant inverse correlation between the k_1 and the fasting plasma glucose, it would be reasonable to conclude that the insulin resistance contributed to the fasting hyperglycaemia.

The effect of insulin on glucose disposal in NIDDM fits in well with the notion of insulin resistance in NIDDM, a proposal first mooted half a century ago (Himsworth, 1936) and reinforced by the observation that

FIGURE 5.2



patients with mild glucose intolerance often display hyperinsulinism in response to glucose loading (Fajans et al, 1976). By contrast, the effect of administered insulin on adipose tissue is inconsistent with any concept of insulin resistance. These findings suggest that the adipocytes' lipase retained sensitivity to insulin and confirms the differential effect of insulin on the adipocytes as compared to other tissues (glucose intolerance), an observation made earlier by Rubenstein et al (1969) in healthy South African Indian males. Similar observations were also made in severely hypoinsulinaemic Pima Indian patients with NIDDM (Howard et al, 1979). Jialal and Joubert (1985) used the endogenous insulin response, in patients with NIDDM, evoked by glucose to demonstrate the differential response of glucose and FFA to an insulin stimulus.

Of abiding interest, of course, must be the basis of the apparent contradictory responses. As previously demonstrated by Jialal and Joubert (1982c) the apparent insulin resistance in patients with NIDDM is not related or based on the hormonal antagonists of insulin.

Although fasting glucagon and cortisol levels were marginally but significantly increased in the patients, the insulin/glucagon molar ratios were unaffected in patients as compared with the reference subjects and hence the two antagonists appear unimportant both in qualitative and molar ratio terms (Unger and Orci, 1981; Shamoon et al, 1980). Indeed, adipose tissue which is also a sensitive target organ for the action of these two hormones appeared unaffected by the recorded increases in their fasting levels; even more telling, GHG levels which were significantly decreased in patients with NIDDM, albeit in quantitative terms, marginally so, should arguably promote insulin sensitivity (Luft and Guillemin, 1974). Manifestly, the role of the insulin counter-regulatory hormones is not of great consequence in the

phenomenon of apparent differential sensitivity of tissue to insulin action.

A more plausible basis for the observations may well be found at the molecular level of insulin action. As discussed in Section 1.3.4.2 insulin action is, in the first instance, dependent on its binding to a cell surface receptor before it can initiate its specific biological actions. As also discussed (Section 1.3.4.2.) the kinetics of binding suggest that an excessive insulin stimulus is associated with receptor downregulation on the cell surface.

In the present study, it should be borne in mind that the response of blood glucose levels to administered insulin was compared with FFA responses in patients and reference subjects. Bearing in mind that the glucose intolerance is apparently dominantly inherited, it is possible that a receptor defect with differential expression in various tissues is a plausible explanation for the observed differential responses of tissues to insulin action. On the other hand, it may simply be that the post-receptor binding action of insulin on glucose metabolism and activation of lipoprotein lipase are not comparable in quantitative terms. The fact is that insulin levels were sufficient to prevent ketoacidosis in the patients with NIDDM, but inadequate to maintain normal carbohydrate metabolism. As Bagdade et al (1968) suggested when they made similar observations, adipose tissue may well require much lower concentrations of insulin to maintain triglyceride metabolism.

Obviously, a study of the receptors on the cell surface of the various tissues is a prerequisite to further enquiry.

5.1.7 CONCLUSION

The differential effect of insulin on plasma glucose and FFA levels in

patients with NIDDM could be interpreted as possible differences in insulin receptors or, quantitatively, different post-receptor binding responses in glucose and lipid metabolism.

5.2. INSULIN BINDING TO CIRCULATING ERYTHROCYTES AND MONOCYTES IN NIDDM.

5.2.1. Purpose of Study

One possible explanation suggested from the results of the study described in Section 5.1 was abnormalities in the insulin receptor; not only is such a proposal attractive from the point of view of the apparent insulin resistance in NIDDM but it does suggest a molecular basis for a dominantly inherited disorder and is readily amenable to direct experimental study.

Whereas it would, of course, be ideal to obtain representative samples of a number of tissues to study the insulin receptors, the choice of tissue is essentially limited to that which is readily accessible with minimum trauma to the patient; in practice, blood. There is indeed good evidence that red blood cell (RBC) receptors are representative of the general insulin receptor status in an individual (Robinson et al. 1979; De Pirro et al. 1980; Dons et al. 1981) and monocytes serve as yet an alternative tissue with some evidence that the receptor status of these cells are similar to that of adipose tissue. (Olefsky, 1976; Joost et al 1982). Certainly, if there are any structural or other abnormalities of insulin receptors in NIDDM which are genetically transmitted, it may be expected that these would be expressed in all tissues requiring insulin for one or other reason, albeit, not necessarily to the same degree in quantitative terms.

In the following study, the kinetics of insulin binding to RBC and

monocyte receptors in patients with NIDDM are reported as a first approximation to the problem of receptor status in NIDDM.

5.2.2. Patients and Reference Subjects

The same ten patients and reference subjects were studied (Section 3.2.2). All patients were women and they were studied in the follicular phase of the menstrual cycle. No patient was on oral contraceptives.

In each patient and reference subject, complete blood counts and RBC indices were entirely normal and reticulocyte counts were $<1\%$.

5.2.3. Study Procedure

The preparation of patients and reference subjects in respect of diet and oral hypoglycaemic agents were as described in Section 3.1.3. After an overnight fast, 120 mls of blood was withdrawn from patients and controls into tubes containing lithium heparin: samples for glucose and insulin assay were taken in the fasting state. In addition samples for serum progesterone measurement were also taken.

5.2.4 METHODS

The radioreceptor assay of erythrocyte and monocyte receptors is described in detail in Appendix A-11 and A-12. Glucose and insulin methods are outlined in Appendix A-1 and A-5.

The kinetics of ^{125}I -insulin binding to insulin receptors on erythrocytes and monocytes respectively, are presented in three ways: the percentage of total radioactivity which was specifically bound to receptors was plotted as a function of the logarithm of total insulin concentration; receptor number (R_0) was calculated according to Scatchard analysis (Scatchard, 1949); total concentration of unlabelled insulin

required to achieve 50% displacement of labelled insulin from the receptor (ID_{50}) was used as an index of the affinity of the insulin receptor for insulin (Grunberger et al, 1983; Taylor, 1984).

5.2.5. RESULTS

The mean fasting plasma glucose levels and fasting plasma insulin levels were significantly greater in the diabetics than in the reference subjects ($11,4 \pm 0,8$; $4,1 \pm 0,1$ $\mu\text{mol/l}$; $p < 0,001$ and $23,0 \pm 2,8$; $11,8 \pm 0,5$ $\mu\text{U/ml}$; $p < 0,001$ respectively). Serum progesterone values were similar in diabetics and controls ($0,8 \pm 0,2$; $0,7 \pm 0,1$ ng/ml ; $p > 0,5$) confirming the follicular phase of menstruation.

The mean maximum percentage of total radioactivity specifically bound to monocyte receptors was significantly lower in the diabetics as compared to their matched reference subjects ($2,2 \pm 0,3$; $4,0 \pm 0,3\%$; $p < 0,02$). This is graphically depicted in Figure 5.3. A significant inverse correlation was obtained between the maximum specific binding and fasting plasma insulin levels ($r = -0,62$; $p < 0,001$).

Scatchard analysis revealed that all 10 diabetics displayed decreased receptor numbers as compared individually to their matched reference subjects (Figure 5.4 to 5.13). ID_{50} values in all the diabetic subjects fell within the reference range, as determined in this laboratory: 2,0-10,0 ng/ml . Receptor affinities for insulin are given in Table 5.1 and indicate that there were no differences between the two groups.

The percentage of total radioactivity specifically bound to erythrocyte receptors was similar in both groups studied ($9,7 \pm 0,5$; $8,9 \pm 0,5\%$; $p > 0,5$). These findings are graphically displayed in Figure 5.14.

Scatchard analysis revealed that all diabetics had similar receptor numbers as their controls (Figure 5.15); receptor affinity for insulin

TABLE 5.1 BIOCHEMICAL FEATURES OF NIDDIY AND CONTROL SUBJECTS
(MONOCYTE RECEPTORS)

| PATIENTS | FASTING INSULIN μ U/ml | FASTING PLASMA GLUCOSE mmol/l | % MAXIMUM SPECIFIC BINDING (0,2ng/ml) | ID ₅₀ ng/ml | Ro (ng/ml) | Ro (in controls) (ng/ml) |
|-----------------------|----------------------------------|--|--|---------------------------|---------------|-----------------------------|
| A ₂ (A.N.) | 17 | 12 | 3,1 | 10,0 | 1,7 | 3,0 |
| B ₁ (A.S.) | 27 | 12 | 1,3 | 10,0 | 0,6 | 1,2 |
| B ₂ (C.S.) | 31 | 12 | 1,5 | 8,0 | 0,7 | 2,2 |
| C ₁ (M.C.) | 17 | 14 | 2,0 | 2,1 | 1,2 | 1,9 |
| C ₃ (H.C.) | 39 | 7 | 1,1 | 5,0 | 0,6 | 2,1 |
| C ₄ (J.M.) | 32 | 13 | 4,1 | 5,4 | 0,5 | 2,3 |
| D ₁ (S.D.) | 13 | 11 | 2,3 | 4,0 | 2,5 | 3,0 |
| D ₂ (K.D.) | 12 | 7 | 1,6 | 7,0 | 2,5 | 3,6 |
| E (A.P.) | 21 | 13 | 2,5 | 2,2 | 1,2 | 2,1 |
| F (T.E.) | 20 | 13 | 2,1 | 5,2 | 1,0 | 1,4 |
| Diabetics Mean | 23,0 \pm 2,8 | 11,4 \pm 0,8 | 2,2 \pm 0,3 | 5,9 \pm 0,9 | 1,3 \pm 0,2 | |
| Range | (12-39) | (7-14) | (1,1-4,1) | (2,1-10) | (0,5-2,5) | |
| Controls Mean | 11,7 | 4,1 | 4,0 | 4,6 | 2,8 | |
| Range | (5-20) | (3,3-6,0) | (2,6-6,2) | (2,0-10,0) | (1,2-3,6) | |

FIGURE 5.3

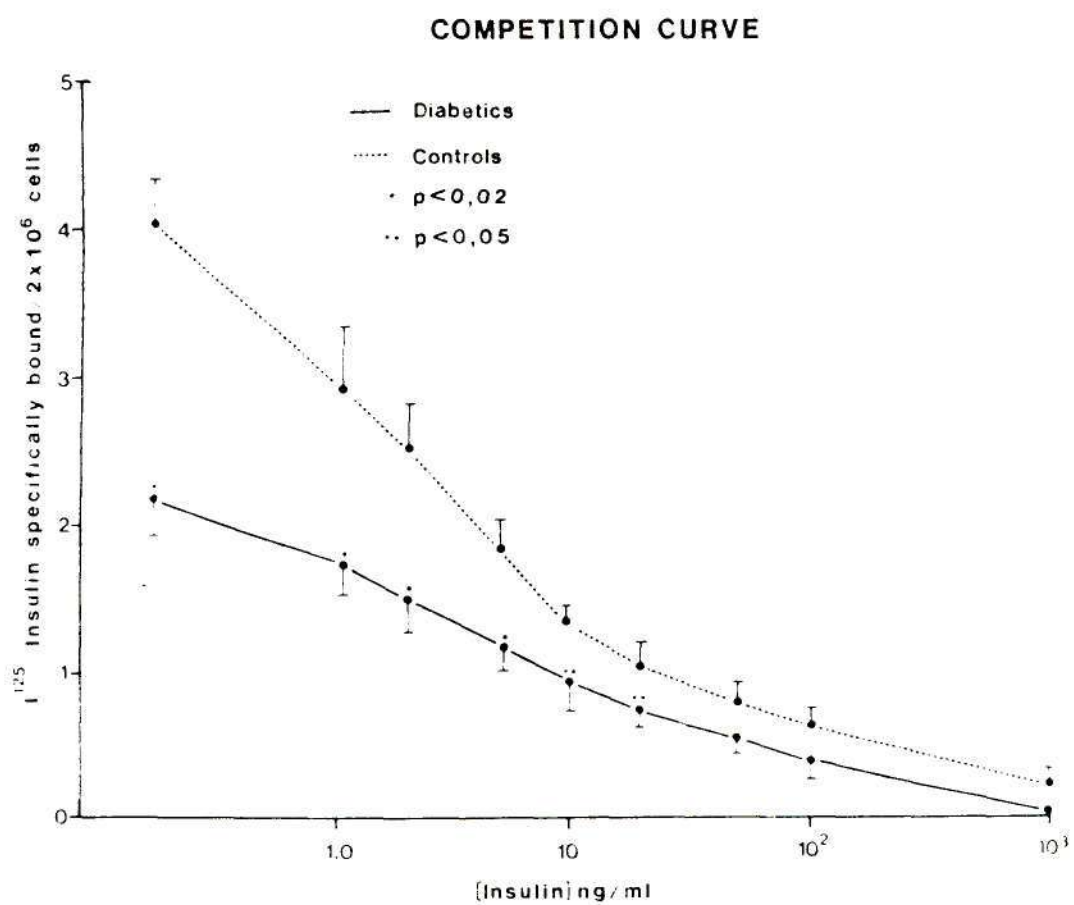


FIGURE 5.4

Scatchard plot (Monocytes)

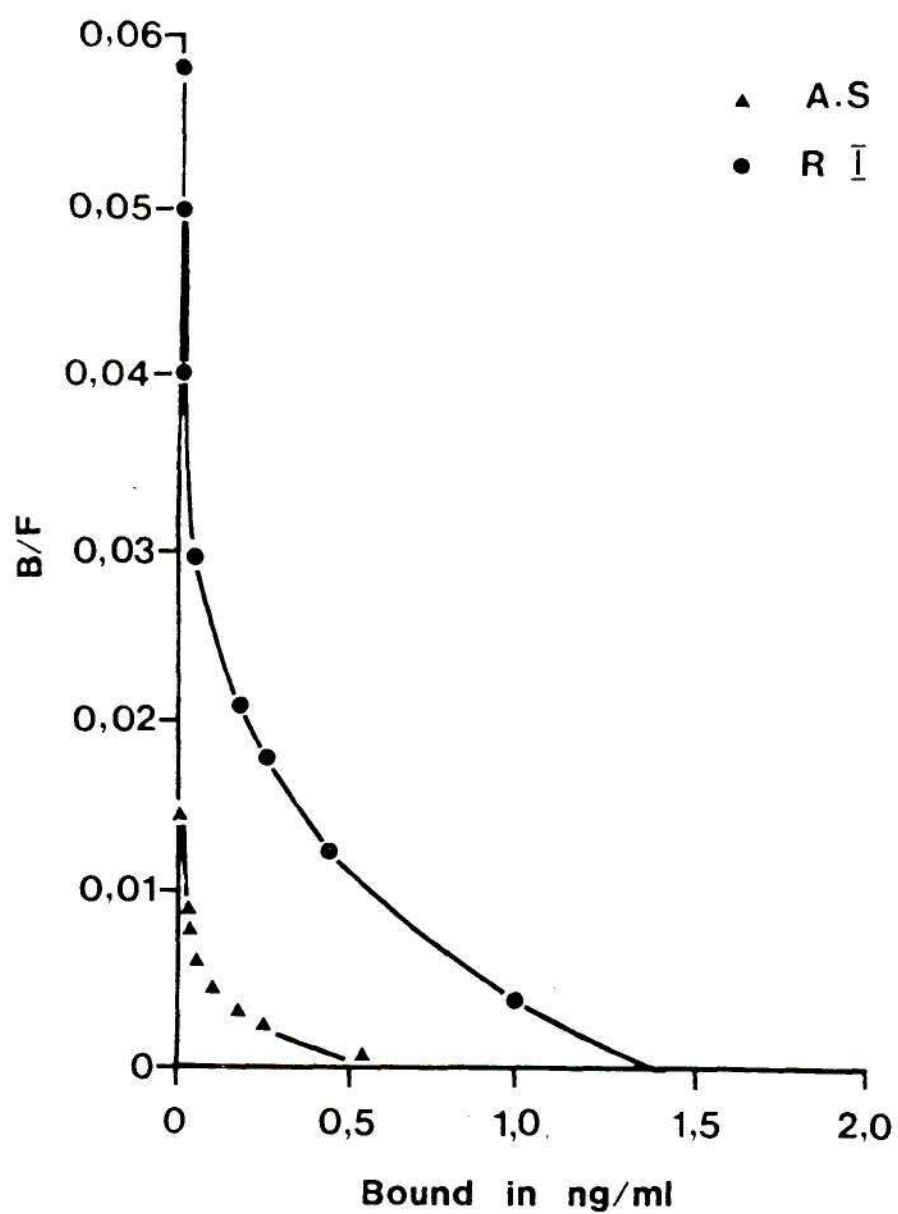


FIGURE 5.5

Scatchard plot (Monocytes)

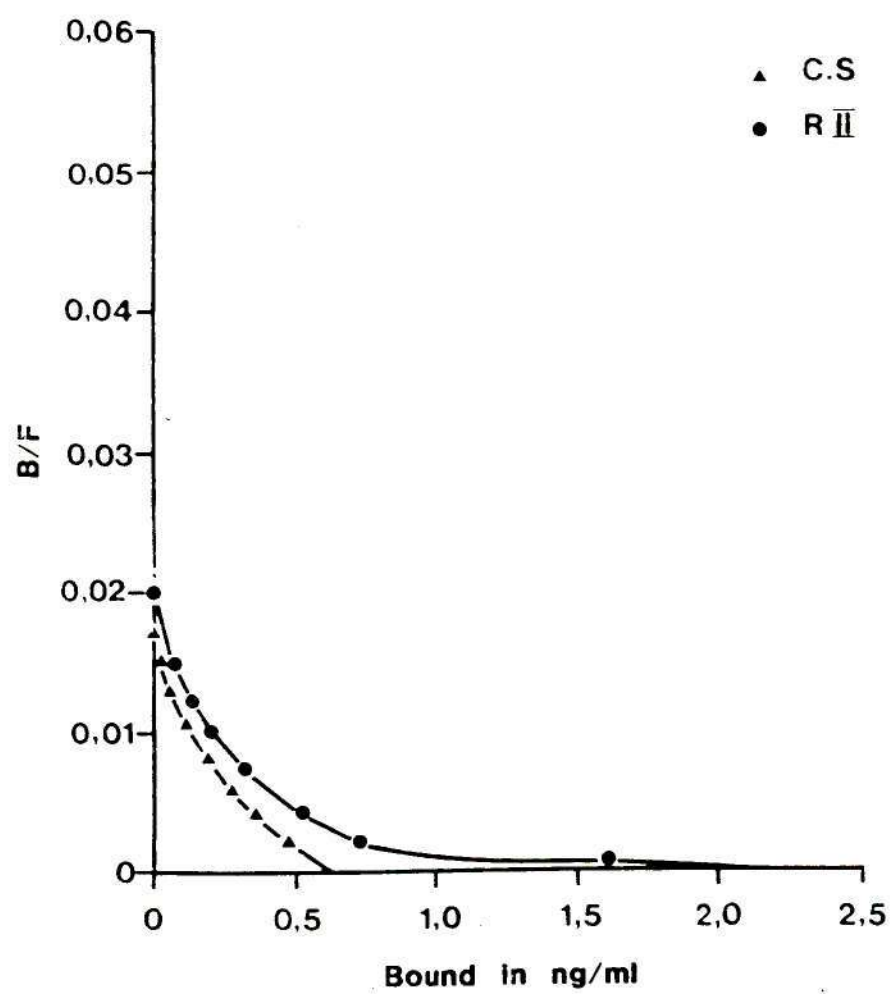


FIGURE 5.6

Scatchard plot (Monocytes)

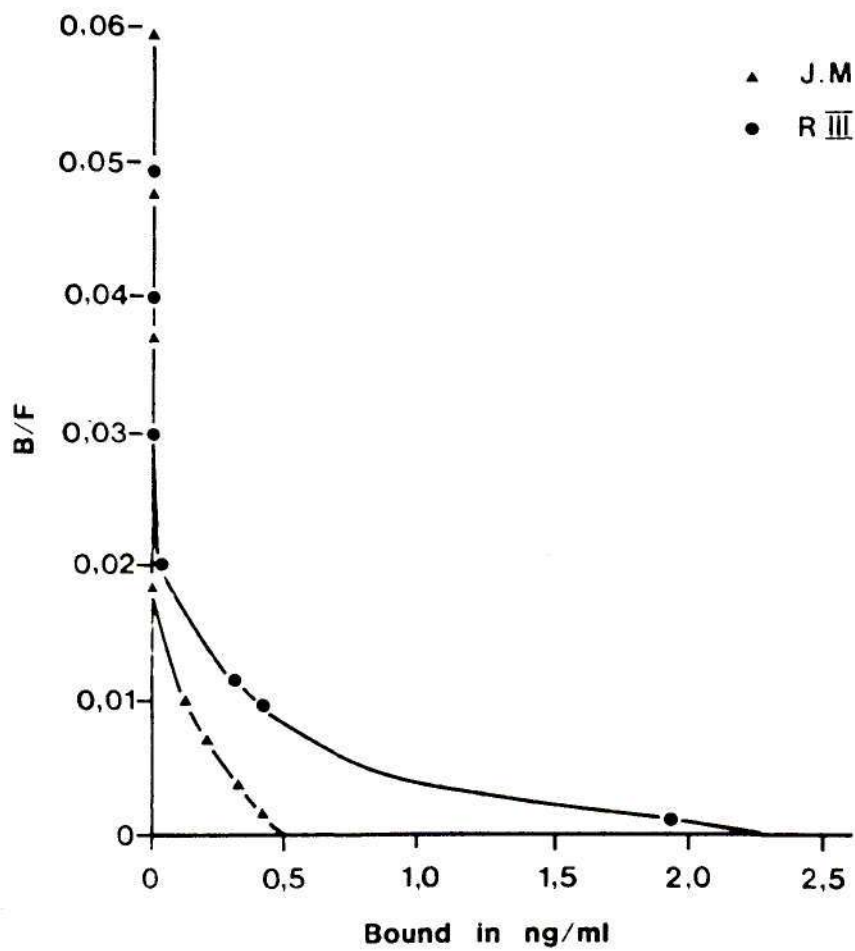


FIGURE 5.7

Scatchard plot. (Monocytes)

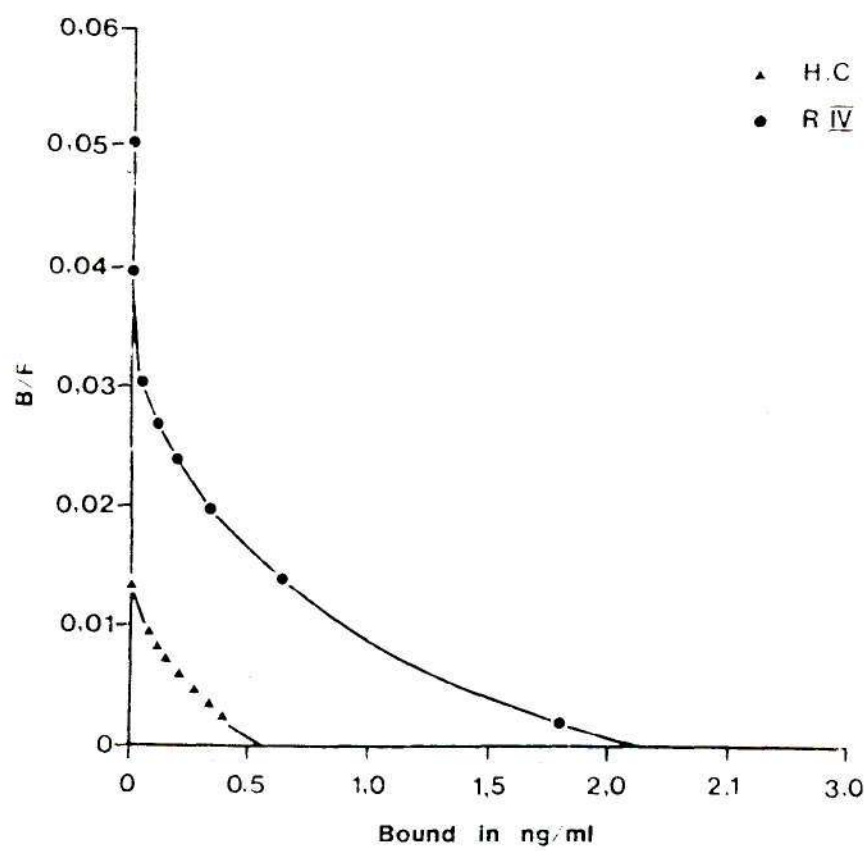


FIGURE 5.8

Scatchard plot (Monocytes)

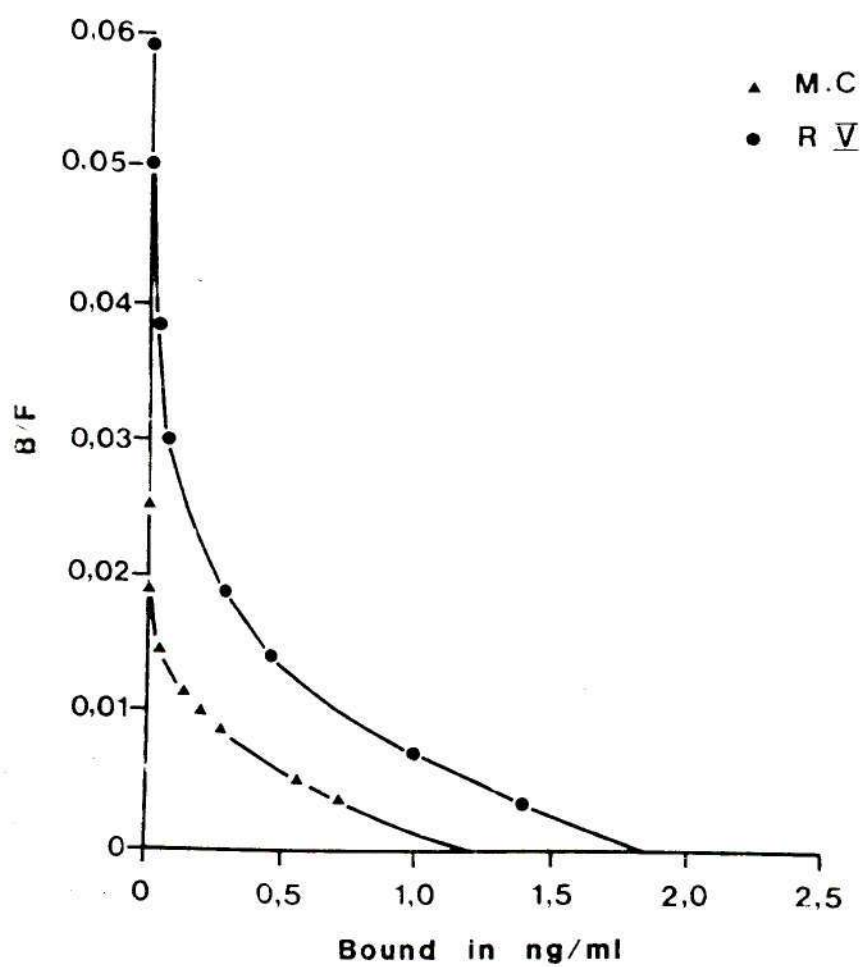


FIGURE 5.9

Scatchard plot (Monocytes)

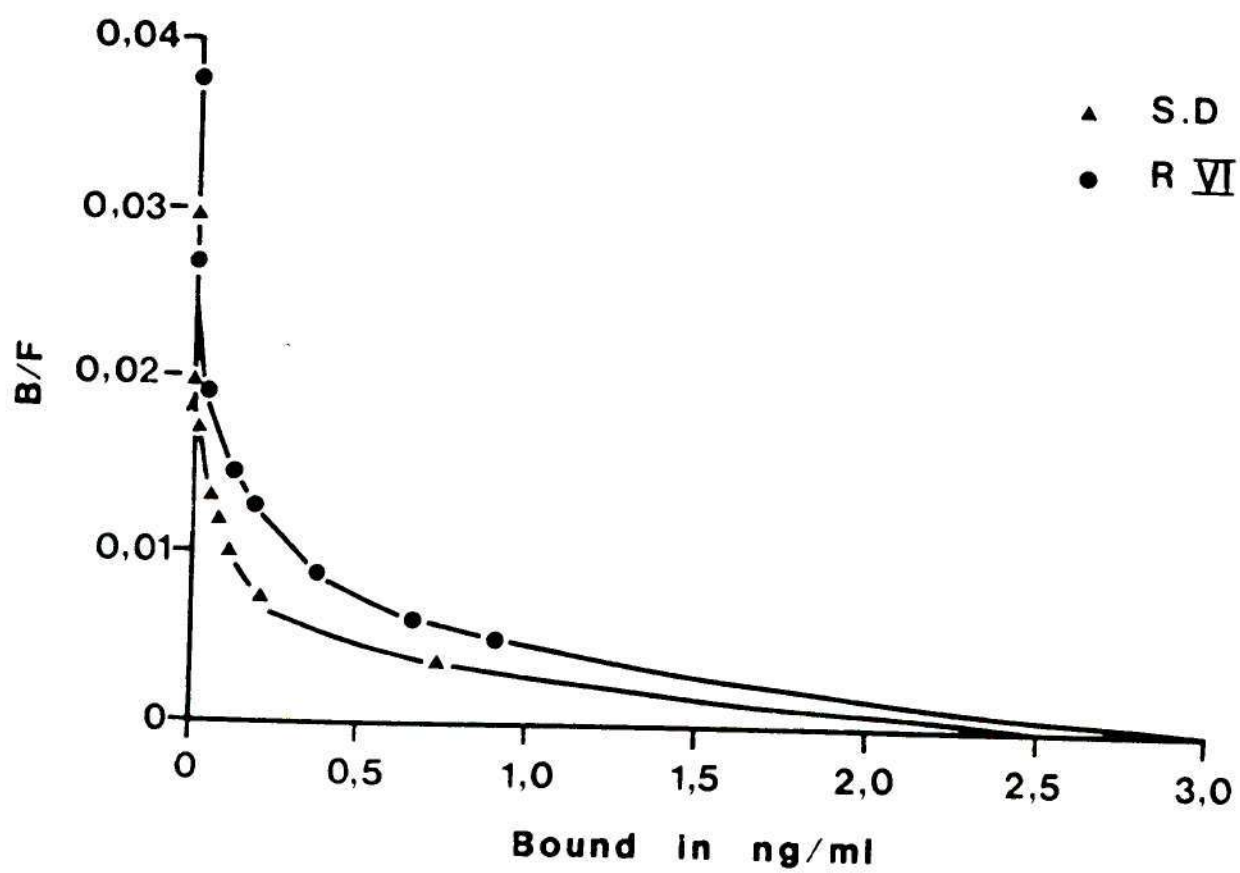


FIGURE 5.10

Scatchard plot (Monocytes)

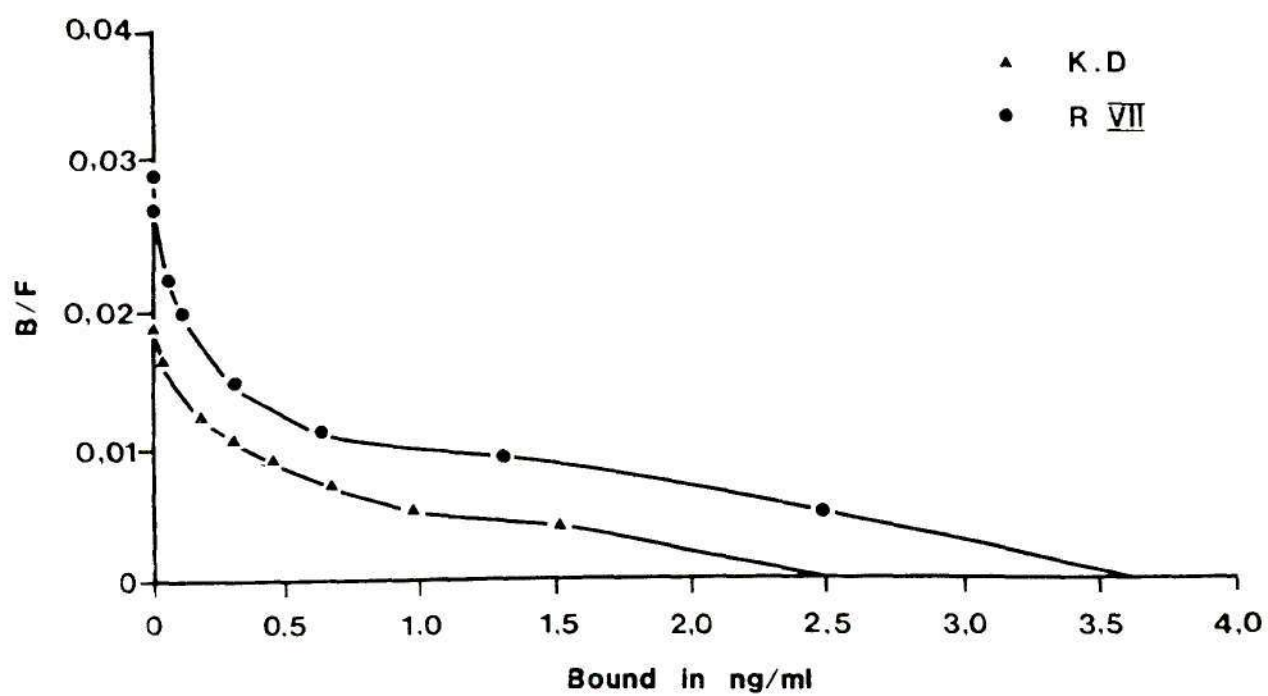


FIGURE 5.11

Scatchard plot (Monocytes)

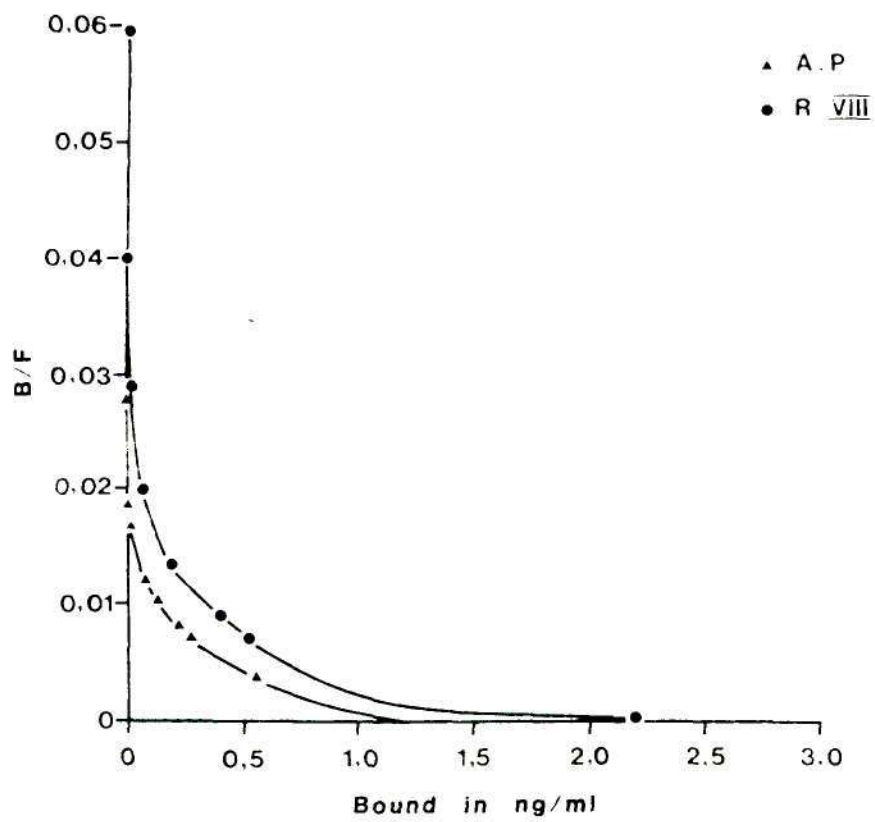


FIGURE 5.12

Scatchard plot (Monocytes)

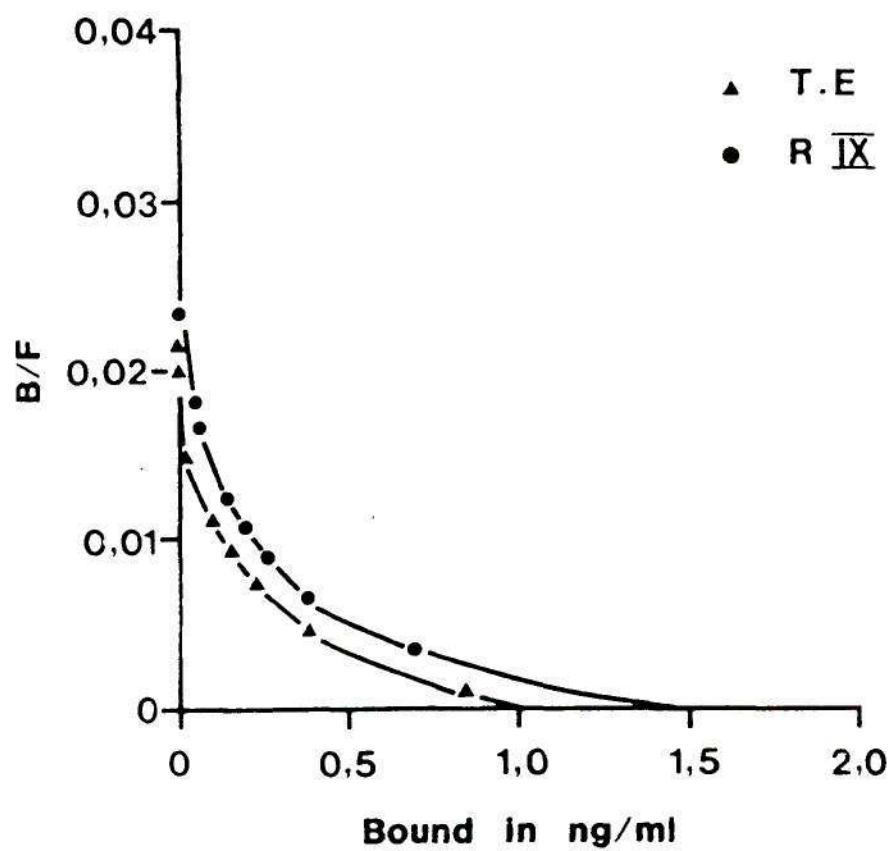


FIGURE 5.13

Scatchard plot (Monocytes)

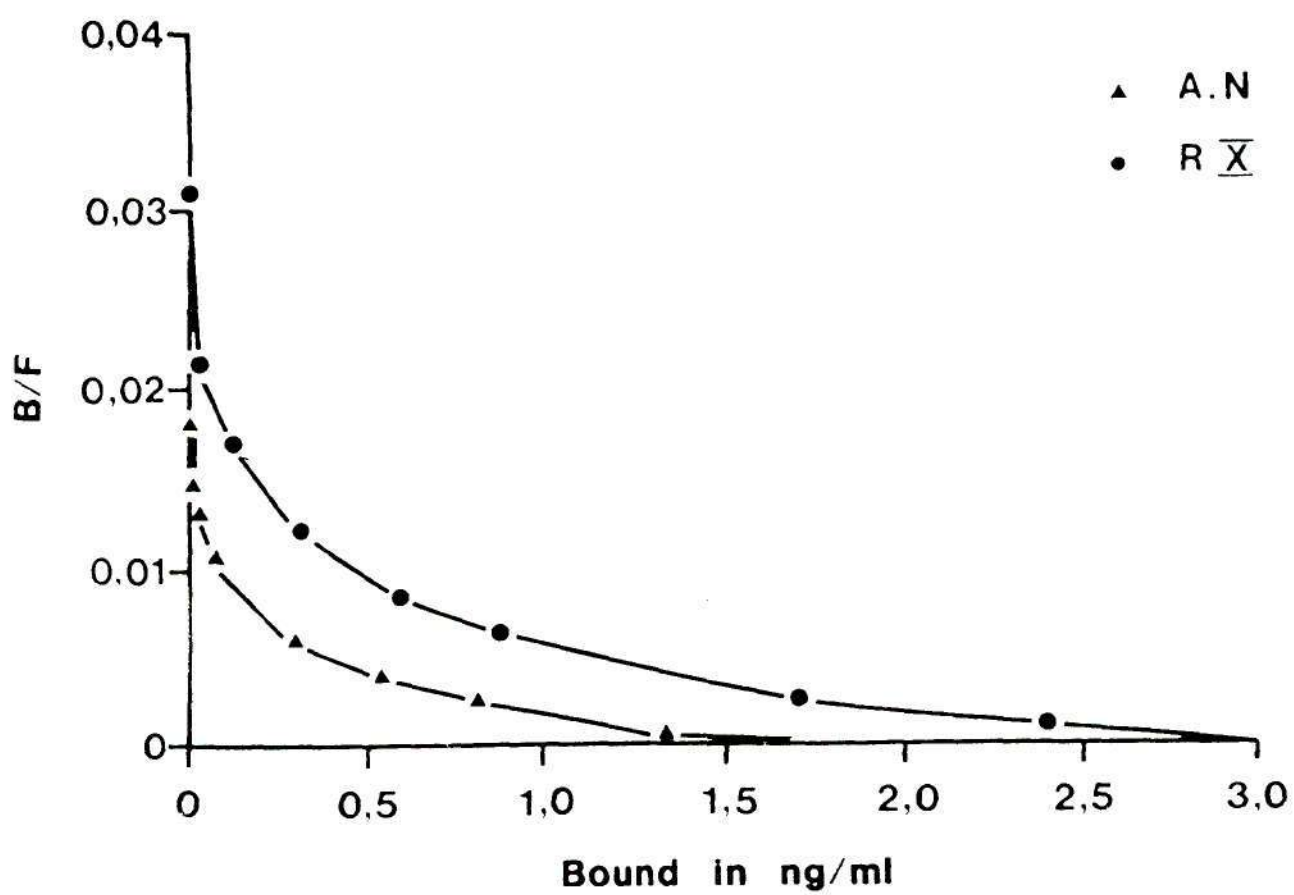


FIGURE 5.14

Competition Curve (erythrocytes).

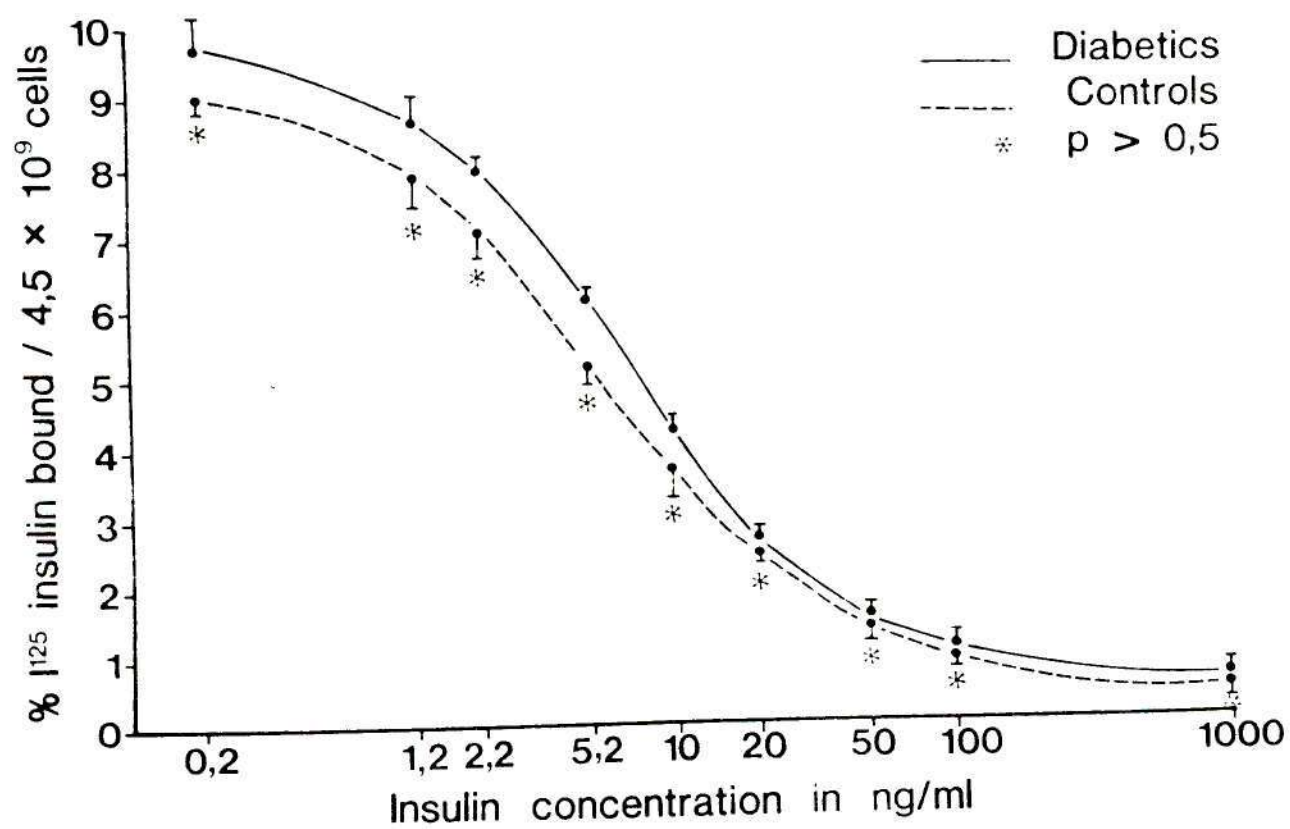
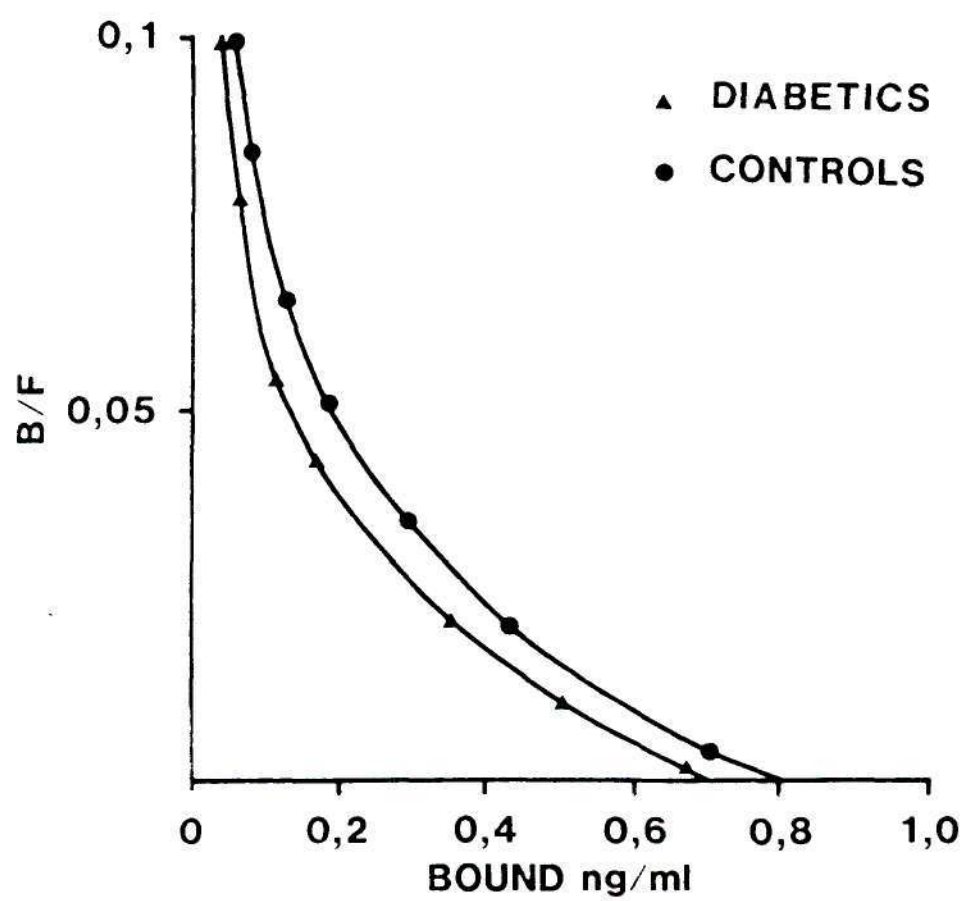


FIGURE 5.15

Mean Scatchard plots (erythrocytes)



fell within the reference range (2,0-10,0 ng/ml).

5.2.6 DISCUSSION

This study has demonstrated that patients with NIDDM display normal insulin binding kinetics to the erythrocyte receptors, whereas their binding to circulating monocytes was decreased as compared to reference subjects.

The study of insulin binding kinetics is one way to establish whether insulin resistance has as its physiological basis, a receptor defect, but the physiological phenomenon of downregulation has to be taken into account in interpreting results. Bearing this in mind, it may be expected that an inverse relationship between the fasting plasma insulin concentration and insulin binding exists. Indeed, such an inverse correlation has been demonstrated between the insulin receptors on monocytes and fasting insulin levels. In other words, the decreased binding to monocytes probably reflects the downregulatory action of insulin on its receptors. If this interpretation is correct three inferences appear to be valid: the higher immunoreactive insulin levels measured in the patients with NIDDM in the fasting state is biologically active; the insulin receptors on monocytes respond appropriately to hyperinsulinism and thus should be regarded as functionally normal; the fasting insulin levels rather than the excursions in plasma insulin are most closely related to the percentage of insulin binding, since it has previously been shown that NIDDM have a delayed and attenuated insulin response to oral glucose (Chapter 3).

The decrease in insulin binding to circulating monocytes was due solely to a decrease in receptor number as affinity values were entirely normal, confirming similar findings by Olefsky and Reaven (1977) who studied patients with NIDDM.

If plasma insulin levels do regulate insulin receptors then the sequence, hyperinsulinaemia leading to decreased insulin receptor numbers, which in turn, lead to decreased percentage insulin binding and thus apparent insulin resistance may be feasible. Or, it could be that decreased receptor numbers lead to apparent insulin resistance and hyperinsulinaemia.

However, the major purpose of this study was to establish whether there is a structural defect in the receptors and hence the RBCs were particularly useful cells to study: they are not dependent on insulin receptors for the entry and utilization of glucose (Dustin et al, 1984); as a non-nucleated cell, the receptor complement cannot be augmented by synthesis; the binding characteristics of insulin to the erythrocyte receptors can be defined with a high degree of accuracy and precision (Appendix A-11); the receptors are readily solubilized and can be studied further in this state.

As could possibly be expected of a cell which is not dependent on its insulin receptors for glucose metabolism and which is non-nucleated, downregulation of insulin receptors observed in the monocytes of patients with NIDDM, was not seen in the case of the RBC insulin receptors. Both the affinity of the receptor for insulin and the number of insulin receptors were similar in the patients with NIDDM and the control group. But these findings are contrary to the experience in NIDDM (Robinson et al, 1979; De Pirro et al, 1980; Dons et al, 1981) in which condition, about a 50% decrease in receptor numbers on erythrocytes was reported; the affinity of insulin binding to the receptors was not affected in the NIDDM patients as was the case in patients with NIDDM. What is even more surprising, is that the patients with NIDDM did not have fasting hyperinsulinism! As has been clearly demonstrated in the present study, in nucleated monocytes, receptor number correlates in-

versely with fasting insulin levels.

Obviously the present study does not confirm the findings of Robinson et al (1979), De Pirro et al (1980) and Dons et al (1981) in patients with NIDDM, and it is even more difficult to concur with their proposal that some structural abnormality of the insulin receptor in NIDDM is inherited.

5.2.7. CONCLUSION

Insulin binding to monocyte insulin receptors were decreased in patients with NIDDM but the binding affinity was unaffected. These findings were interpreted as the normal physiological process of receptor downregulation as a result of the increased fasting insulin levels in patients with NIDDM. Seen in conjunction with the fact that the binding kinetics of insulin to RBC receptors were similar to that of controls, a reasonable conclusion is that the insulin receptors are certainly functionally intact and thus presumably structurally intact.

5.3 ¹²⁵I-INSULIN BINDING TO THE SOLUBILIZED ERYTHROCYTE

MEMBRANE RECEPTOR

5.3.1. Purpose of Study

Present findings of insulin binding to RBC receptors did not support those of others (Robinson et al, 1979; De Pirro et al, 1980; Dons et al, 1981) in NIDDM and the possibility of technical errors could account for these disparate findings. Inasmuch as the solubilized RBC receptor is held to be the biological significant receptor (Grigorescu et al, 1983), RBC insulin receptors were solubilized in the non-ionic detergent Triton X-100 and its binding kinetics to insulin studied. In this section the results are reported.

5.3.2. Patients and Reference Subjects

The ages and body mass indices of the ten patients and controls has been set out in Section 3.2.2.

The red blood cell indices were entirely normal in all subjects and reticulocyte counts were < 1%.

5.3.3. Study Procedure

Precautions regarding diet and oral hypoglycaemic agents were similar to that described in Section 3.1.3. After an overnight fast, 120 mls of blood was withdrawn from the patients and reference subjects into tubes containing lithium heparin. Samples for glucose and insulin were also taken in the fasting state.

5.3.4. METHODS

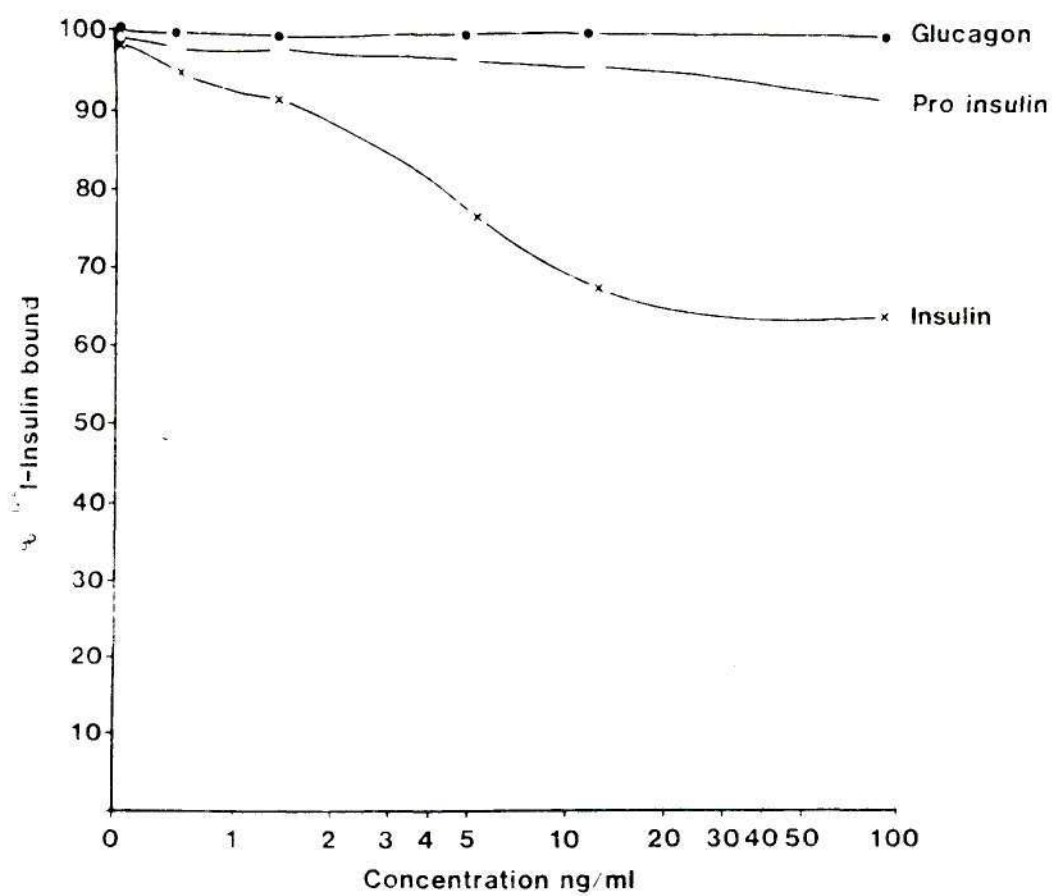
The solubilization of RBC insulin receptors and the radioreceptor assay for ^{125}I -insulin binding to the soluble erythrocyte membrane receptor is described in Appendix A-13. Glucose and insulin methods are set out in Appendix A-1 and A-5.

The data on insulin binding to receptors is expressed in three ways (described in Section 5.2.4.)

To determine whether the receptors on the membrane were specific for insulin, binding studies were also carried out in the presence of unlabelled proinsulin and glucagon. Approximately 65% of total bound insulin was displaced by 10 ng of unlabelled insulin. Only 5% was displaced by the same concentration of proinsulin. There was no displacement of bound insulin by glucagon at this concentration level. (Figure 5.16).

FIGURE 5.16

Displacement of ^{125}I -insulin by unlabelled insulin, proinsulin and glucagon. (RBC membrane).



5.3.5 RESULTS

The relevant clinical and biochemical information of the patients and reference subjects is set out in Table 5.2

The mean fasting plasma insulin and glucose levels were significantly greater in the diabetics than in the reference group ($23,5 \pm 2,3$; $10,8 \pm 1,2$ mU/ml; $p < 0,01$; $12,3 \pm 0,7$; $4,3 \pm 0,1$ mmol/l; $p < 0,001$ respectively).

The mean percentage of total radioactivity specifically bound to receptors was lower in the diabetics as compared to their reference subjects at insulin concentrations of 0,2 ng/ml only ($7,8 \pm 0,7$; $9,9 \pm 0,7\%$; $p < 0,05$). At higher concentrations of insulin the percentage binding did not differ in the two groups (Figure 5.17)

Scatchard analysis of insulin binding revealed that all the diabetics had similar receptor numbers to their control subjects (Figure 5.18). The ID_{50} values of all the diabetics fell within the reference range (2,0-10,0 ng/ml)

5.3.6 DISCUSSION

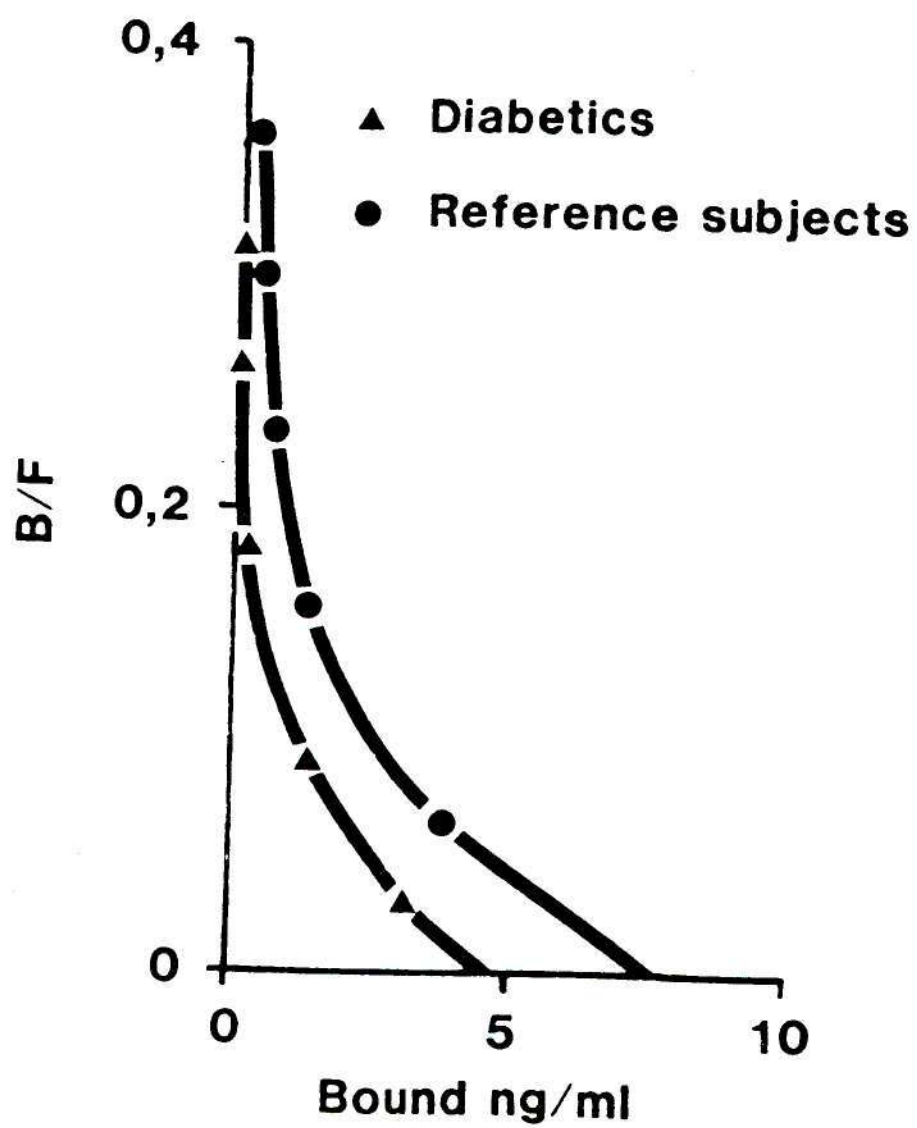
The membrane preparation solubilized in Triton X-100 bound insulin specifically: labelled insulin was displaced by excess unlabelled insulin; only a small percentage of the bound insulin was displaced by proinsulin; glucagon did not displace any bound insulin at all. Further, the binding properties of the soluble receptor were similar to those of the intact erythrocyte as regards specificity, affinity, pH optima and curvilinear Scatchard plot. Thus the solubilized receptor can be regarded as a "true" insulin receptor. It may also be concluded that the insulin receptor, despite being part of a biological membrane is not dependent on the membrane lipids for the recognition of insulin.

TABLE 5.2 BIOCHEMICAL FEATURES OF NIDDY AND CONTROL SUBJECTS
(ERYTHROCYTE MEMBRANE RECEPTOR)

| PATIENTS | FASTING PLASMA INSULIN (μ U/ml) | FASTING PLASMA GLUCOSE (mmol/l) | ID 55 (ng/ml) | % MAXIMUM SPECIFIC BINDING | |
|-----------------------|---|--|------------------|-------------------------------|----------|
| | | | | NIDDY | CONTROLS |
| A ₂ | 17 | 14 | 4,5 | 10,1 | 11,2 |
| B ₁ | 28 | 11 | 6,2 | 5,3 | 7,8 |
| B ₂ | 30 | 12 | 9,6 | 6,2 | 10,0 |
| C ₁ | 18 | 15 | 5,5 | 6,3 | 9,0 |
| C ₃ | 34 | 8 | 2,0 | 4,2 | 5,6 |
| C ₄ | 32 | 14 | 2,8 | 10,7 | 14,0 |
| D ₁ | 18 | 13 | 9,2 | 8,2 | 11,0 |
| D ₂ | 12 | 10 | 2,0 | 7,1 | 8,4 |
| E | 24 | 14 | 4,2 | 9,6 | 11,0 |
| F | 21 | 12 | 3,3 | 9,8 | 10,8 |
| Mean | 23,5 \pm 2,3 | 12,3 \pm 0,7 | 4,9 \pm 0,8 | 7,8 \pm 0,7 | |
| Range | (13-34) | (8-14) | (2,0-9,2) | (4,2-10,7) | |
| Reference Subjects | | | | | |
| Mean | 10,8 | 4,3 | 4,5 | 9,9 | |
| Range | (5-20) | (3,3-6,0) | (2,0-10,0) | (5,6-14) | |

FIGURE 5.18

Mean Scatchard plots (erythrocyte membrane).



Given the circumstances and the findings that the binding kinetics of insulin were measured in a preparation that seemed to bind insulin very specifically, the differences between reference subjects and patients with NIDDM are negligible and confirm the findings of insulin binding to intact erythrocytes. The observations of the workers who studied NIDDM (Robinson et al, 1979; De Pirro et al, 1980; Dons et al, 1981) cannot be confirmed. Indeed, as has already been argued, every aspect of insulin binding to the receptor points to normal binding kinetics of insulin to receptors in NIDDM. The argument seems to hold that if the binding kinetics of a receptor to the ligand are not disturbed, structural abnormalities in the receptor are unlikely.

However, there is still a further test which can be applied. It has been proposed that the decreased binding of insulin to circulating monocytes in patients with NIDDM is due to decreased receptor numbers which is consistent with downregulation in response to the increased fasting insulin levels in NIDDM. If tissues derived from patients with NIDDM were to be cultured in a milieu free from excessive insulin concentrations, this hypothesis can be tested. In the following section, experience with cultured fibroblasts are described.

5.3.7 CONCLUSION

All the evidence on insulin binding to receptors in patients with NIDDM point to normal binding kinetics and even normal function. It is concluded that a structural defect in the receptor is unlikely and hence the abnormality which is inherited in NIDDM is not directed at the insulin receptor.

5.4 ¹²⁵I-INSULIN BINDING TO FIBROBLASTS IN CULTURE IN NIDDM

5.4.1 Purpose of Study

In the previous section it was argued that if tissue from NIDDM patients were cultured in a milieu free from the metabolic abnormalities associated with the condition, the binding kinetics of insulin to the receptor could be a further test to determine whether the receptors are structurally and functionally sound. Put differently the one defect unequivocally found, namely, decreased insulin binding to the monocytes of patients with NIDDM, can be interpreted as an acquired defect. Indeed, this is a functionally normal response to raised fasting insulin levels in NIDDM.

In the section following, insulin binding to cultured fibroblasts from patients with NIDDM will be described.

5.4.2. Patients and Reference Subjects

Four of the ten diabetics with NIDDM and four age, sex and race matched control subjects consented to having a skin biopsy. All patients were female.

5.4.3. Study Procedure

Fibroblasts were obtained by a punch biopsy from the volar aspect of the forearm in both patients and controls. The specimen was immediately placed in a sterile solution containing minimal essential medium (MEM) and subsequently cultured in monolayer. Details of fibroblast culture are set out in Appendix A-14.

5.4.3. Methods

The radioreceptor assay for ^{125}I -insulin binding to fibroblasts in culture is described in Appendix A-14. The data on insulin binding to fibroblasts is expressed in three ways (described in Section 5.2.4.)

5.4.5. RESULTS

The mean percentage of total radioactivity bound to receptors was similar in the diabetics and reference subjects at insulin concentrations ranging from 0,2 to 10^4 ng/ml (maximum specific binding, $1,5 \pm 0,3$; $1,3 \pm 0,3$; $p > 0,5$). This data is graphically expressed in Figure 5.19.

Scatchard analysis of insulin binding revealed that all diabetics had similar receptor numbers to their control subjects (Figure 5.20)

The ID_{50} values in the diabetics were within the reference range (2,0-8,0 ng/ml).

5.4.6. DISCUSSION

This study has shown that human fibroblast monolayers contain specific, high affinity (2,0-8,0 ng/ml) insulin receptors that displayed all the binding characteristics of insulin receptors on erythrocytes and monocytes. Scatchard analysis revealed curvilinear plots, similar to those of circulating blood cells.

The binding of 125 I-insulin to fibroblasts was similar in diabetics and reference subjects as regards binding kinetics, receptor number and receptor affinity. These findings accord with those of Prince et al (1981), who studied patients with NIDDM, and the findings of Gelehrter et al (1981) who investigated patients with MODY. Gelehrter's study was more limited than the present one, in that the four patients studied by his group were from a single family with MODY. All four patients used in this study were from four different families with three generation transmission of non-insulin-dependent diabetes.

It is abundantly clear that when grown in the artificial milieu of tissue culture, the insulin receptors of fibroblasts derived from

FIGURE 5.19

Competition Curve (fibroblasts)

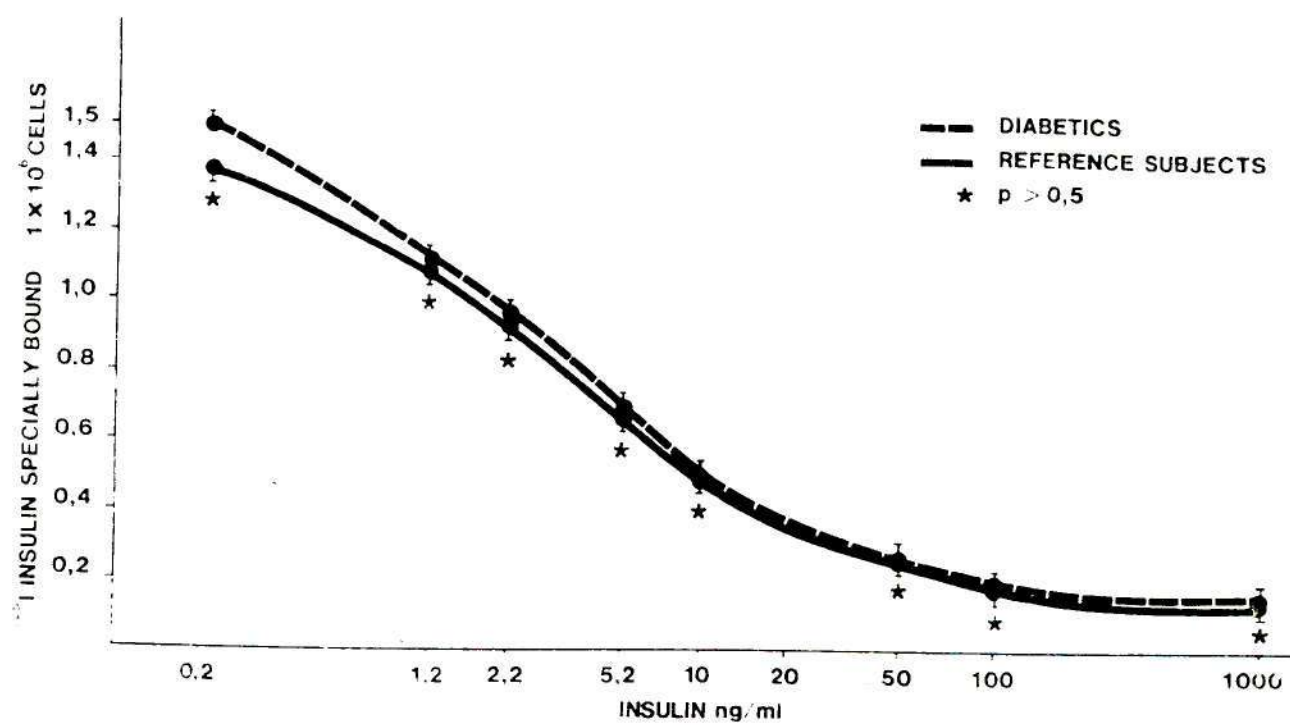
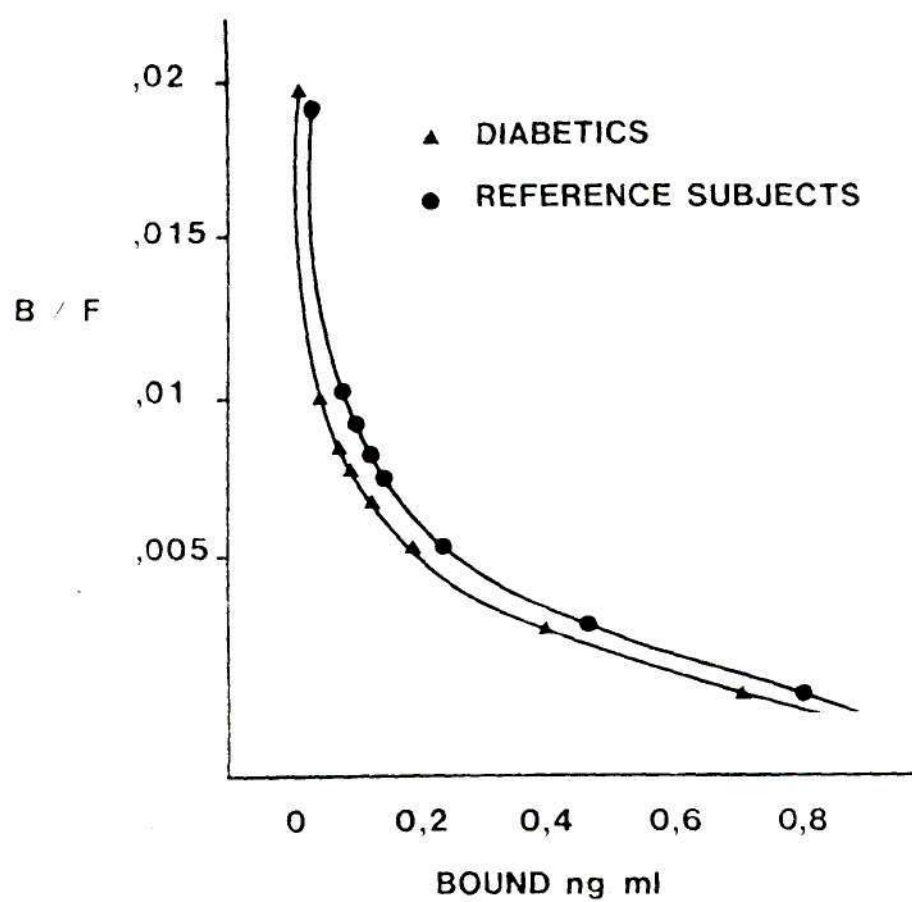


FIGURE 5.20

Mean Scatchard plots (fibroblasts)



patients with NIDDM differed in no way from normal patients as far as the binding kinetics of insulin to the receptor is concerned. Since the fibroblast is a nucleated cell one would have expected that any putative structural defect of the insulin receptor in NIDDM, would have been replicated in tissue culture and would have been manifest in the functional aspects of insulin binding kinetics. This has not been found and reinforces the argument that the decreased binding of insulin to monocyte receptors in patients with NIDDM is nothing more than simply the normal functional expression of downregulation in response to increased fasting levels of the ligand, a phenomenon common to many other polypeptide hormone receptors which are surface located.

Seen in this light the findings of normal binding of insulin to RBC receptors and to the solubilized receptors from patients with NIDDM seem entirely credible and correct.

If these insulin receptors in patients with NIDDM are unequivocally functionally intact and it may reasonably be inferred, structurally intact as well, some inference may be drawn from the study. Raised fasting insulin levels are unquestionably inversely correlated with decreased insulin binding (section 5.2) and is almost certainly the result of downregulation of the insulin receptors. But once insulin receptors are downregulated, it is self-evident that the patient will manifest insulin resistance; indeed, this is the physiological purpose of the downregulatory response, functioning the metabolic response required.

But also, the teasing question remains unanswered: What starts off the fasting hyperinsulinism?

5.4.7 CONCLUSIONS

This is the first study on insulin resistance, in which both the diabetics and reference subjects have been studied under optimal conditions. Once the diabetic fibroblasts have been removed from the diabetic milieu, they expressed binding characteristics similar to their reference subjects.

From this experiment it can be concluded that receptor defects contributing to the insulin resistance are secondary to the constant hyperglycaemia and hyperinsulinaemia.

5.4.8 SUMMARY AND GENERAL CONCLUSIONS ON INSULIN RESISTANCE IN NIDDM

In vivo and in vitro techniques have been used to compute insulin resistance in NIDDM. The insulin tolerance test was used as an in vivo method and the glucose disposal constant (k_1) was used as an index of insulin resistance. As the k_1 was lower in the diabetics than the reference subjects and it correlated inversely with the fasting hyperglycaemia, it was concluded that diabetics were insulin resistant. A peculiar finding in the Indian diabetic was the fact that the adipocytes remained sensitive to the action of insulin, explaining in part the lack of ketosis in this group of patients. Although insulin levels were sufficient to prevent ketoacidosis, they were inadequate to maintain normal carbohydrate metabolism. This would indicate that adipose tissue requires lower levels of insulin for lipid metabolism supporting the view of Bagdade et al (1968).

Receptor studies on circulating blood cells: monocytes, erythrocytes and the solubilized erythrocyte membrane were used as in vitro tests of insulin resistance. While binding to erythrocytes was normal in the diabetics, binding to monocytes were diminished. These decreases

in insulin binding were due only to a decrease in receptor number, with no change in affinity. This led to the postulate that hyperinsulinism downregulates the receptor numbers. As the intact erythrocyte has no nucleus, it is held to be incapable of affecting downregulation and thus its binding kinetics were normal. If this hypothesis was correct, fibroblasts grown in an environment that is removed from the abnormal metabolic and endocrine status of the diabetic, should display normal binding characteristics. In culture, insulin receptors on fibroblasts from diabetics had exactly the same insulin binding characteristics as those of matched non-diabetics. These findings support the interpretation that the binding of insulin to the erythrocytes and monocytes from patients with NIDDM were appropriate and the insulin in these patients was biologically active.

SUMMARY AND GENERAL CONCLUSIONS

Using strictly defined criteria, this study only included patients with three generation transmission of non-insulin-dependent diabetes via one parent. However, only 36% of the siblings tested were diabetic, suggesting autosomal dominant inheritance with incomplete penetrance and/or only heterozygote transmission.

Thirty one percent of the diabetics were obese as opposed to 20% of the non-diabetic family members, a finding which suggests that obesity is a possible cofactor in the development of diabetes.

Complications were generally mild in these patients, the commonest being peripheral neuropathy. A surprising finding was that not a single diabetic presented with ketosis despite the fact that they were prone to intercurrent infections.

No association could be found between the HLA system and the hyperglycaemic trait in these families with NIDDM.

In response to an oral glucose load, patients with NIDDM displayed diminished insulinaemic and C-peptide responses as compared to the non-diabetic family members and reference subjects. On the other hand, not a single non-diabetic family member had any evidence of glucose intolerance. There was no evidence to suggest that the lowered insulin response (in the diabetics) was the end result of an initial hyperinsulinaemic response to oral glucose, as suggested by Reaven (1980).

The diabetics had no first phase insulin release in response to iv glucose, while the response of the non-diabetic family members were similar to that of the reference subjects. The normality of the insulin responses in the non-diabetic family members and the fact that they were of a similar age as the diabetics, enhances the suggestion of an autosomal dominant mode of inheritance.

Although the overall insulin responses to oral and intravenous glucose stimuli were substantially lower in the diabetics, one has to account for the fact that the patients had a considerable fasting hyperglycaemia. This high fasting plasma glucose level is a constant stimulus to the β cell and hence the acute storage pool of insulin can be expected to be diminished. The demonstrated poor β - cell response to further increments in hyperglycaemia is thus hardly surprising.

The C-peptide: insulin molar ratios were used as an index of the hepatic extraction of insulin. As they were within normal limits in the patients with NIDDM, it was concluded that the hypoinsulinaemic responses in the diabetics were not due to a defect in the extraction of insulin by the liver.

In response to two non-glucose secretagogues: glucagon and tolbutamide, NIDDM had higher insulin responses than that attained with glucose stimuli per se. However, these responses were still lower than that achieved in the control group. After IV arginine administration, NIDDM and reference subjects had similar insulin responses. This was proven to be the result of the potentiation of the insulin response to a non-glucose stimulus by hyperglycaemia. But it does prove that the β -cells can respond appropriately and substantially to certain stimuli.

The greater insulinaemic responses with non-glucose stimuli, merely confirms that the mechanisms of insulin release are different to that of glucose. But it does also suggest that the poor insulinaemic response to glucose itself is a reflection of the excessive stimulation of this mechanism as a result of constant hyperglycaemia. Certainly, insulin storage is not greatly affected and the release mechanisms are intact though overloaded. It is the constant hyperglycaemia with conse-

quential continued β -cell stimulation which deplete the acute storage pool of insulin. Eventually a self-perpetuating cycle ensues which results in a progressive decrease in β -cell performance and apparent insulin deficiency. This hypothesis of "exhaustion" (for want of a better term) seems a plausible one, since at the time of writing, three of the patients in the present series had become insulin dependent (Patients A⁻, C₂ and F).

Using both in vivo and in vitro methods, insulin resistance in NIDDM is a real factor. Insulin tolerance as a challenge in patients revealed resistance to insulin-induced disposal of glucose, but, normal sensitivity of the adipose tissue to insulin stimulation. The insulin: glucagon molar ratios were also normal in NIDDM, partly explaining the ketosis resistance in this group of patients.

In the in vitro studies, ¹²⁵I-insulin binding to erythrocytes, monocytes, the erythrocyte membrane and fibroblast receptors were used. While the binding to circulating erythrocytes were normal in the patients with NIDDM, the insulin binding to monocytes was significantly decreased. This decrease in binding was due solely to a decrease in receptor numbers, as affinity values were normal. Because fasting insulin levels and receptor numbers were inversely correlated, the decreased binding was attributed to the downregulatory effect of insulin on the monocyte receptors. Since the mature erythrocyte is a non-nucleated cell, it was not expected to manifest downregulation and hence is not considered an accurate marker of insulin resistance. This was proven in the case of RBC receptors either on the intact erythrocyte or solubilized from RBC membranes.

To further test whether the receptor defect was secondary to the hyperinsulinism, that is, acquired, fibroblasts from diabetics were cultured

in a milieu free from the metabolic and hormonal status of diabetics. Under these circumstances, diabetic cells bound insulin to the same extent as non-diabetic cells confirming that decreased binding to monocytes was secondary to the fasting hyperinsulinaemia. As fibroblasts faithfully express the genetic endowment of the donor, hereditary defects leading to insulin resistance at the receptor level can be excluded.

From the findings in this thesis, it is clear that both insulin deficiency and insulin resistance exist in patients with NIDDM. The physiological basis of the insulin deficiency in response to glucose stimulation appears to be, in the initial stages, the sustained stimulation of the β cells by fasting hyperglycaemia; the hyperglycaemia in turn is sustained in part, at least, by the physiologically induced downregulation of insulin receptors in some tissues. And thus a vicious self-sustaining cycle is set up.

The proposal that the inheritance of this form of diabetes, which so clearly suggests a Mendelian type of autosomal dominant inheritance pattern, could be related to an insulin receptor defect was not confirmed by direct measurement. Indeed, in NIDDM, receptors on intact erythrocytes, receptors solubilized from RBC membranes, monocyte receptors and receptors on fibroblasts cultured in monolayer, all proved to have normal binding kinetics for insulin, to the point of demonstrating the physiological phenomenon of downregulation in the case of monocytes.

Sadly, therefore the receptors in NIDDM did not prove to be an actual or potential marker of the abnormality inherited in this condition, but the studies based on the hypothesis have produced a wealth of valuable information on NIDDM.

APPENDIX A - METHODS

APPENDIX A-1 DETERMINATION OF PLASMA GLUCOSE

(Centrifichem Methodology)

A-1.1. Reference

Trinder, P (1969)

A-1.2. Principle

Is based on the following chemical reaction: glucose + O₂ + H₂O

glucose oxidase gluconate + H₂O₂

2H₂O₂ + 4 aminophenazone + phenol peroxidase 4 - (p-benzoquinone - mono-imino) phenazone + 4 H₂O.

A-1.3. Reagents

- (1) 4 aminophenazone
- (2) Tween 20
- (3) Phenol/Sodium Chloride

A-1.4. Procedure

Glucose levels were measured on a discrete centrifugal analyser (Centrifichem system 500). Briefly, a special rotor is used which has 30 identical radial cavities. Each cavity is constructed with 2 separate wells which do not communicate when the rotor is stationary. In each cavity one well holds a diluted standard or unknown sample, the other well holds the necessary reagents. When the rotor has been fully charged, the centrifugal force causes the mixing of the sample and reagents. As the rotor revolves, the inbuilt calorimetric cuvettes measure the absorbance of each sample (520nm). Three standards (high, normal and low) are incorporated into each assay. Range of the assay was 0 - 30 mmol/l. If samples were > 30 mmol/l, a one/three dilution was done. In addition 2 controls were incorporated into each batch of samples.

A-1.5 Comment

Blood was collected into tubes containing a fluoride-oxalate mixture. Fluoride is an anti-glycolytic agent which inhibits the glycolytic enzymes in red blood cells. The samples were centrifuged and glucose determinations carried out on plasma as soon as possible after collection of specimens.

Precision in this assay and all other assays used in this study was assessed by calculating the intra-assay coefficient of variation (control samples done in quintuplicate) and inter-assay coefficient of variation (control sample done in at least 5 consecutive assays).

For the glucose assay the intra- assay coefficient of variation was 1,1% and the inter-assay coefficient of variation was 1,2%.

APPENDIX A 2 : DETERMINATION OF SERUM CREATININE

Auto Analyser Methodology

A-2.1 References

Technicon Auto Analyser Handbook (1970)

Tietz (1976)

Varley et al (1980)

A-2.2 Principle

The method used for the estimation of creatinine utilises the Jaffe reaction which results in the production of a red tautomer of creatinine picrate when an alkaline picrate solution is added to a solution containing creatinine. The change in absorbance was measured at 505 nm using a flow cuvette with a 15 mm light path.

A-2.3 Reagents

(1) Sodium Chloride Solution (1,8%)

18 g NaCl

Distilled H₂O q.s. 1000 ml

Brij-35, 30% solution 1,0 ml.

(2) Sodium Hydroxide (0,5 molar)

NaOH 20,0 g

Distilled water q.s. 1000 ml.

(3) Saturated Picric Acid

Picric Acid 13,0 g

Distilled water q.s. 1000 ml

(4) Standard Creatinine Solution (10 mmol/l)

1,13 g of creatinine was dissolved in 0,1 molar HCL and made up to 1000 ml.

(5) Working Standards

The stock solution was diluted with distilled water as follows:

| ml of stock solution | Diluted to | $\mu\text{mol/l}$ |
|----------------------|------------|-------------------|
| 2 ml | 100 ml | 200 |
| 4 ml | 100 ml | 400 |
| 6 ml | 100 ml | 600 |
| 8 ml | 100 ml | 800 |
| 10 ml | 100 ml | 1000 |
| 12 ml | 100 ml | 1200 |

A-2.4 Procedure

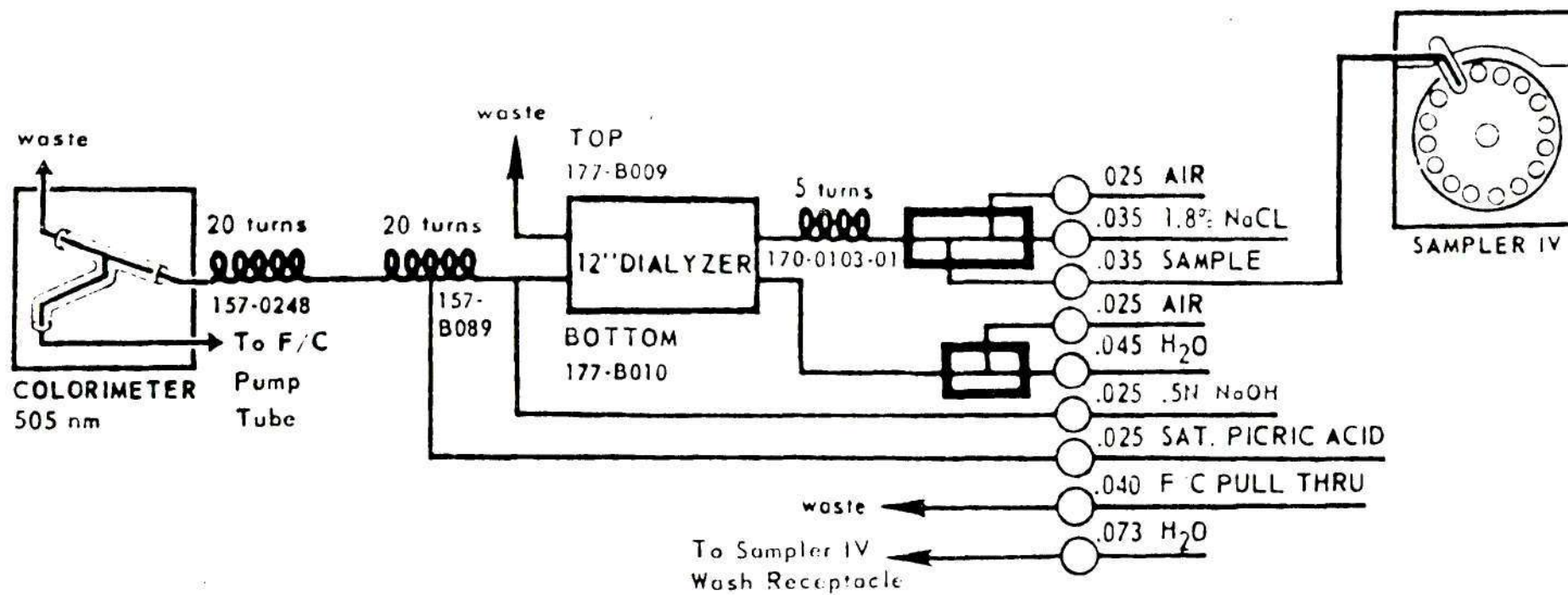
The flow diagram of the methodology employed is given in Figure A-2.

A-2.5 Comment

Samples consisted of clear unhaemolysed serum. In the Auto Analyser, dialysis performs much the same function as Lloyd's reagent in isolating creatinine since the interfering chromogens pass less readily across the membrane. Since colour development is slower with other chromogens and the colour can be read at a point before maximum extinc-

FIGURE A-2

FLOW DIAGRAM OF CREATININE METHODOLOGY.



tion is reached in a controlled fashion, this minimises the interference of the other chromogens. The intra-and inter-assay coefficients of variation of the creatinine assay were 4,61% and 5,01% respectively.

APPENDIX A-3 : QUANTITATIVE ESTIMATION OF URINARY PROTEIN

A-3.1 References

Wootton (1974)

Tietz (1976)

A-3.2 Principle

Protein is precipitated out of solution with trichloroacetic acid. This allows for isolation of the protein from substances in the urine which interfere with the biuret reaction. The precipitated protein is then dissolved in sodium hydroxide and measured calorimetrically using the biuret reaction: alkaline copper solution (Biuret reagent) reacts with peptide linkages of amino acids in proteins producing a violet colour which is proportional to the amount of protein present.

A-3.3. Reagents

- (1) Trichloroacetic acid 20%
20 g per 100 ml redistilled water
- (2) Sodium Hydroxide 0,2 molar
8 g NaOH per 1000 ml water.
- (3) Biuret reagent (alkaline copper reagent)
9 g sodium tartrate was dissolved in about 400 ml of 0,2 M NaOH; 3 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was then added and dissolved by stirring. When dissolved, 5 g potassium iodide (KI) was added and the mixture made up to a 1000 ml with 0,2 M NaOH. This solution was stored in a polyethylene bottle.

(4) Protein Standard:

A commercial control (Validate A-General Diagnostics) in which the protein concentration is accurately known was diluted 1 : 20.

(5) Quality Control.

A second commercial control (Wellcome control, Wellcome laboratories) was also diluted 1: 20.

A-3.4. Procedure

After recording the 24 hour volume, an aliquot of urine was centrifuged to remove particulate matter. Thereafter a qualitative test for protein was performed on the specimen (Albustix Ames). If a result of absent, trace or + was obtained, 2 ml of urine was pipetted into a centrifuge tube. If a result of ++ or more was obtained, 1 ml of urine was pipetted into a centrifuge tube. An equal volume of 20% TCA was then added to each test tube. After allowing the protein to precipitate for 30 min the tubes were centrifuged for 10 min. The supernatant was poured off and the tubes were inverted over a piece of filter paper and permitted to drain completely (5 min).

Thereafter 5 ml of 0,2 M NaOH was added to each tube to dissolve the precipitate.

The test schedule was then set up as follows:

| | Unknown | Std. | Control | Blank |
|-------------------|---------|------|---------|-------|
| Std. (1 : 20) | - | 3 ml | - | - |
| Control (1 : 20) | - | - | 3 ml | - |
| Unknown | 3 ml | - | - | - |
| Redistilled water | - | - | - | 3 ml |
| Biuret reagent | 5 ml | 5 ml | 5 ml | 5 ml |

After mixing all tubes well they were incubated in a waterbath at 37°C for 30 minutes.

The absorbance of each sample was then read against the blank using a glass cuvette with a 1 cm light path at 550 nm on a Beckman spectrophotometer.

A-3.5 Calculation

$$\text{Protein concentration of unknown (test)} = \frac{\text{Absorbance(test)}}{\text{Absorbance(std)}} \times \frac{\text{Concentration std}}{1}$$

Corrections were made for the appropriate dilutions of test, standard and control.

A-3.6 Comment

All determinations were carried out in duplicate.

An assay was only accepted if the control value obtained was within the assigned reference range. The intra- and inter-assay coefficients of variation of the assay were 2,56% and 5,3% respectively.

APPENDIX A-4 : DETERMINATION OF GLYCOSYLATED HAEMOGLOBIN LEVELS

Glycosylated haemoglobin (HbA₁) levels were quantitated by a micro-chromatographic procedure using a commercial kit (Helena Laboratories, Texas, USA).

A-4.1 Principle

The method used to measure HbA₁ was based on the fact that a negatively charged cation exchange resin exhibits an affinity for positively charged molecules. At selected ionic strength and pH, the glycosylated haemoglobins (HbA₁) are less positively charged than haemoglobin A.

Therefore the HbA₁ molecules bind to the negatively charged resin less tightly than HbA. With the application of a fast fraction buffer, glycosylated haemoglobins are eluted while the other haemoglobins are retained. The absorbance of the HbA₁ eluate and the diluted total haemolysate are read on a spectrophotometer and the percentage HbA₁ calculated.

A-4.2 Reagents

(1) Quick Columns

Each column was prepacked with at least 300 mg of cation exchange resin equilibrated in phosphate buffer pH 6,7.

(2) Fast Fraction Developer Elution Buffer

100 ml

(3) Haemolysate Reagent

20 ml

(4) Lyophilized HbA₁ Control

The lyophilized HbA₁ control was reconstituted with 100 μ l of redistilled water.

A-4.3 Procedure

The assay was performed at 22°C. Prior to the assay the whole blood sample collected in K-EDTA was haemolysed by adding 300 μ l of the haemolysate reagent to 20 μ l of whole blood. The tubes were then vortexed vigorously and allowed to stand for 5 min to ensure complete haemolysis of the sample.

One quick column per sample was placed in a column rack and the contents of the column resuspended using a pasteur pipette. The bottom cap closure of the column was removed and the supernatant allowed to drain into a test tube. A small collecting tube (12 x 75 mm) was then placed under each quick column (F.F. tube). From the haemolysed sample

prepared, 100 μ l of the haemolysate was loaded onto the quick column and a further 100 μ l was pipetted into a large (16 x 125 mm) collection tube, the T.F. tube.

Following the absorption of the sample into the resin bed, 1,5 ml of Fast Fraction Buffer was applied to each column.

After the complete elution of the fast fraction (10 - 15 mins) the contents of the test tube in which this fraction was collected (F.F. tube) was made up to 3 ml with redistilled water and mixed on a vortex. Also the contents of the total fraction tube (T.F. tube) was made up to 15 ml with redistilled water and the contents mixed thoroughly.

Thereafter the absorbances of the F.F. tube and T.F. tube were read against redistilled water at 415 nm using a glass cuvette with a 1 cm light path on a Beckman Acta V Spectrophotometer.

A-4.4 Calculation

The percent HbA₁ for each sample was calculated as follows:

$$\text{HbA}_1 (\%) = \frac{\text{Absorbance F.F. tube}}{5 (\text{Absorbance T.F. tube})} \times \frac{100}{1}$$

5 = dilution factor (15 ml T.F. tube/3 ml F.F. tube = 5)

A-4.5 Comment

Assays were done on the same day the sample was taken.

For an assay to be acceptable, the control value obtained had to be in the assigned reference range.

The intra-and inter-assay coefficients of variation for the HbA₁ assay were 3,9% and 7,2% respectively.

APPENDIX A-5 : DETERMINATION OF SERUM INSULIN CONCENTRATIONS

Insulin concentrations were determined by a radioimmunoassay technique using the Phadebas Insulin Test from Pharmacia Diagnostics, Uppsala, Sweden.

A-5.1 Principle

Specific antibodies to insulin are covalently bound to sephadex particles as the solid phase. The concentration of insulin in an unknown sample is evaluated by its capacity to compete with a fixed amount of labelled insulin for the binding sites on the insulin antibodies. After incubation, the particles are sedimented by means of centrifugation, washed and the radioactivity bound to the sephadex particles is measured. The quantity of insulin in the sample is inversely proportional to the bound radioactivity.

A-5.2 Reagents

(1) Buffer solution

Dissolve the 4,2 g buffer substance (dry powder) in 200 ml redistilled water. The pH of the buffer solution was 7,4.

(2) Physiological Saline

9 g per 1000 ml of redistilled water.

(3) ^{125}I -Insulin

The 8 ng (111 kBq) of lyophilized ^{125}I -Insulin was reconstituted in 10 ml of buffer solution.

(4) Sephadex - Anti-insulin complex

The sephadex anti-insulin antibody complex was transferred to a beaker containing a magnetic stirring rod and dissolved in 100 ml of buffer in portions.

(5) Insulin Standard

The insulin standard (porcine insulin) was calibrated against

research standard A (human insulin) for immunoassay. With the anti-serum used in the Phadebas Insulin test, the calibrated porcine insulin and the WHO insulin standard yielded superimposable standard curves.

After reconstitution with 4,0 ml redistilled water, the lypophilized insulin standards contained 320 μ U/ml of insulin. Standard solutions, final concentration 320, 160, 80, 40, 20, 10 and 5 μ U/ml, were prepared from the stock solution (320 μ U/ml) by dilution in buffer according to the following schedule:

| Final Std. Concentration | Buffer Solution | Std. Solution |
|--------------------------|-----------------|--------------------|
| A 320 μ U/ml | - | 4 ml |
| B 160 μ U/ml | 500 μ l | + 500 μ l of A |
| C 80 μ U/ml | 500 μ l | + 500 μ l of B |
| D 40 μ U/ml | 500 μ l | + 500 μ l of C |
| E 20 μ U/ml | 500 μ l | + 500 μ l of D |
| F 10 μ U/ml | 500 μ l | + 500 μ l of E |
| G 5 μ U/ml | 500 μ l | + 500 μ l of F |

A-5.3 Procedure

A Phadebas reference serum (control) was incorporated in each assay.

The test schedule was as follows:

| Reagents | Total | N.S.B. | Bo | Std. | Control/ Samples |
|--------------------------------------|-------|--------|-------------|------|---------------------|
| Buffer | - | 1,10ml | 100 μ l | - | - |
| Std (μ l) | - | - | - | 100 | - |
| Control/Samples (μ l) | - | - | - | - | 100 |
| 125 I-Insulin (μ l) | 100 | 100 | 100 | 100 | 100 |
| Sephadex-anti-insulin complex (ml) | - | - | 1,0 | 1,0 | 1,0 |

All tubes were mixed and incubated on a mechanical shaker for 3 hours at room temperature.

Thereafter all tubes except the totals were centrifuged at $2,000 \times g$ for 2 minutes and the supernatant aspirated. To all tubes except the totals, 2 ml of 0,9% saline was added. The tubes were then centrifuged at $2,000 \times g$ for 2 minutes and the supernatant aspirated. The washing procedure with 0,9% saline was performed thrice.

The radioactivity in each tube was determined using a gamma counter (5 min counts).

A-5.4 Calculation

The count rates for each of the standards was expressed as a percentage of the mean count rate of the 'zeros' (B/Bo). The B/Bo for each insulin standard was plotted against its respective insulin concentration on lin log paper and a standard curve constructed. A composite standard curve generated from 10 consecutive assays is shown in Figure A-5.

The count rates of each of the unknowns (control and samples) was expressed as a percentage of the 'zeros' (B/Bo) and the concentration of insulin was read off directly from the standard curve for each unknown sample.

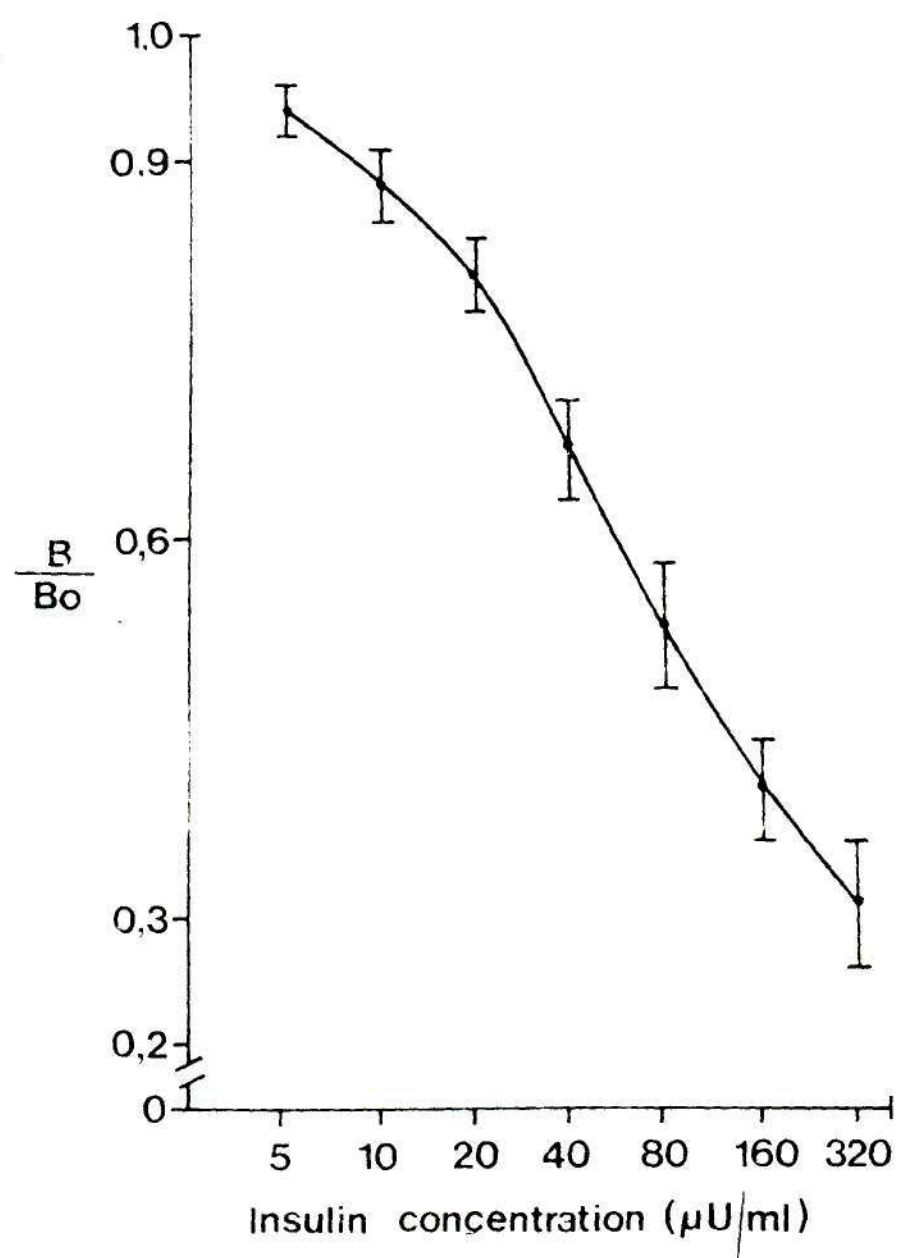
Also the count rates of the zeros (Bo) and non specific binding (NSB) tubes were expressed as a percentage of the total counts.

A-5.5 Comment

Serum was separated from blood as soon as possible after collection and stored at -20°C until assayed.

All determinations were carried out in duplicate.

FIGURE A-5 COMPOSITE STANDARD CURVE GENERATED FROM 10 CONSECUTIVE ASSAYS (MEAN + 1SD)



For an assay to be acceptable the following criteria had to be fulfilled:

- a) Maximum binding (B_0/Total) had to be between 20 - 25%.
- b) Nonspecific binding (NSB) had to be less than 2%.
- c) The control value obtained should be in the assigned reference range.

The intra-and inter-assay coefficients of variation for the insulin assay were 4,4% and 4,6% respectively.

APPENDIX A-6 : DETERMINATION OF SERUM C-PEPTIDE CONCENTRATIONS

C-peptide concentrations were determined by a radioimmunoassay technique using the Biodata C-peptide kit from Serono Diagnostics, Chavannes de Bogis, Switzerland.

A-6.1 Principle

The antigen whether from standard or from samples competes with a constant amount of radioactive tracer (^{125}I -C-peptide) for the binding sites of the antibody. After incubation, the amount of tracer bound is inversely proportional to the amount of hormone present in sample or in the standards. The immunocomplex is precipitated by adding polyethyleneglycol solution and collected by centrifugation. The radioactivity is then read in a γ -counter. By interpolating on the standard curve, it is possible to obtain the C-peptide sample concentrations.

A-6.2 Reagents

- (1) Diluent for C-peptide.

Containing C-peptide free serum, freeze-dried with preservatives. This was reconstituted in 12,5 ml of distilled water.

- (2) C-peptide antiserum.

Containing anti C-peptide serum raised in rabbits, freeze-dried in phosphate buffer with preservatives. The antiserum

was mixed with 12,5 ml distilled water.

(3) C-peptide standards.

Containing protein solution, preservatives and C-peptide freeze-dried at the following concentrations.

0; 0,3; 0,6; 1,0; 2,0; 4,0; 8,0 and 20 ng/ml while the 0 standard was mixed with 2 ml of distilled water, the others were reconstituted with 1 ml of distilled water.

(4) ^{125}I -C-peptide

Containing C-peptide labelled with ^{125}I freeze dried in phosphate buffer with preservatives. These contents were reconstituted with 12,5 mls distilled water.

(5) PEG 20% TW solution

Containing polyethyleneglycol MW 6000 in solution with tween 20 and preservatives.

(6) Serotest M

Containing human serum, freeze dried with preservative. This was reconstituted with 1 ml of distilled water. All above reagents were stored at 4°C until use.

A-6.3 Procedure

All reagents were allowed to reach ambient temperature and mixed thoroughly before use. A control serum (serotest) was incorporated in each assay.

| Reagents | Sample | Bo | Standard | NSB | Total |
|-----------------------------|--------|-----|----------|-----|-------|
| Sample | 0,1 | - | - | - | - |
| 0 Standard | - | 0,1 | - | 0,1 | - |
| Distilled Water | - | - | - | 0,1 | - |
| Standards | - | - | 0,1 | - | - |
| ^{125}I -C-peptide | 0,1 | 0,1 | 0,1 | 0,1 | 0,1 |
| C-peptide antiserum | 0,1 | 0,1 | 0,1 | - | - |

Thereafter all tubes were mixed thoroughly and incubated for 24 hrs at 4°C.

PEG 20% (1 ml) was added to all tubes except the totals.

All tubes except the totals were centrifuged at 2,000 xg for 5 minutes.

The radioactivity in each tube was determined using a gamma counter (1 minute counts)

A-6.4 Calculation

The count rates for each of the standards was expressed as a percentage of the mean count rate of the 'zeros' (B/Bo). The B/Bo for each C-peptide standard was plotted against its respective C-peptide concentration on lin-log paper and a standard curve constructed. A typical standard curve generated from 5 consecutive assays is shown in Fig A-6.

The count rates of each of the unknowns (control and samples) was expressed as a percentage of the 'zeros' (B/Bo) and the concentration of C-peptide was read off directly from the standard curve for each unknown sample.

The count rates of the zeros (Bo) and non-specific binding (NSB) tubes were expressed as a percentage of the total counts.

A-6.5 Comment

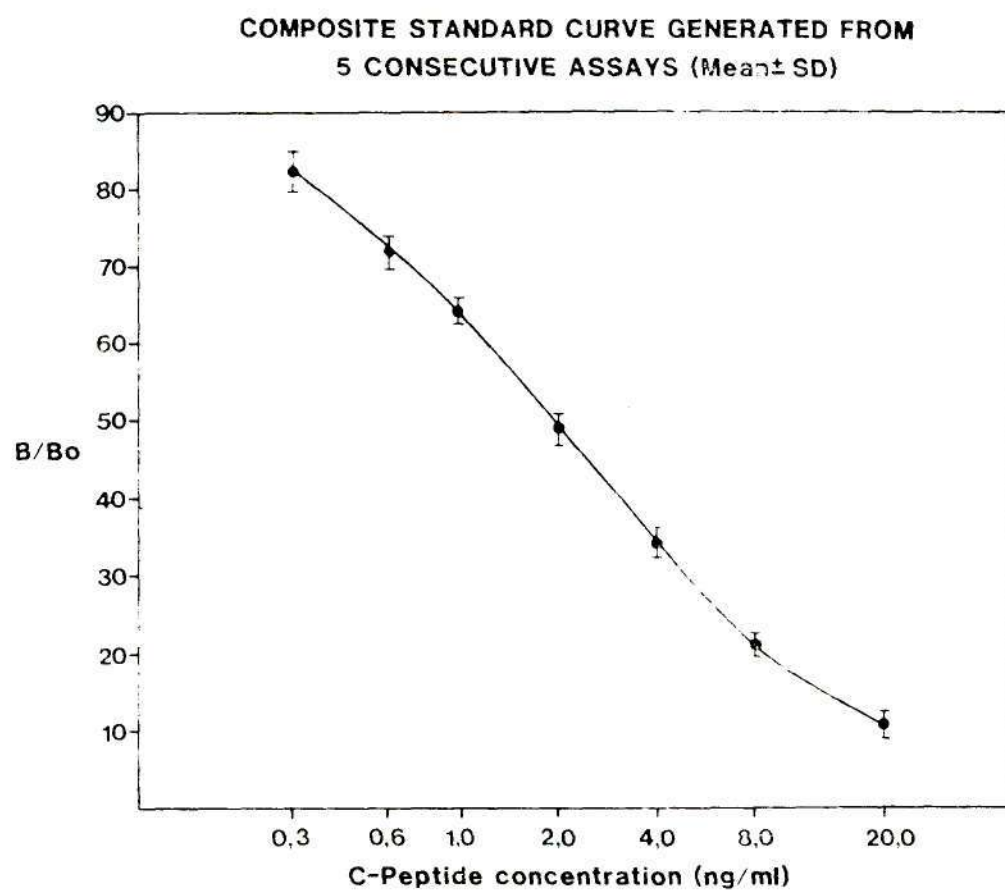
Serum was separated from blood as soon as possible after collection and stored at -20°C until assayed.

All determinations were carried out in duplicate.

For an assay to be acceptable the following criteria had to be met:

- a) Maximum binding (Bo/Total) had to be between 35 and 40%.
- b) Non-specific binding (NSB) had to be less than 5%.

FIGURE A-6



- c) The control value obtained should be in the assigned reference range.

Intra- and inter-assay coefficients of variation for CPR were 4,8% and 7,3%.

APPENDIX A-7 : DETERMINATION OF PLASMA FREE FATTY ACIDS

Plasma free fatty acids (FFA) were determined calorimetrically using a commercial Kit (Boehringer Mannheim GmbH Diagnostica)

A-7.1 Reference

Duncombe (1964)

A-7.2 Principle

Free fatty acids are converted to chloroform-soluble copper salts. The copper in the organic layer is subsequently measured calorimetrically after adding diethyldithiocarbamate which forms a coloured complex with copper. By comparing the intensity of colour developed in unknown samples with the colour developed in a known standard, FFA concentrations can be determined in the unknowns.

A-7.3 Reagents

- (1) Copper reagent (Solution 1)

Cupric nitrate 0,27 mol/l

Triethanolamine buffer 0,45 mol/l pH 7,8.

- (2) Diethyldithiocarbamate solution (Solution 2)

9 mmol/l diethyldithiocarbamate in redistilled butanol

- (3) Chloroform

This was washed with water on the day of the test to remove all traces of alcohol and dried with sodium sulphate and filtered.

- (4) Stock Standard Solution (500 $\mu\text{mol/l}$)
 12,82 mg of palmitic acid in 100 ml chloroform. This stock solution was stored in the dark at room temperature.

A-7.4 Procedure

The extraction was carried out in 15 ml ground glass stoppered centrifuge tubes. The test schedule was as follows:

| | Blank | Standard | Sample |
|-------------------------------------|-------|----------|--------|
| Chloroform (ml) | 5,0 | 5,0 | 5,0 |
| Standard (μl) | - | 200 | - |
| Plasma (μl) | - | - | 200 |
| Redistilled water (μl) | 200 | - | - |
| Copper reagent (Soln 1) (ml) | 1,0 | 1,0 | 1,0 |

The tubes were shaken vigorously for 10 minutes on a mechanical shaker followed by centrifugation for 5 minutes. Thereafter the blue green aqueous layer, together with the protein layer, was carefully removed by means of a fine-tipped pipette connected to a water-jet aspirator. Care was taken to remove the layers completely. 2 ml of the chloroform layer (blank, standard and sample) was pipetted into clean, dry test tubes and 0,2 ml of diethyldithiocarbamate reagent was added. The contents of the tubes were mixed and after 10 minutes the absorbance (A) of the samples and standard was read against the blank at 436 nm in a glass cuvette with a 1 cm light path on a Beckman Acta V spectrophotometer.

A-7.5 Calculation

The concentration of FFA in the samples was calculated as follows:

$$\text{concentration of FFA sample} = \frac{A \text{ sample}}{A \text{ standard}} \times \frac{500}{1} \quad (\mu\text{Eq/l})$$

A-7.6 Comment

Samples for FFA estimation were taken in potassium EDTA tubes on ice centrifuged immediately at 4°C and the plasma stored at -20°C until assayed. All determinations were carried out in duplicate.

The intra- and inter-assay coefficients of variation of the stock standard solution were 3,8% and 4,2% respectively.

APPENDIX A-8 : DETERMINATION OF SERUM CORTISOL CONCENTRATIONS

Serum cortisol levels were determined by a radioimmunoassay technique using the Gammacoat ¹²⁵I-Cortisol RIA Kit (Travenol Laboratories, Massachusetts).

A-8.1 Principle

Serum cortisol concentrations were determined by radioimmunoassay using a coated tube technique. The unknown samples and standards are incubated with ¹²⁵I-cortisol tracer in antibody coated tubes where the antibody is immobilised onto the lower inner wall of the Gammacoat tube. After incubation, the contents of the tubes are aspirated and the tube counted. The quantity of cortisol in the sample is inversely proportional to the bound radioactivity.

A-8.2 Reagents

- (1) Phosphate Buffered Saline (PBS)
100 ml
- (2) Cortisol Serum Blank
600 µl
- (3) Rabbit Anti-Cortisol Serum Coated Tubes
100 tubes

(4) ^{125}I -Cortisol Tracer

148 kBq ^{125}I -cortisol tracer was reconstituted in 100 ml of PBS buffer

(5) Cortisol Serum Standards

Cortisol serum standards containing 55,2; 138; 276; 690 and 1656 nmol/l in 600 μl aliquots were mixed gently without foaming.

A-8.3 Procedure

All reagents were allowed to reach ambient temperature and mixed thoroughly without foaming before use.

A radioassay control serum (Lyphochek) was incorporated in each assay. 10 μl volume of serum blanks, standards and samples were pipetted in duplicate into antibody-coated tubes together with 1,0 ml of ^{125}I -cortisol tracer solution. All tubes were mixed and incubated for 45 minutes in a waterbath at 37°C. Thereafter all tubes were aspirated and the radioactivity determined in a gamma counter (1 min counts). Total tubes consisting of 1,0 ml tracer only were also counted.

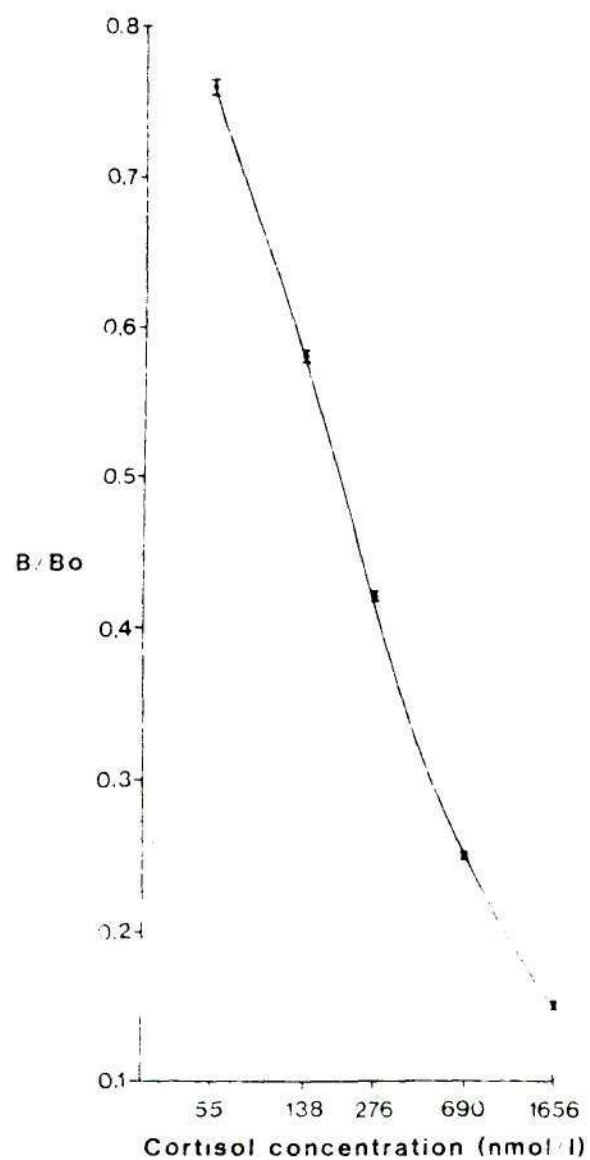
A-8.4 Calculation

The count rates for each of the standards were expressed as a percentage of the mean count rate of the 'zeros' (B/Bo). The B/Bo for each cortisol standard was plotted against its respective cortisol concentration on lin-log paper and a standard curve constructed. A typical standard curve generated from 10 consecutive assays is shown in Figure A-8.

The count rates of each of the unknowns (control and samples) was expressed as a percentage of the mean count rate of the 'zeros' (B/Bo) and the concentration of cortisol was read off directly from the standard curve for each unknown sample.

FIGURE A-8

COMPOSITE CORTISOL STANDARD CURVE
GENERATED FROM 10 CONSECUTIVE
ASSAYS (Mean \pm 1 SD)



Also the count rates of the zeros (B_0) was expressed as a percentage of the total counts.

A-8.5 Comment

Serum was separated from blood as soon as possible after collection and stored at -20°C until assayed.

All determinations were carried out in duplicate.

For an assay to be acceptable the following criteria had to be fulfilled.

- a) Maximum binding (B_0/Total) had to be between 45-55%.
- b) The control value obtained should be in the assigned reference range.

The intra- and inter-assay coefficients of variation for the cortisol assay were 2,4% and 7,7% respectively.

APPENDIX A-9 : DETERMINATION OF PLASMA GLUCAGON CONCENTRATIONS

Plasma glucagon levels were determined by a radioimmunoassay technique using the Glucagon PEG Kit (Serono Diagnostics, Switzerland).

A-9.1 Principle

The assay is based on the competitive binding principles of radioimmunoassay. Standards and unknown samples are incubated with ^{125}I -glucagon tracer and fixed amounts of anti-glucagon antibody. At the end of the incubation period, the radioactive antigen-antibody complex is precipitated with the addition of a polyethyleneglycol (PEG) solution and collected by centrifugation. The quantity of glucagon in the sample is inversely proportional to the bound radioactivity.

A-9.2 Reagents

- (1) Trasylol (Bayer)

10,000 KIU/ml (10ml)

(2) Assay Buffer

120 ml phosphate buffer pH 7 - 7,5.

(3) Rabbit Anti-Glucagon Antibody

The lyophilized antibody after being reconstituted with 12,5 ml buffer had a titre of 1 : 1800. This antibody is claimed to be highly specific for pancreatic glucagon; 100% cross-reactivity with pancreatic glucagon and absent cross reactivity with enteroglucagon up to 10 µg/ml.

(4) ¹²⁵I-glucagon

The lyophilized ¹²⁵I-glucagon, 5 - 10 mg. (specific activity 6,7 - 8,9 MBq/µg was dissolved in 12,5 ml of buffer and mixed gently.

(5) Carrier Serum

The lyophilized Bovine Gamma globulin (300 mg) was dissolved in 12,5 mls of buffer. Concentration of gamma globulin after reconstitution was 24 mg/ml.

(6) Polyethyleneglycol solution (20%)

120 ml of 20% PEG (6000) in phosphate buffer

(7) Glucagon Standard

20 ng lyophilized glucagon was reconstituted in 10 ml of buffer. This stock standard solution contained 2000 pg/ml of glucagon. Standard solutions, final concentrations 2,000; 1,000; 500; 250; 125; 62,5 pg/ml were prepared from the stock solution (2000 pg/ml) by diluting in buffer according to the following schedule:

| | Final Standard Concentration | Buffer Soln. (ml) | Std. Soln. (ml) |
|---|------------------------------|-------------------|-----------------|
| A | 2000 pg/ml | - | 10 |
| B | 1000 pg/ml | 1 | + 1 A |
| C | 500 pg/ml | 1 | + 1 B |
| D | 250 pg/ml | 1 | + 1 C |
| E | 125 pg/ml | 1 | + 1 D |
| F | 62,5 pg/ml | 1 | + 1 E |

A-9.3 Procedure

A pooled serum control was incorporated in each assay. The test schedule was as follows:

| Reagents | Total | N.S.B. | Bo | Std. | Control/ Samples |
|------------------------|-------|--------|-----|------|---------------------|
| Trasylol (μl) | - | 100 | 100 | 100 | 100 |
| Buffer (μl) | - | 200 | 100 | - | - |
| Glucagon Std. (μl) | - | - | - | 100 | - |
| Samples/Control (μl) | - | - | - | - | 100 |
| Glucagon Antisera (μl) | - | - | 100 | 100 | 100 |

Tubes mixed and incubated at 4 C for 24 hours.

| | | | | | |
|--------------------------------|-----|-----|-----|-----|-----|
| ¹²⁵ I-Glucagon (μl) | 100 | 100 | 100 | 100 | 100 |
|--------------------------------|-----|-----|-----|-----|-----|

Tubes mixed and incubated at 4 C for 24 hours

| | | | | | |
|--------------------|---|-----|-----|-----|-----|
| Carrier Serum (μl) | - | 100 | 100 | 100 | 100 |
|--------------------|---|-----|-----|-----|-----|

Tubes mixed

| | | | | | |
|---------------------------------|---|-----|-----|-----|-----|
| Cold PEG solution (4°C) (ml) | - | 1,0 | 1,0 | 1,0 | 1,0 |
|---------------------------------|---|-----|-----|-----|-----|

Tubes mixed thoroughly

All tubes except the totals were centrifuged at 2,000 xg for 15 mins and the supernatant decanted.

Thereafter the precipitate in all tubes except the totals was washed with 2 mls of distilled water without shaking the tubes. The tubes were centrifuged at 2,000 xg for 5 minutes and the supernatant decanted.

The radioactivity in each tube including the totals was determined using a gamma counter (5 min counts).

A-9.4 Calculation

The count rates for each of the standards was expressed as a percentage of the mean count rate of the 'zeros' (B/Po). The B/Bo for each glucagon standard was plotted against its respective glucagon concentration on lin-log paper and a standard curve constructed. A typical standard curve generated from 10 consecutive assays is shown in Figure A-9.

The count rates of each of the unknowns (control and samples) was expressed as a percentage of the 'zeros' (B/Bo) and the concentration of glucagon was read off directly from the standard curve for each unknown sample.

Also the count rates of the zeros (Bo) and nonspecific binding (NSB) tubes were expressed as a percentage of the total counts.

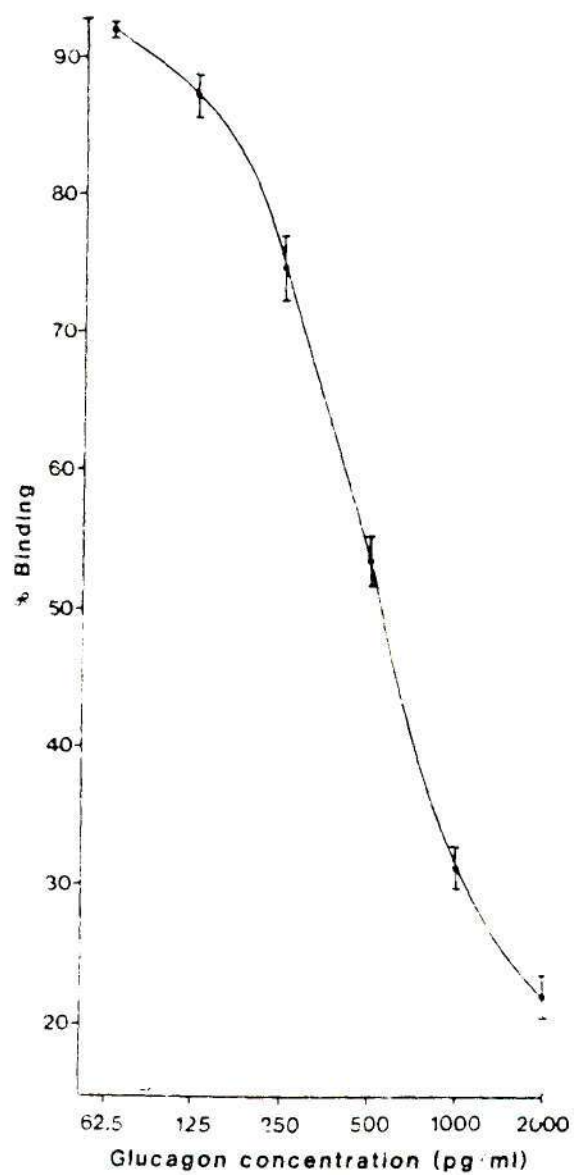
A-9.5 Comment

To minimise proteolytic degradation of glucagon, blood was drawn into chilled tubes containing 1000 IU Trasylol and 2 mg EDTA per ml of blood, centrifuged immediately at 4°C and the plasma stored at -20°C until assayed.

All determinations were carried out in duplicate.

FIGURE A-9

COMPOSITE GLUCAGON STANDARD CURVE
GENERATED FROM 10 CONSECUTIVE
ASSAYS (Mean \pm 1 SD)



For an assay to be acceptable the following criteria had to be fulfilled:

- a) Maximum binding (B_0 /Total) had to be between 35-45%.
- b) Non specific binding (NSB) had to be less than 5%.
- c) The control value obtained should be in the assigned reference range.

The intra- and inter-assay coefficients of variation for the glucagon assay were 0,9% and 9,2% respectively.

APPENDIX A-10 : DETERMINATION OF SERUM GROWTH HORMONE CONCENTRATIONS

Growth hormone levels were determined by a radioimmunoassay technique using the Phadebas Human Growth Hormone Kit (Pharmacia Diagnostics, Uppsala, Sweden).

A-10.1 Principle

The method used for quantitating human growth hormone (HGH) is a solid phase radioimmunoassay using a 'sandwich' technique. HGH antibodies covalently coupled to paper discs (solid phase) react during the first incubation with the unknown sample. After washing, a fixed amount of ^{125}I -labelled immunosorbent purified HGH-antibodies are added, forming a specific complex with the HGH molecules in the sample which are bound to the antibodies on the paper discs during the previous incubation. The radioactivity of this complex is then measured and is directly proportional to the quantity of HGH in the sample.

A-10.2 Reagents

(1) HGH-Free Diluent

The lyophilized diluent was reconstituted by adding 5 ml of redistilled water.

(2) ^{125}I -Anti-HGH solution.

0.8 μg (222 kBq) of ^{125}I -anti-HGH was reconstituted by adding 10,0 ml of redistilled water.

(3) Anti-HGH discs

The antibody bound to discs were supplied in cassettes.

(4) HGH Standards

The HGH standard is calibrated against the First International Reference Preparation of Growth Hormone. After reconstitution with 1,5 ml redistilled water, the lyophilized HGH standard had a final concentration of 75 ng/ml. Standard solutions at final concentrations: 75 ng/ml, 25 ng/ml, 7,5 ng/ml, 0,75 ng/ml and 0,25 ng/ml were prepared from the stock solution (75 ng/ml) by diluting in HGH-free diluent according to the following schedule:

| Final Standard Concentration | | Diluent | Standard | |
|------------------------------|------------|-------------------|----------|------------------------|
| A | 75 ng/ml | - | + | 1,5 ml |
| B | 25 ng/ml | 800 μl | + | 400 μl of A |
| C | 7,5 ng/ml | 900 μl | + | 100 μl of A |
| D | 2,5 ng/ml | 900 μl | + | 100 μl of B |
| E | 0,75 ng/ml | 900 μl | + | 100 μl of C |
| F | 0,25 ng/ml | 900 μl | + | 100 μl of D |

A-10.3 Procedure

A Phadebas reference serum (control) was incorporated in each assay.

The test schedule was as follows:

| | Total | Standard | Control/ Samples |
|-----------------|-------|-------------------|---------------------|
| Anti-HGH disc | - | + | + |
| Standards | - | 100 μl | - |
| Control/Samples | - | - | 100 μl |

All tubes were covered with plastic film and incubated for 3 hours at room temperature.

Thereafter all the liquid in each tube was aspirated using a pasteur pipette coupled to an aspirator.

2,5 ml of 0,9% saline was added to all tubes except the totals and the tubes were allowed to stand for 10 minutes, after which the liquid was aspirated from the tubes. This washing procedure with 0,9% saline was performed thrice.

After the washing procedure, 100 μ l of 125 I-anti-HGH solution was added to all tubes including the totals. The tubes were covered with plastic film and incubated for 20 hours at room temperature.

Thereafter, all the liquid in each tube (except totals) was aspirated using a pasteur pipette coupled to an aspirator and the washing procedure with 2,5 ml 0,9% saline was performed thrice as described above.

The radioactivity in each tube was determined using a gamma counter (5 min counts).

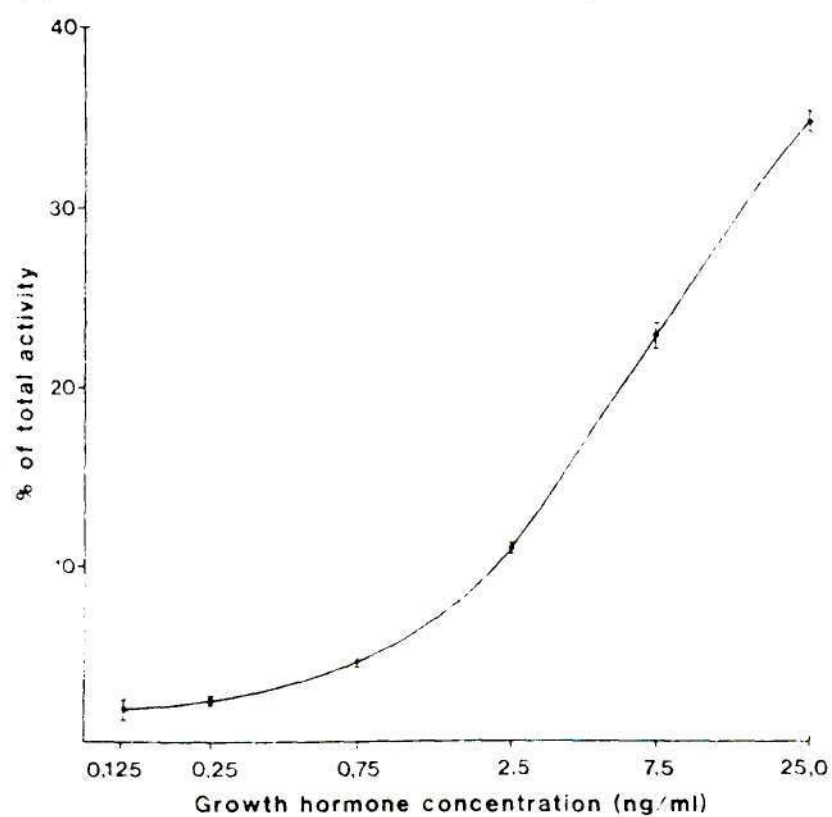
A-10.4 Calculation

The count rates for each of the standards, control and samples were expressed as a percentage of the mean count rate of the total activity tubes (B/T%). The percentage values obtained for each of the standards was plotted against its respective HGH concentration on lin-log paper and a standard curve constructed. A typical standard curve generated from 10 consecutive assays is shown in Figure A-10.

Using the (B/T %) for the control and samples the concentration of HGH was read off directly from the standard curve.

FIGURE A-10

COMPOSITE GROWTH HORMONE STANDARD CURVE GENERATED
FROM 10 CONSECUTIVE ASSAYS (Mean : 1SD)



A-10.5 Comment

Serum was separated from blood as soon as possible after collection and stored at -20 C until assayed.

All determinations were carried out in duplicate.

For an assay to be acceptable the control value obtained should be in the assigned reference range. The detection limit of the assay was found to be 0,25 ng/ml. The intra- and inter-assay coefficients of variation for the HGH assay were 5,6% and 9,4% respectively.

APPENDIX A-11 : MEASUREMENT OF ¹²⁵-INSULIN BINDING TO INSULIN RECEPTORS ON ERYTHROCYTES.

A-11.1 Reference

Gambhir et al (1977)

A-11.2 Principle

The measurement of insulin receptors is conceptually similar to a conventional radioimmunoassay and is based on the principle that ligand and receptor bind specifically and reversibly but with the equilibrium grossly in favour of bound ligand and receptor complex. Inasmuch as a finite number of receptors exist, saturation binding by increasing quantities of ligand can be achieved.

Insulin receptors are, of course, located on the cell surface in common with receptors for other peptide hormones. When the usual technique of saturation analysis is applied, that is, incubation of the receptor with increasing concentrations of unlabelled ligand and fixed amounts of labelled ligand, it is inevitable that a highly polarized molecule like insulin will adhere to complementary polarized cell surface elements on a non-saturable basis. The specific binding can however, be assessed by relying on the reversibility of ligand-receptor binding and

the fact that the receptors can be saturated. A gross excess of unlabelled ligand will displace the labelled ligand and by measuring how much labelled ligand is displaced, the specific binding can be assessed.

Separating bound and free ligand is no problem for a receptor located on the cell surface; simple centrifugation will achieve separation.

A-11.3 Reagents

(1) Ficoll-Hypaque solution (Pharmacia, Uppsala, Sweden).

(2) RBC buffer (buffer G) pH 8,0

| | |
|-----------------------------------|-----------|
| Hepes - n - 2 -- hydroxy | 50 mmol/l |
| Tris (hydroxymethyl) aminomethane | 50 mmol/l |
| MgCl ₂ | 10 mmol/l |
| EDTA | 2 mmol/l |
| Dextrose | 10 mmol/l |
| CaCl ₂ | 10 mmol/l |
| NaCl. | 10 mmol/l |
| KCl | 5 mmol/l |
| Bovine Serum Albumin (BSA) | 1 g/l |

All reagents were of certified quality.

(3) Dibutylphthalate (Sigma Chemical Company, Missouri, U.S.A)

(4) Unlabelled insulin (porcine) obtained from Novo Industries, Denmark was made up in 0,01 molar HCl at a concentration of 1mg/ml and stored at -20°C. This solution was the stock standard from which all other standards were made up.

(5) ¹²⁵I-insulin (porcine, labelled in position Tyrosine A₁₄; from Amersham International, Buckinghamshire, United Kingdom). Specific activity 200-300 µCi/µg.

A-11.4 Procedure

Twenty millilitres of blood was drawn from subjects into tubes containing lithium heparin. After centrifugation of the blood (10 minutes, $400 \times g$, 20°C), the supernatant plasma was aspirated and the compacted erythrocytes were diluted with normal saline (1:1) and then layered onto a 10ml Ficoll-Hypaque gradient. This sample was centrifuged in a Beekman J2-21 centrifuge (15 minutes, $400 \times g$, 20°C). The supernatant was aspirated and the RBC's were resuspended in 10 ml of saline and once again placed on a Ficoll gradient and centrifuged as before. After aspirating the supernatant, the cells were resuspended in 10 ml of chilled buffer G and centrifuged at $400 \times g$ (4°C ; 15 minutes). The supernatant was aspirated and the erythrocytes resuspended in 4-5 ml of buffer G and counted in a Coulter counter. The cell count varied between $3,5 - 5,6 \times 10^9$ cells /ml.

Viability of cells was assessed by the trypan blue dye exclusion technique in which one ml of cells suspension was incubated with 1% trypan blue. This was left to stand for 5 minutes. Less than 2% of cells contained trypan blue indicating a viability of greater than 98%.

To set up the radioimmunoassay; a series of insulin standards were prepared freshly in buffer G from the stock solution of porcine insulin in 0,01 molar HCl, with a concentration of 1 mg/ml. The dilutions were as follows:-

20 μl insulin stock (1mg/ml) \pm 180 μl buffer G \rightarrow insulin 100 $\mu\text{g/ml}$

50 μl insulin (100 $\mu\text{g/ml}$) \pm 450 μl buffer G \rightarrow insulin 10 $\mu\text{g/ml}$.

50 μl insulin (10 $\mu\text{g/ml}$) \pm 450 μl buffer G \rightarrow insulin 1 $\mu\text{g/ml}$.

50 μl insulin (1 $\mu\text{g/ml}$) \pm 450 μl buffer G \rightarrow insulin 100 ng/ml.

50 μl insulin (100 ng/ml) \pm 450 μl buffer G \rightarrow insulin 10 ng/ml.

^{125}I -iodoinsulin was diluted to a concentration of 0,2ng/ml (30,000-

40,000 cpm).

An assay was set up in which a constant number of cells and total radioactivity were incubated with increasing concentrations of unlabelled insulin over the concentration range of 1,2 ng/ml to 1×10^5 ng/ml. Appropriate blanks were included in the assay. Details and the sequence in which the various components were added to the tubes were as follows:-

| UNLABELLED INSULIN | STANDARD USED | BUFFER G | 125 INSULIN 0,2 NG/ML ($\pm 3 \times 10^4$ CPM/ TUBE) | CELL SUSPEN- SION | TOTAL INSULIN CONCENTRATION |
|-----------------------|------------------|------------|--|-------------------------|-----------------------------------|
| - | - | 50 μ l | 50 μ l | 400 μ l | 0,2 |
| - | - | 50 μ l | - | - | 0,2 |
| 50 μ l | 10 ng/ml | - | - | - | 1,2 |
| 10 μ l | 100 ng/ml | 40 μ l | - | - | 2,2 |
| 25 μ l | 100 ng/ml | 25 μ l | - | - | 5,2 |
| 50 μ l | 100 ng/ml | - | - | - | 10 |
| 10 μ l | 1 μ g/ml | 40 μ l | - | - | 20 |
| 25 μ l | 1 μ g/ml | 25 μ l | - | - | 50 |
| 50 μ l | 1 μ g/ml | - | - | - | 100 |
| 50 μ l | 10 μ g/ml | - | - | - | 1000 |
| 50 μ l | 1 mg/ml | - | - | - | 100,000 |

The tubes were incubated at 15°C for 90 minutes with gentle agitation every 30 minutes. At the end of the incubation period, the cells in each tube were resuspended by gentle mixing and a 200 μ l portion was withdrawn and added to chilled microfuge tubes containing 200 μ l of buffer G and 200 μ l dibutylphthalate and centrifuged for 2 minutes (Beckman microfuge). The supernatant fluid was removed by aspiration

and the radioactivity in the cell pellet was counted in a Berthold Multi Crystal gamma counter (LB 2100); the remaining cell suspension in all the assay tubes were pooled and a 200ul portion was withdrawn and counted to obtain total counts.

Non specific binding (NSB) was the counts bound to erythrocytes in the presence of 10^5 ng/ml unlabelled insulin.

All assays were carried out in duplicate and only freshly drawn erythrocytes were used.

The degradation of insulin occurring during incubation was determined by trichloroacetic acid (TCA) solubility, in which equal volumes of TCA and supernatant containing ^{125}I -iodoinsulin incubated at 4°C for 1/2 hour and then centrifuged ($400 \times g$, 4°C , 30 minutes), the supernatant was decanted into a tube and both the precipitate and the supernatant were counted in a gamma counter. Percentage TCA soluble was calculated using the following formula:

$$\frac{\text{Radio activity supernatant}}{\text{Radioactivity supernatant plus precipitate}} \times 100$$

Less than 5% of ^{125}I -insulin was TCA solubilized i.e degraded.

A-11.5 Calculations

Erythrocyte counts were corrected to 4.5×10^9 cells/ml by dividing 4.5×10^9 by the RBC suspension count used in the assay; the ratio is referred to as the correction factor.

$$\% \text{ total bound} = \frac{\text{cpm pellet}}{\text{total cpm}} \times 100 \times \text{correction factor}$$

$$\% \text{ specifically bound} = \frac{\text{cpm pellet} - \text{cpm NSB}}{\text{total cpm}}$$

Specifically bound radioactivity was plotted as a function of the logarithm of total insulin concentration (Figure A-11.1).

Receptor number was calculated according to Scatchard (1949) Bound/Free hormone was plotted against bound hormone using the following formulae:

$$\text{cpm bound corrected} = (\text{mean cpm-NSB}) \times \text{correction factor}$$

$$\text{Free insulin} = \text{Total counts} - \text{cpm bound corrected}$$

$$\frac{\text{Bound Insulin}}{\text{Free Insulin}} = \frac{\text{cpm bound corrected}}{\text{total counts}} \times \text{concentration insulin ng/ml/tube}$$

A typical Scatchard plot is presented in Figure A-11.2. The total concentration of unlabelled insulin required to achieve 50% displacement of labelled insulin from the receptor (ID_{50}) was used as an index of the affinity of the insulin receptor. Values ranged from 2,0 - 10,0 ng/ml.

A-11.6 Comment

Despite the small amount of radioactivity bound in these assays, measurement of the receptors proved to be remarkably reliable. Mean NSB was only 1,7% of total cpm (range 1,2 - 2,2%). The interassay coefficient of variation was 9,7%.

Provided the insulin is labelled in position A-14 and the product is less than three weeks old, no particular difficulties were experienced with the assay.

APPENDIX A-12. MEASUREMENT OF ^{125}I -INSULIN BINDING TO INSULIN RECEPTORS ON CIRCULATING MONOCYTES

A-12.1 Reference

Bar et al (1976)

FIGURE A-11.1 COMPETITION CURVE OF ^{125}I -INSULIN BINDING TO
CIRCULATING ERYTHROCYTES

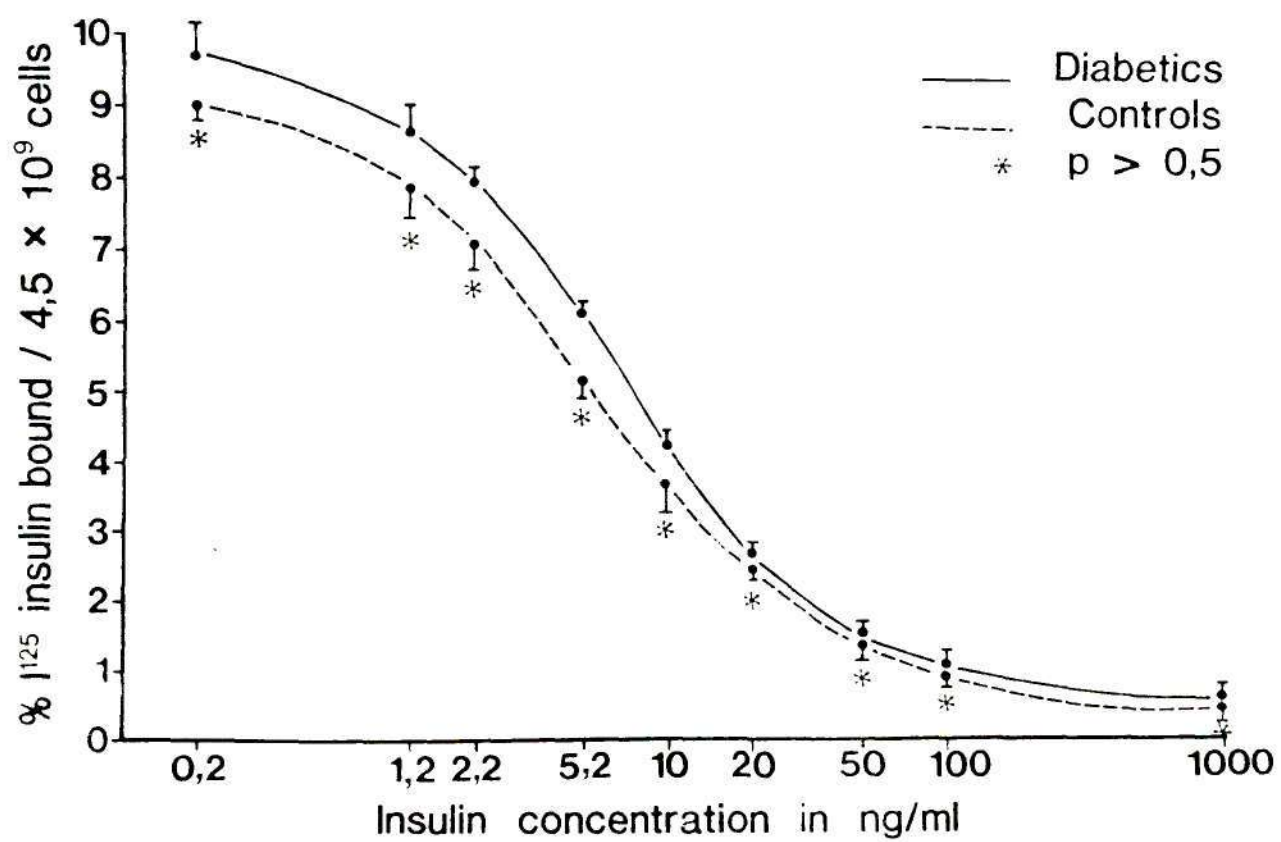
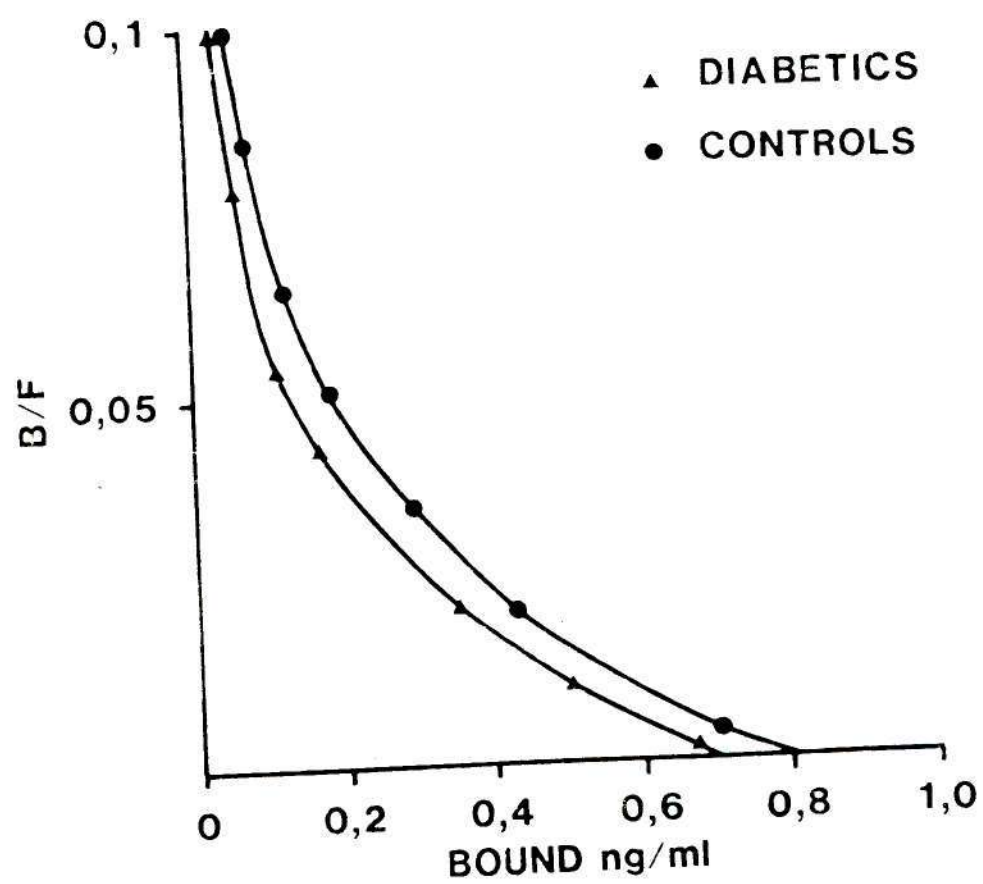


Table A-11.2

Scatchard plot of erythrocytes.



A-12.2 Principle

In principle the measurement of surface insulin receptors on monocytes differ in no way from that described for RBC's in Section A-11.2; the isolation procedure of the monocytes is based on ficoll-Hypaque gradient centrifugation.

A-12.3 Reagents

(1) Ficoll-Hypaque solution (Pharmacia, Uppsala, Sweden).

(2) Monocyte buffer

| | |
|---------------------|------------|
| Hepes - n-2-hydroxy | 100 mmol/l |
|---------------------|------------|

| | |
|----------|-----------|
| Dextrose | 10 mmol/l |
|----------|-----------|

| | |
|-------|------------|
| Na Cl | 120 mmol/l |
|-------|------------|

| | |
|-------------------|------------|
| MgSO ₄ | 1,2 mmol/l |
|-------------------|------------|

| | |
|------|----------|
| EDTA | 7 mmol/l |
|------|----------|

| | |
|-----|------------|
| KCl | 2,5 mmol/l |
|-----|------------|

| | |
|-----|------|
| BSA | 1g/l |
|-----|------|

All reagents were of certified quality.

(3) Latex beads (LB-11) in an aqueous suspension from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

(4) Unlabelled insulin (porcine) obtained from Novo Industries, Denmark was made up in 0,01 molar HCl at a concentration of 1mg/ml and stored at -20°C. This solution was the stock standard from which all other standards were made up.

(5) ¹²⁵I-insulin (porcine; labelled in position Tyrosine A14, Amersham International, Buckinghamshire, UK) specific activity 200-300 µCi/µg.

A-12.4 Procedure

One hundred ml of blood obtained from fasting individuals was withdrawn into tubes containing lithium heparin and centrifuged 10 minutes,

400 x g, 20°C); the plasma was aspirated; the buffy coat and the RBC's were diluted with normal saline in a ratio of 1:1. Twenty ml quantities were layered onto a 10ml Ficoll-Hypaque gradient in separate tubes. These samples were centrifuged (20°C, 400 x g, 20 minutes), the supernatant carefully aspirated without disturbing the mononuclear layer. The mononuclear cells were carefully aspirated and mixed with 10 ml of saline; the preparation was centrifuged at 400 x g, 20°C for 20 minutes. The pellet was resuspended in 10ml of Hepes buffer and centrifuged as before. The final cell pellet was resuspended in 4-5 ml of hepes buffer and counted in a haemocytometer. Cell counts usually came to about $45-50 \times 10^6$ mononuclear cells/ml. For assay purposes the suspension was diluted to contain 10×10^6 mononuclear cells/ml.

Inasmuch as lymphocytes have virtually no insulin receptors and constitute the bulk of mononuclear cells isolated, the exact number of monocytes present in the preparation had to be determined. This was done by relying on the phagocytosis of latex beads by the monocytes. Two hundred μ l of mononuclear cell suspension was added to 5 ml of 2% BSA and centrifuged at 4°C, 400 x g for 10 minutes. The supernatant was aspirated and the cell pellet was resuspended in 0.5 ml of foetal calf serum and 10 μ l of latex bead suspension. This was incubated for 30 minutes at 37°C. The sample was once again centrifuged, the supernatant was aspirated and the cells in the infranatant were resuspended in 1ml of saline and counted. Cells which had 3 or more latex beads in the cytoplasm were identified as monocytes. The percentage varied between 13 and 20% (mean 17%).

The radio-receptor assay using the mononuclear cell preparation, is precisely as described for RBC's (Section A-11.4)

The incubation period for the monocyte assay was 3 hours (room temp-

perature: -20°C -25°C) Thereafter the cells in each tube were resuspended by gentle mixing and a 200 μ l portion was withdrawn and added to chilled microfuge tubes containing 200 μ l Hepes buffer and centrifuged in a microfuge for 2 minutes. The supernatant fluid was removed by aspiration and the radioactivity in the cell pellet was counted in a gamma counter, the remaining cell suspension in all the assay tubes were pooled and a 200 μ l portion was withdrawn and counted to obtain total counts.

NSB was the counts bound to monocytes in the presence of 10^5 ng/ml unlabelled insulin.

All assays were carried out in duplicate and only freshly drawn cells were used.

The degradation of insulin as determined by TCA solubility was < 5% during incubation and > 98% of monocytes were viable as assessed by the trypan blue dye exclusion technique.

A-12.5 Calculations

The only difference in calculating results concerns the correction factor which was derived as follows: Number of mononuclear cells in suspension \times percentage monocytes estimated by latex bead ingestion gives the true monocyte count/ml. To standardize assays the results were corrected to 2×10^6 cells.

$$\text{Correction factor} = \frac{2 \times 10^6}{\text{actual monocyte count.}}$$

All other calculations and plotting of results were as set out in Section A-11.5

The dose response curves and Scatchard plots are set out graphically in Figures A-12.1 and A-12.2.

FIGURE A-12.1

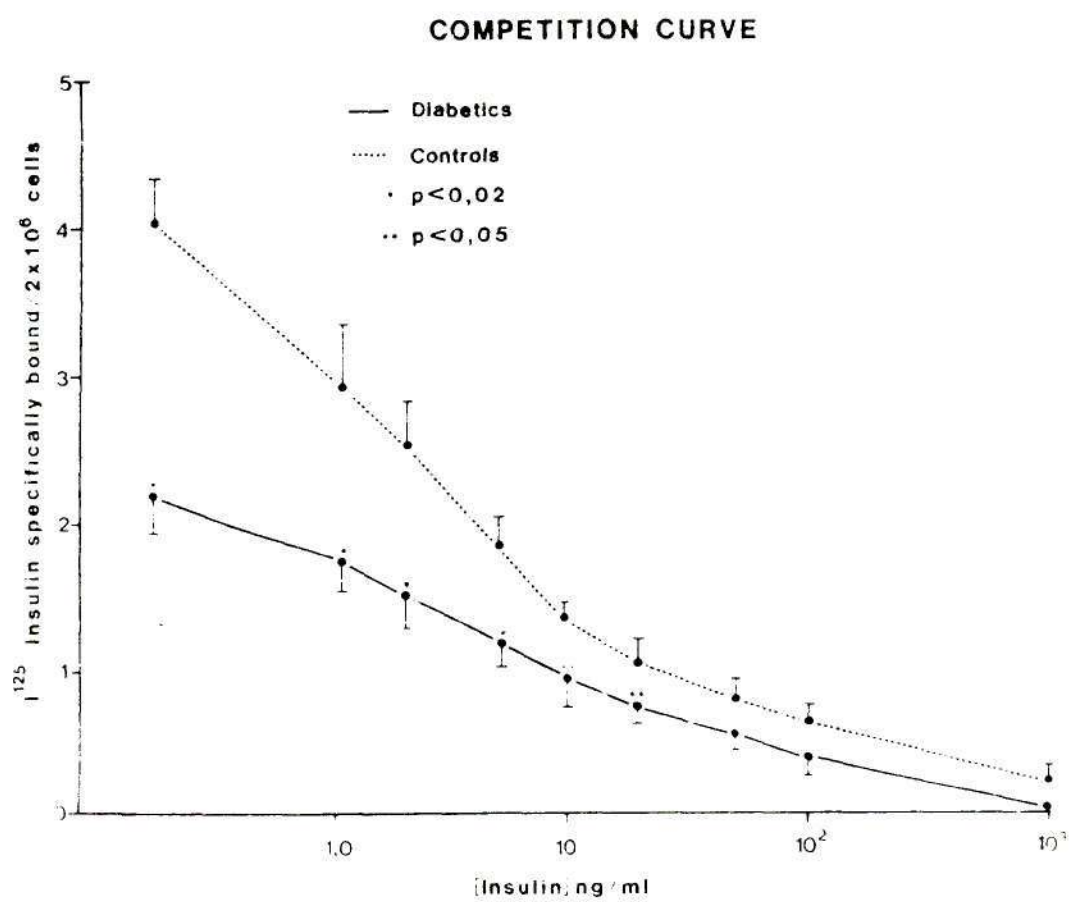
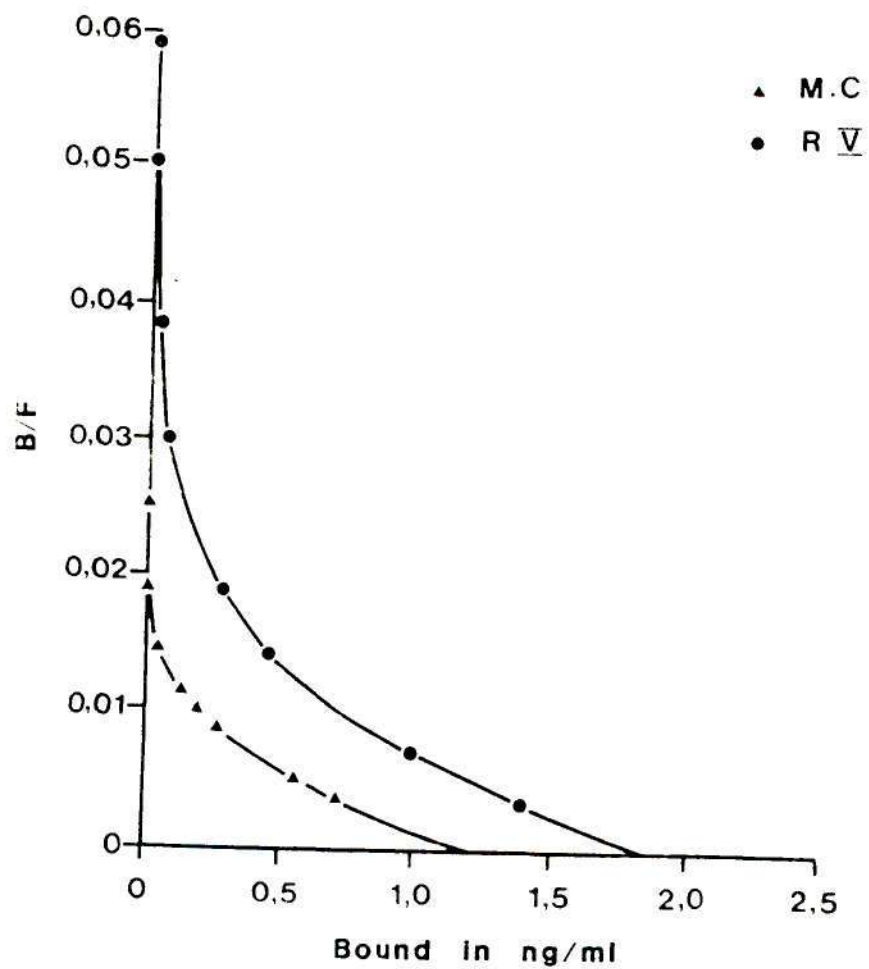


FIGURE A-12.2 A TYPICAL SCATCHARD PLOT (MONOCYTES) OF A DIABETIC ▲
AND REFERENCE SUBJECT ●



The affinity values ranged from 2.0 - 10,0 ng ml.

A-12.5 ~~Comment~~

Although monocytes are not target cells for insulin action, insulin binding to these cells has correlated with binding studies on adipocytes.

The assay was precise, the interassay of coefficient being 13%. Mean non-specific-binding was only 1,6% of total cpm (range 1,2 - 2,3%).

The radioactive insulin used was labelled in position Tyrosine A-14.

APPENDIX A-13. MEASUREMENT OF ^{125}I -INSULIN BINDING TO INSULIN RECEPTORS ON THE SOLUBILIZED ERYTHROCYTE MEMBRANE

A-13.1 Reference

Grigorescu et al (1983)

A-13.2 Principle

Assay of the insulin receptors on the solubilized RBC membrane is in principle the same as described in Section A-11.2. Insulin receptors were extracted from red cell ghosts using Triton x -100 and ^{125}I -insulin binding measured under equilibrium conditions (4°C for 18 hours).

A-13.3 Reagents

- (1) Ficol-Hypaque solution (Pharmacia, Uppsala, Sweden).
- (2) 5 mmol/l Tris (hydroxymethyl) aminoethane phosphate buffer
pH 8,0
- (3) Trasylol (10,000 KIU/ml, Bayer, West Germany).
- (4) Phenylmethyl sulphonylfluoride (Sigma Chemical Company,

Missouri, USA).

- (5) 50 mmol/l Hepes buffer (pH 7,6)
11,92 g/l hepes - n-2-hydroxy
- (6) Triton x -100 solution (Sigma Chemical Company, Missouri, USA).
- (7) Assay buffer
NaCl 0,88
Triton x -100 solution (80 μ l)
50 mM Hepes buffer (100 mls)
- (8) Unlabelled insulin (porcine) obtained from Novo Industries, Denmark was made up in 0,01 molar HCL at a concentration of 1mg/ml and stored at -20 °C. This solution was the stock standard from which all other standards were made up.
- (9) 125 I-insulin (porcine, labelled in position Tyrosine A14, from Amersham International, Buckinghamshire, U.K.) specific activity 200-300 μ Ci/ μ g.

A-13.4 Procedure

After an overnight fast 100 ml blood was withdrawn from patients and controls into tubes containing lithium heparin. The procedure for the isolation of erythrocytes which entails Ficoll-Hypaque density gradient centrifugation has been described in detail in Section A-11.4. Thereafter the compacted erythrocytes were mixed with 40 ml of 5mM Tris phosphate buffer containing 3 ml phenylmethylsulphonylfluoride (0,3%) and 10 ml trasylol (10,000 KIU/ml). The RBC's were incubated for 1 hour at 4 °C to lyse the cells in the hypotonic buffer and the membranes were spun down in a J2-21 Beckman centrifuge at 20,000 x g(4 °C) for 20 minutes. The membrane pellet was resuspended in 40 ml of 0,5 mM Tris phosphate buffer (pH 8,0) with protease inhibitors. The centrifugation

process was then repeated 4 times. The final wash of erythrocyte membrane was in Hepes buffer (pH 7,6) and the membrane pellet was resuspended in 11,5 mls of Hepes buffer and 100 μ l of Triton X-100 solution. The undissolved material was immediately separated by ultracentrifugation at 200,000 \times g at 4°C for 90 minutes. The supernatant (which contained the solubilized receptor) had a protein concentration that ranged from 1-2 mg/ml (Lowry et al, 1951).

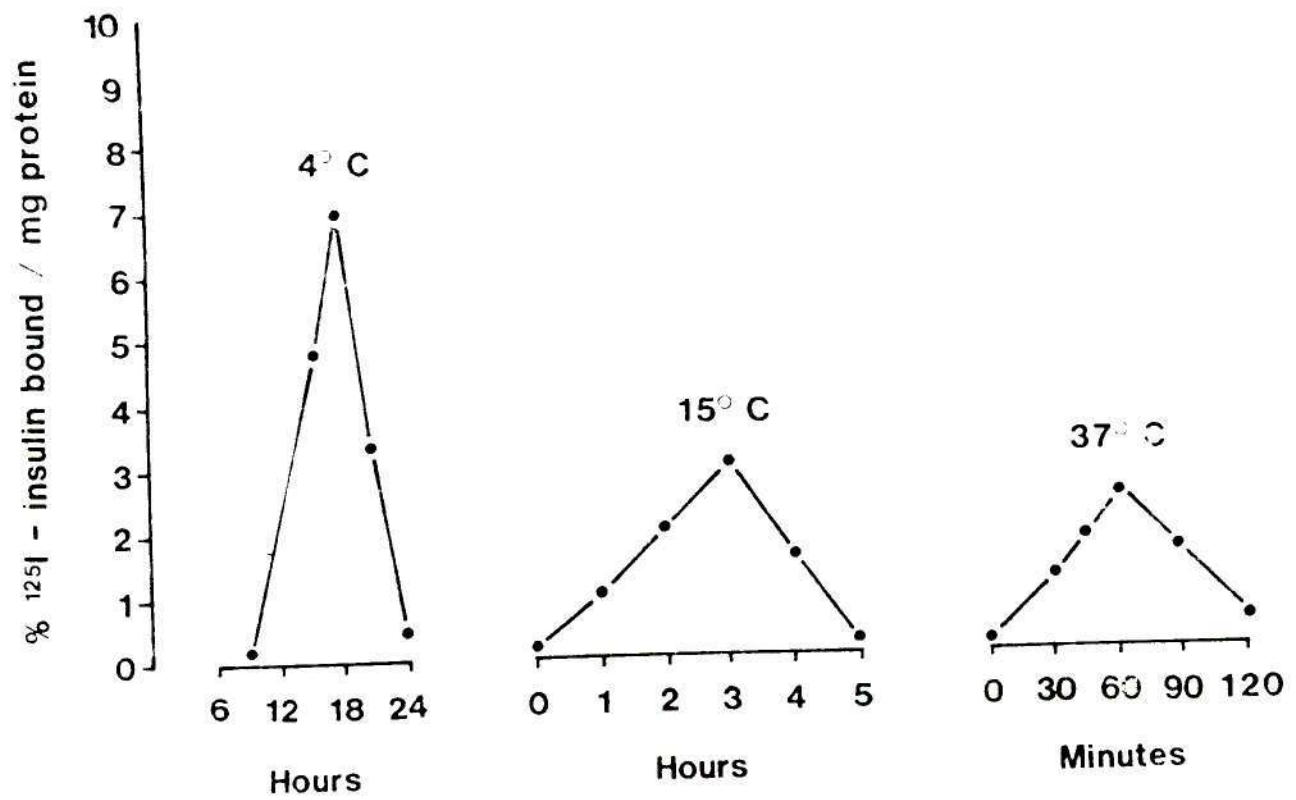
For the characterization of insulin binding, the solubilized receptor was diluted to give a protein value of 60 μ g/ml. To set up the radio-receptor assay a series of insulin standards were prepared freshly in assay buffer from the stock solution of porcine insulin (1mg/ml) as detailed in Section A-11.4.

125 I-iodoinsulin was diluted to a concentration of 0,2 ng/ml (30,000-40,000 cpm/tube).

An assay was set up in which a constant amount of membrane protein and total radioactivity was incubated with increasing concentrations of unlabelled insulin over the concentration range of 1,2 ng/ml through to 1×10^4 ng/ml as described in Section A-11.4.

A time course plot revealed that maximum binding was obtained after an incubation period of 18 hours at 4°C (Figure A-13.1). At the end of incubation 200 μ l of γ globulin and 500 μ l polyethyleneglycol (25%) were added to the assay tubes and the contents centrifuged in a Beckman microfuge (5 minutes); the supernatant was aspirated and the pellet was resuspended in 1 ml of 10% polyethyleneglycol and centrifuged for 5 minutes. The supernatant was once again aspirated and the pellet counted in a Berthold Gamma Counter. A 50 μ l portion of diluted 125 I-insulin was counted in duplicate to obtain the total counts.

FIGURE A-13.1 TIME COURSE (rbc MDMBRANE)



NSB was the counts bound to the erythrocyte membrane in the presence of 10^4 ng/ml unlabelled insulin.

Binding was specific for insulin only (Figure A-13.2)

The degradation of insulin as determined by TCA solubility was $< 5\%$ during incubation.

All assays were done in duplicate and only freshly drawn blood was used.

A-13.5 Calculations

Binding of membrane receptors was expressed per mg of protein.

Scatchard plots were drawn as described in Section A-11.5: receptor affinity was calculated as detailed in Section A-11.5.

The dose response curve and Scatchard plots are presented in Figure A-13.3 and A-13.4 respectively.

A-13.6 Comment

Insulin receptors were successfully extracted from inverted erythrocytes with the aid of a non-ionic detergent Triton X-100. These were 'true' insulin receptors in that Scatchard plots and affinity values were similar to those of erythrocytes and monocytes. Also, the receptor bound insulin with a high degree of specificity, in that there was no binding to unlabelled glucagon and binding to proinsulin was negligible.

The assay was carried out with much precision the interassay coefficient of variation being $5,3\%$. Mean non-specific binding was only $2,7\%$ (range $1,7 - 3,0\%$ of total cpm).

FIGURE A-13.2 BINDING OF INSULIN, PROINSULIN AND GLUCAGON TO THE INSULIN RECEPTOR ON THE ERYTHROCYTE MEMBRANE

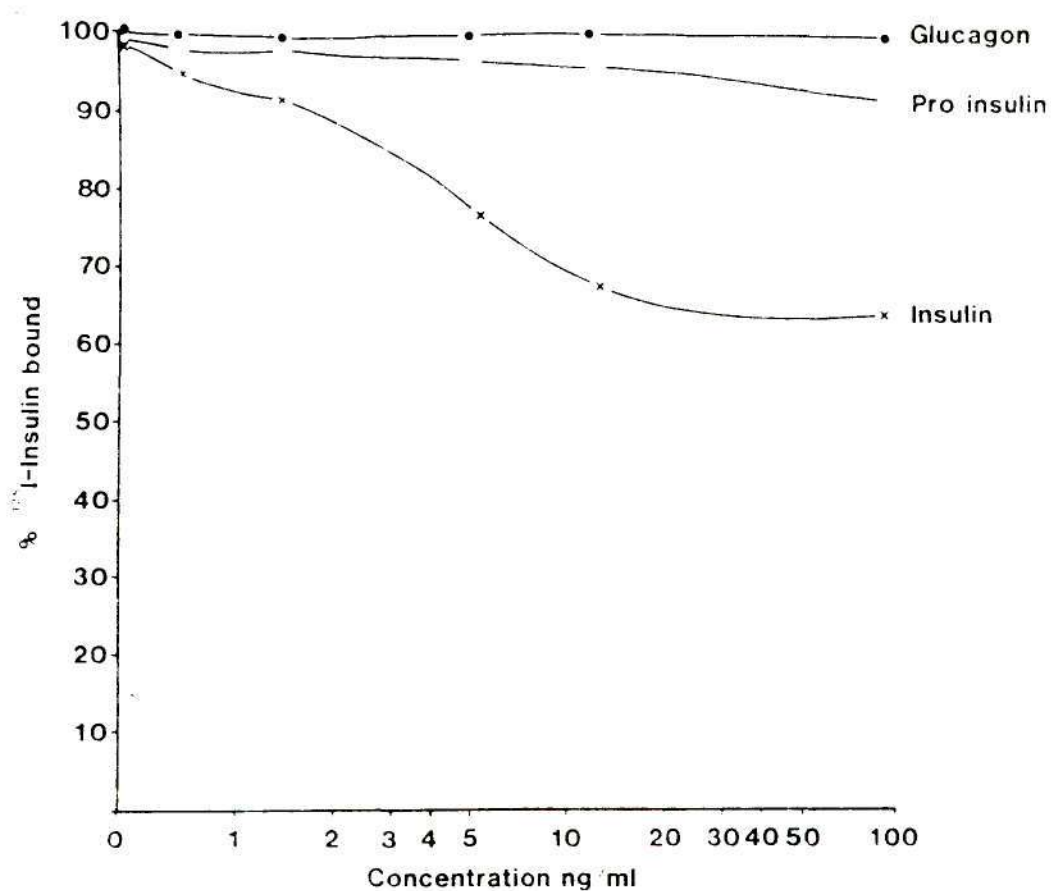


FIGURE A-13.3 COMPETITION CURVE (ERYTHROCYTE) MEMBRANE

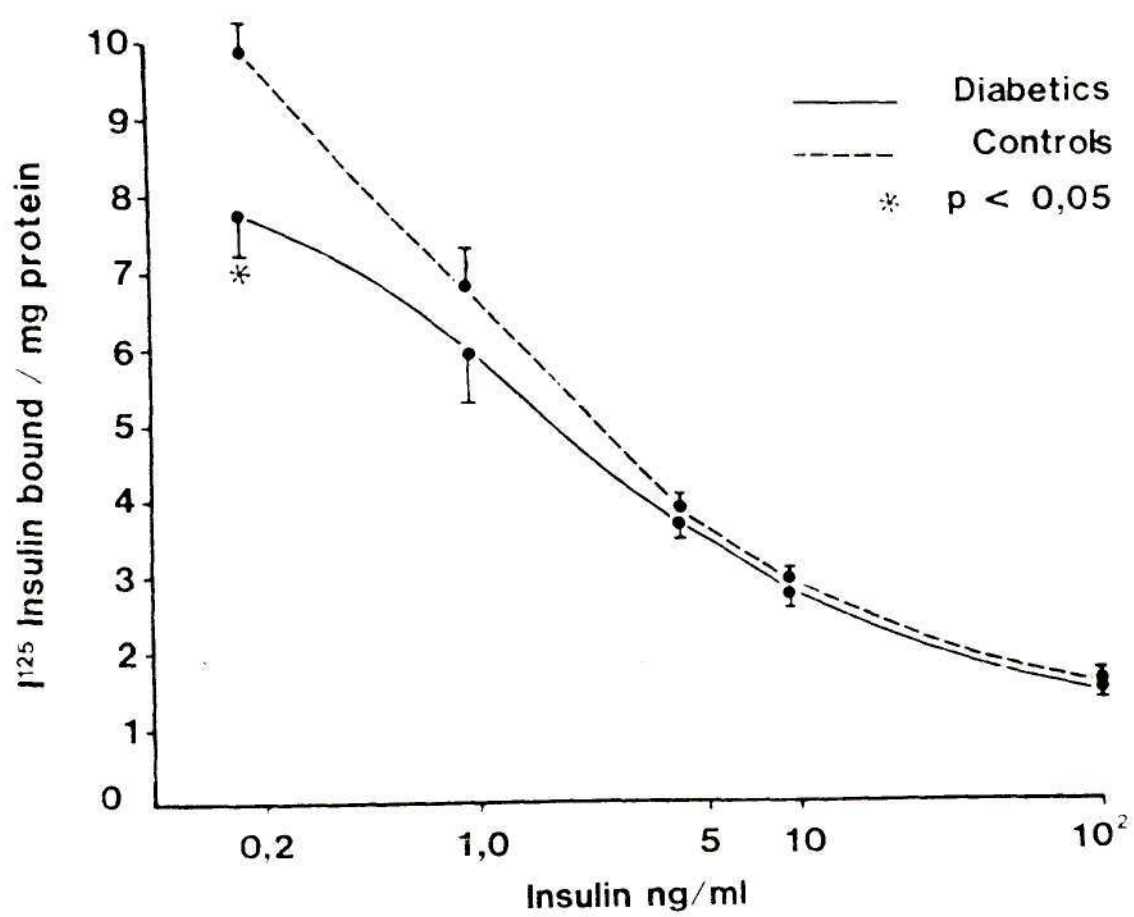
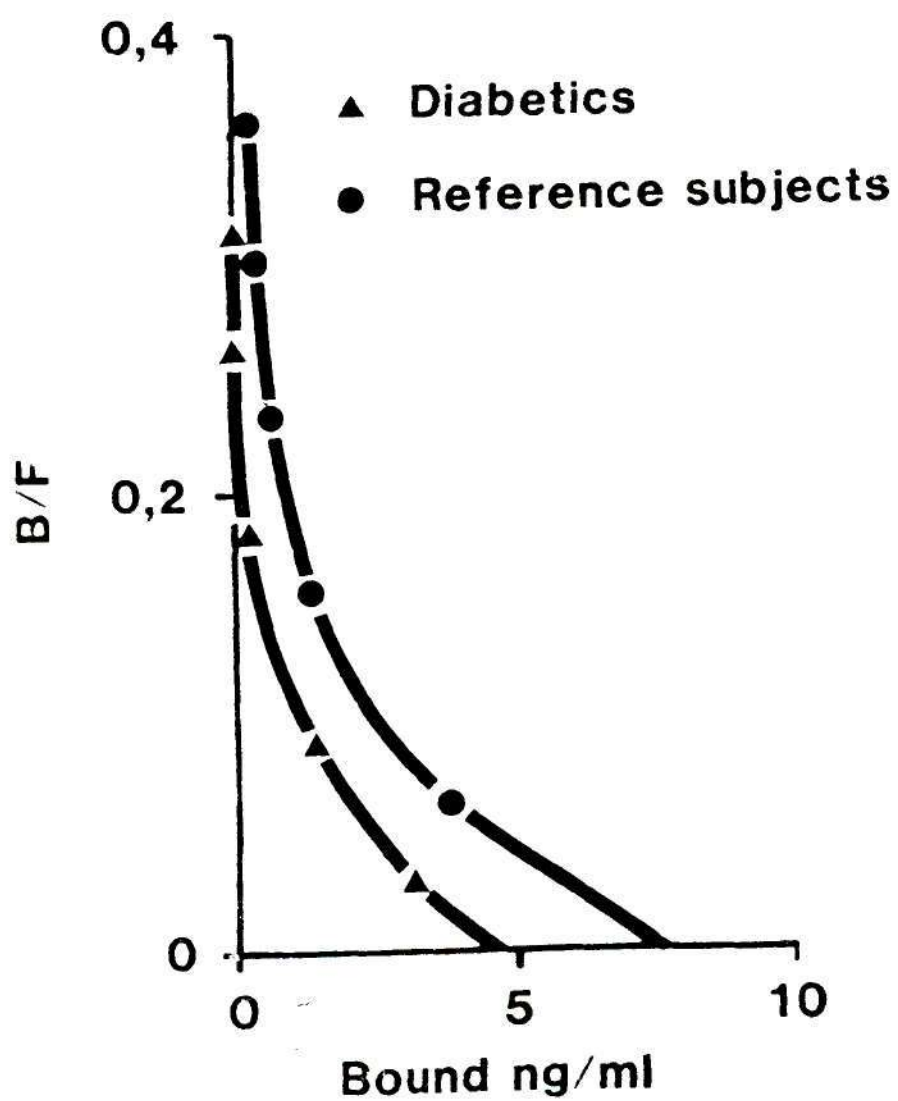


FIGURE A-13.4 SCATCHARD ANALYSIS OF 10 DIABETICS ▲ AND 10
REFERENCE SUBJECTS ● (RBC MEMBRANE)



APPENDIX A-14. MEASUREMENT OF ^{125}I -INSULIN BINDING TO INSULIN RECEPTORS ON CULTURED HUMAN FIBROBLASTS.

A-14.1 References

Rechler and Fodskalny (1976)

Gelehrter et al (1981)

A-14.2 Principle

The radioreceptor assay principle has been described in Section A-11.2.

A-14.3 Reagents

(1) Minimal Essential Medium (MEM -- alpha, Flow Laboratories, Scotland, United Kingdom).

(2) Trypsin 0,01% (Flow Laboratories, Scotland, U.K.)

(3) Soybean trypsin inhibitor 0,02% (Sigma Chemical Company, Missouri, U.S.A.)

(4) Hepes binding buffer (HBB) pH 8,0

| | |
|-------------------|------------|
| Hepes-n-2-hydroxy | 100 mmol/l |
|-------------------|------------|

| | |
|-------|------------|
| Na Cl | 1,8 mmol/l |
|-------|------------|

| | |
|-----|----------|
| KCl | 5 mmol/l |
|-----|----------|

| | |
|-------------------|-----------|
| MgSO ₄ | 12 mmol/l |
|-------------------|-----------|

| | |
|----------|-----------|
| Dextrose | 88 mmol/l |
|----------|-----------|

| | |
|-----|-------|
| BSA | 1 g/l |
|-----|-------|

(5) Phosphate buffered saline (PBS)

| | |
|-----|---------|
| KCl | 0,2 g/l |
|-----|---------|

| | |
|---------------------------------|---------|
| KH ₂ PO ₄ | 0,2 g/l |
|---------------------------------|---------|

| | |
|-------|-------|
| Na Cl | 8 g/l |
|-------|-------|

| | |
|----------------------------------|---------|
| Na ₂ HPO ₄ | 2,16g/l |
|----------------------------------|---------|

| | |
|------|---------|
| EDTA | 0,19g/l |
|------|---------|

- (6) Unlabelled insulin (porcine) obtained from Novo Industries, Denmark and made up in 0,01 molar HCl at a concentration of 1mg/ml. and deep frozen as the stock standard. .
- (7) 125 I-insulin (porcine, labelled in position Tyrosine A₄ , from Amersham International, Buckinghamshire , U.K.) specific activity 200 - 300 μ Ci/ μ g.

A-14.4 Procedure

Fibroblasts were obtained from the volar surface of the forearm by punch biopsies of the skin. The specimens were placed immediately into MEM. Thereafter monolayer cultures were maintained (in 25 cm² flasks) in MEM supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U penicillin/ml and 100 μ g streptomycin/ml, in an atmosphere of 5% CO₂ and 95% air. Cultures were split 1 to 3 and fed twice weekly. Routine cell counts were made on trypsinized preparations with a haemocytometer. All studies were performed on cultures between the 3rd and 10th passage.

Insulin binding to fibroblasts was carried out on cultures that were fed with medium 7 days prior to use. For each assay 10 flasks were utilized (each containing $\pm 1 \times 10^6$ cells). The medium was aspirated and cells in each flask were washed with 10 ml of PBS buffer. Three ml of trypsin were then added to each flask; the flasks were incubated at 37°C, for 5 minutes following which the cells were viewed under a microscope to check whether they had been detached. Five ml of soybean trypsin inhibitor were next added to all flasks to stop the process of trypsinisation and the contents of all flasks pooled and centrifuged for 2 minutes (Beckman J2-21, 400 x g.) The supernatant was aspirated and the cell pellet was resuspended in 10 ml of PBS and once again centrifuged for 2 minutes; the supernatant aspirated and the pellet

was suspended in 10 ml of HBB, following which cells were spun down at 20°C for 2 minutes at 400 \times g. The final fibroblast pellet was reconstituted in 4-5 ml of HBB.

A series of insulin standards were prepared freshly in HBB from the stock solution of porcine insulin (1mg/ml). Details of dilutions have been described (Section A-11.4)

125 I-iodoinsulin was diluted to a concentration of 0,2 ng/ml (30,000 - 40,000 cpm).

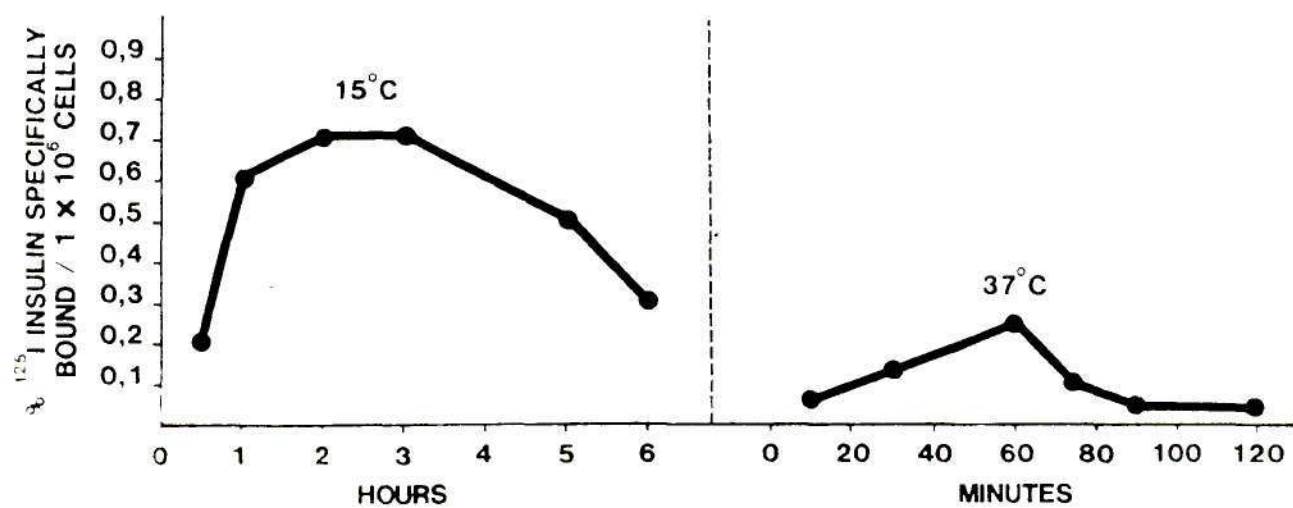
The assays were set up by adding 400 μ l of cell suspension and a fixed amount of radioactivity with increasing concentrations of unlabelled insulin over the concentration range of 1,2 ng/ml to 1×10^5 ng/ml. Details and the sequence and volume in which the various components were added to the tubes and given in Section A-11.4.

The tubes after gentle mixing were incubated at 15°C for 3 hours (Figure A-14.1). At the end of the incubation period, 200 μ l portions from each tube were withdrawn and added to microfuge tubes containing 200 μ l of HBB and centrifuged for 2 minutes. The supernatant fluid was removed by aspiration and the radioactivity in the cell pellet was counted in a Berthold multihead gamma counter. A 50 μ l portion of the diluted 125 I-insulin was also counted to obtain the total counts.

NSB was taken as the counts bound to fibroblasts in the presence of 10^5 ng/ml unlabelled insulin.

All assays were carried out in duplicate. The degradation of insulin as determined by TCA solubility was < 5% during the incubation period.

FIGURE A-14.1 TIME COURSE (FIBROBLASTS)



A-14.5 Calculations

Fibroblasts were counted with the aid of a haemocytometer. The count was expressed 1×10^6 cells.

Binding characteristics and affinity values were worked out according to formulae set out in Section A-11.5.

The dose response curve and Scatchard plots are depicted in Figures A-14.2 and A14.3 respectively.

A-14.6 Comment

All assays were carried out under standardized conditions in that cultures were fed 7 days prior to assay.

A time course revealed that optimum binding was attained at 15°C after 3 hours incubation.

Fibroblast insulin receptors bound insulin with a high degree of affinity; Scatchard plots were similar to that of erythrocytes and monocytes.

The mean NSB for the fibroblast assay was low - 0,28% (0,2 - 0,4% of total cpm) and the interassay coefficient of variation was 12%.

FIGURE A-14.2 COMPETITION CURVE (FIBROBLASTS)

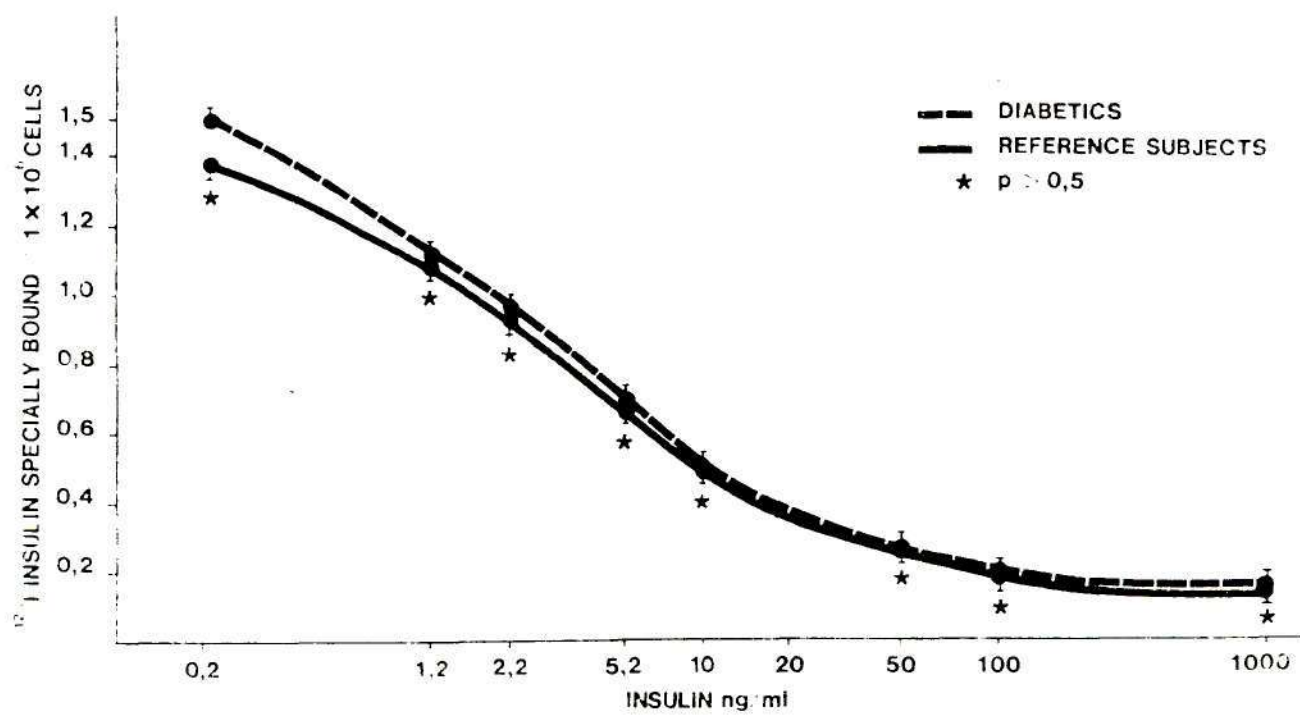
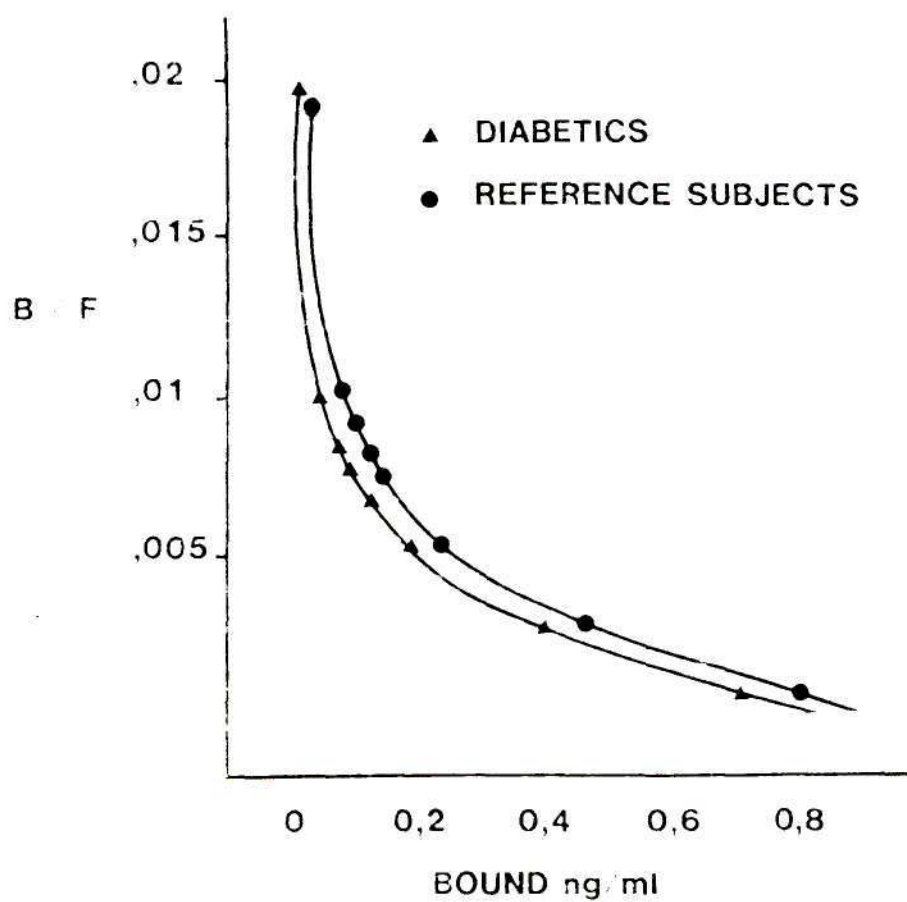


FIGURE A-14.3 SCATCHARD PLOTS OF 4 DIABETICS ▲ AND 4 REFERENCE
SUBJECTS ●



APPENDIX B

STATISTICAL ANALYSES

The raw data for each set of parameters to be analysed was subjected to a Goodness of Fit test to determine whether the data was normally distributed (parametric).

If the data was normally distributed, differences were analysed by the students t test (paired and unpaired).

Non parametric data was analysed by the Mann Whitney U test (corrected for ties). Spearman's rank correlation coefficients (r) were calculated to quantitate the correlation between relevant variables.

Significance was defined at the 5% level ($p < 0,05$) using 2-tailed statistics.

Computations were undertaken on a Sirius Computer (Sirius Systems Technology, California, USA)

Data in this thesis is expressed as mean \pm SEM unless stated otherwise.

APPENDIX C

CONSENT

Informed consent was obtained from all participants (patients and control subjects) in this study. Provocative tests (OGTT, IVGTT, glucagon, tolbutamide, arginine and insulin tolerance tests) were approved by the Ethics Committee of the Faculty of Medicine, University of Natal.

APPENDIX D

UNITS USED

In the present study the SI system was not strictly adhered to in the reporting of results. The reason for this was to facilitate comparisons between results obtained in the present study and that of previous workers.

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