# NON-INSULIN-DEPENDENT DIABETES

# IN YOUNG INDIANS:

# A CLINICAL AND BIOCHEMICAL STUDY

BY

ISHWARLAL JIALAL

SUBMITTED IN PARTIAL FULFILMENT OF REQUIREMENTS FOR THE DEGREE OF DOCTOR OF MEDICINE IN THE DEPARTMENT OF CHEMICAL PATHOLOGY, UNIVERSITY OF NATAL. (1982)

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# A C K N O W L E D G E M E N T S

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# P R E F A C E

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These studies represent original work by the author and have not been submitted in any form to another University.

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 which were relevant to this thesis were published:

1) Non-insulin-dependent Diabetes Mellitus with Early Onset in Blacks and Indians. A.C. Asmal, B. Dayal, I. Jialal, W.P. Leary, M.A.K. Omar, N.L. Pillay and F.T. Thandroyen. South African Medical Journal (1981) 6= : 93 - 96.

2) The insulin and glucose response to an oral glucose load in non-insulin dependent diabetes in the young.
I. Jialal, S.M. Joubert, A.C. Asmal, N. Jenkins. South African Medical Journal (1982) 61 : 351 - 354.

3) Cholesterol, triglycerides and high-density lipoprotein cholesterol levels in non-insulin dependent diabetes in the young.
I. Jialal, S.M. Joubert and A.C. Asmal. South African Medical Journal (1982) 61 : 393 - 395.

4) Serum $beta_2$-microglobulin estimation as an indicator of the glomerular filtration rate.
I. Jialal, B.C. Nathoo, S. Bejai, S.M. Joubert. South African Medical Journal (1982) 61 : 953 - 954.

5) Vascular complications in non-insulin-dependent diabetes in the young.

I. Jialal, N.H. Welsh, S.M. Joubert, M.C. Rajput. South African Medical Journal (1982) 62 : 155 - 157.

6) The effect of oral hypoglycaemic agents on lipid levels in non-insulin-dependent diabetes in the young.

I. Jialal and S.M. Joubert. South African Medical Journal (1982) 62 : 348 - 349.

7) Cortisol, glucagon and growth hormone responses to oral glucose in non-insulin-dependent diabetes in the young.

I. Jialal and S.M. Joubert. South African Medical Journal (1982) 62 : 549 - 552.

8) Fasting plasma glucose and glycosylated haemoglobin levels in the assessment of diabetic control in non-insulin-dependent diabetes in the young.

I. Jialal, S.M. Joubert and D. Kendall. South African Medical Journal (1982) 62 : 889 - 891.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACTH | Adrenocorticotrophic hormone |
| ADP | Adenosine diphosphate |
| Apo A-1 | Apoprotein A-1 |
| Apo B | Apoprotein B |
| Apo C-11 | Apoprotein C-11 |
| ATP | Adenosine triphosphate |
| BMJ | British Medical Journal |
| Bo | Maximum binding |
| BSA | Bovine serum albumen |
| C | Centigrade |
| ECG | Electrocardiogram |
| EDTA | Ethylene diaminotetra acetic acid |
| FFA | Free Fatty Acids |
| GFR | Glomerular Filtration Rate |
| GLI | Glucagon-like immunoreactivity |
| GH | Growth Hormone |
| G | Gram |
| g | Gravitational Force |
| g | gram |
| GTT | Glucose tolerance test |
| $HbA_1$ | Glycosylated haemoglobins |
| HMG Co A Reductase | 3 hydroxyl-3 methylglutaryl coenzyme A reductase |
| HGH | Human growth hormone |
| HDL | High density lipoprotein |
| HLA | Human leucocyte antigen |
| IDDM | Insulin dependent diabetes mellitus |
| IDL | Intermediate density lipoprotein |
| IGT | Impaired glucose tolerance |
| IRG | Immunoreactive glucagon |
| ILA | Insulin-like activity |
| IRI | Immunoreactive insulin |
| IU | International Unit |
| JOD | Juvenile onset diabetes |
| Kg | Kilogram |
| $K_1$ | Glucose disposal constant |
| KBq | Kilo Becquerel |
| LCAT | Lecithin cholesterol acyl transferase |

| | |
|---|---|
| LDL | Low density lipoprotein |
| L | Litre |
| LPL | Lipoprotein lipase |
| Malonyl Co A | Malonyl Coenzyme A |
| mg | milligram |
| mℓ | millilitre |
| mmol | millimole |
| mm | millimetre |
| M | Molar |
| MBq | Mega Becquerel |
| MODY | Maturity onset diabetes in the young |
| $MgCL_2$ | Magnesium chloride |
| MSI | Modified Seltzer insulinogenic index |
| µℓ | microlitre |
| PBS | Phosphate buffered saline |
| PEG | Polyethylene glycol |
| pg | picogram |
| $NAD^+$ | Nicotinamide adenine dinucleotide |
| $NAD^+ + H^+$ | Reduced nicotinamide adenine dinucleotide |
| ng | nanogram |
| NIDDM | Non-insulin dependent diabetes mellitus |
| NIDDY | Non-insulin dependent diabetes in the young |
| NSB | Non specific binding |
| NRS | Normal rabbit serum |
| PLC | Proinsulin-like components |
| SDS | Sodium dodecyl sulphate |
| TCA | Trichloracetic acid |
| TRIS | Tris (hydroxymethyl) aminomethane |
| VLDL | Very low density lipoprotein |
| WHO | World Health Organisation |

# INTRODUCTION

One of the earliest recorded references to polyuria is found in the Papyrus Ebers (1500 BC) and much later the occurrence of "honey urine" was noted by an ancient Hindu physician, Sushrutha, in old Indian Sanskrit (400 BC). However, the first good clinical description of the disease is ascribed to Celsus, although the name "diabetes" was introduced by Aretaeus of Cappadocia. The body of knowledge which has accumulated since these early recordings to the present state of the art reflects a most impressive sojourn, punctuated by many milestones, each adding impetus to future attempts in a relentless endeavour to unravel the aetiopathogenesis of this common malady. However, this "sweet evil" (diabetes) remains an enigma in many ways.

There is little doubt today that there are 2 major types of diabetes: juvenile onset diabetes, presently known as insulin-dependent diabetes mellitus (IDDM) and maturity onset diabetes, referred to as non-insulin dependent diabetes mellitus (NIDDM). In NIDDM aggregation of HLA types, evidence of cell mediated immunity and the presence of circulating islet cell antibodies, which are characteristically associated with IDDM, are not found. There is also a vast difference in concordance of diabetes in the co-twins between the two types of diabetes suggesting that a different mixture of genetic and environmental factors is operative in the pathogenesis of these two types of diabetes.

In 1960, Fajans and Conn drew attention to the existence of a form of diabetes with an onset before the age of 35 years. Their patients showed a substantial improvement in glucose tolerance when treated with an oral hypoglycaemic agent, tolbutamide. Subsequent to this report numerous studies from various parts of the world confirmed this entity of non-insulin dependent diabetes in the young (NIDDY) in White Caucasians. There are, however, several different syndromes

presenting as mild carbohydrate intolerance in the first two to three decades of life.

The classical form of NIDDY is a mild non-insulin requiring form of diabetes in which the disorder is inherited as a dominant trait; there is little progression of glucose intolerance, if any, with time, and the diabetes is rarely accompanied by vascular complications. This subtype of diabetes is referred to as MODY (maturity onset diabetes in the young) and thus constitutes a subset under the broad umbrella of NIDDY. However, recently compelling evidence for heterogeneity within MODY has been presented. This evidence is based on the prevalence of certain HLA antigens, insulin responses to oral glucose, occurrence of vascular complications, progression of hyperglycaemia to the stage of insulin requirement and failure to demonstrate autosomal dominant inheritance in some families studied.

In the South African Indian population which has a high prevalence of diabetes, Campbell was the first to draw attention to NIDDY in Indians more than two decades ago. Since this initial report, nobody has really studied NIDDY in any depth in South Africa and certainly not in the Indian population.

NIDDY in the local Indian population is of particular interest for the obvious reason that diagnostic and management problems arise daily in a population with a high prevalence of non-insulin dependent diabetes. It is vital that the clinical features, endocrine and associated biochemical aberrations be known in detail if this condition is to be managed appropriately and adequately. A study of these aspects therefore became the primary task of this thesis. To pre-empt any challenge that patients were not really diabetic, the strict criteria of the W.H.O. for the diagnosis of diabetes were chosen. It should therefore be borne in mind throughout this study that a group of rather

severe diabetics were selected by design. The patients studied represent the rather extreme end of the spectrum. But, in the event, this selection proved advantageous in that it covered an unstudied part of the spectrum and some light could be shed on the natural history of the disorder.

In the long term the purpose was to prepare the ground for what must become the thrust of future studies, namely the biochemical pathogenesis of NIDDM. If it is true that some forms of NIDDY are inherited dominantly, existing techniques should make it possible to identify a gene(s) locus and if this is done the biochemical basis of this disorder must be identifiable. In the present study direct examination of these aspects were not undertaken, but an attempt was certainly made to pinpoint those biochemical abnormalities which are perhaps primary or central to the whole disorder.

# CHAPTER 1

# THE CLINICAL PRESENTATION OF NON-INSULIN DEPENDENT DIABETES IN THE YOUNG

## 1.1 DEFINITION OF DIABETES

Disease states, as a rule, are categorised in terms of aetiology, pathogenesis, clinical presentation and so forth and not defined. However, despite the enormous investigational effort that has gone into the study of diabetes, the goal of the categorisation of diabetes in classical terms remains distant. Historically, the outstanding feature of diabetes is, of course, the abnormality in glucose homeostasis and the associated polyuria. But, diabetes is much more than an aberration in glucose homeostasis and thus a need has arisen to convey what is understood when the term diabetes is used. Hence the attempts to define the condition.

Many have attempted to define diabetes. None of the definitions are satisfactory. However, as a starting point, the definition of primary diabetes in the textbook Joslin's Diabetes Mellitus (1971) has merit. This definition has it that: "Diabetes mellitus is a chronic, hereditary disease characterised by an abnormally high level of glucose in the blood and the excretion of that sugar in the urine. The basic defect is an absolute or relative lack of insulin which leads to abnormalities of metabolism, not only of carbohydrate but also of protein and fat." To this may be added that, with increasing duration, structural abnormalities supervene in a variety of tissues which manifest as the so-called "complications". No doubt, in time, many more qualifications will be added.

## 1.2 CLASSIFICATION OF DIABETES

The classification and diagnosis of diabetes and glucose intolerance

has undergone a reappraisal by the National Diabetic Data Group (1979) of the National Institutes of Health (Maryland). Primary diabetes can be broadly classified into 2 major types: Type 1, Insulin Dependent Diabetes Mellitus (IDDM) or Juvenile Onset Diabetes, and Type 2, Non-Insulin Dependent Diabetes (NIDDM), or Maturity Onset Diabetes. The Insulin Dependent Diabetic (IDDM) is usually characterised by severe and pathognomonic changes in the pancreatic islets, by an eventual absolute deficiency of endogenous pancreatic insulin secretion, insulinopaenia, abrupt onset, proneness to ketosis and dependency on daily insulin administration for the remaining period of life. Classically, IDDM is first recognised during childhood or adolescence, but it can occur at any age.

Non-insulin dependent diabetes mellitus is quantitatively the commonest type of primary diabetes throughout the world. It usually presents after the age of 40 with an insidious onset. Patients with NIDDM are not insulin dependent or ketosis prone, although insulin therapy may be required for correction of symptomatic or persistent hyperglycaemia. Obesity is present in 60 to 90 percent of patients and serum insulin levels may be normal, elevated or depressed. In NIDDM, aggregation of HLA types, evidence of cell mediated immunity and presence of circulating islet cell antibodies are not found. A different genetic basis is also reflected by a much more frequent familial pattern of occurrence than in IDDM; the strong genetic predisposition of NIDDM is manifested by an almost 100% concordance of diabetes in identical twins. (Tattersall and Pyke, 1972).

A comparison of IDDM and NIDDM is presented in Table 1.1 (Craig 1981).

In this review the emphasis will be on NIDDM, more specifically, NIDDM in the Young.

TABLE 1.1

COMPARISON OF THE TWO COMMON CLINICAL TYPES OF DIABETES MELLITUS

| COMPARISON FACTOR | IDDM INSULIN-DEPENDENT | NIDDM NON-INSULIN DEPENDENT |
|---|---|---|
| Former terminology | Juvenile-onset | Adult or maturity-onset |
| Age at onset | Most often childhood, may occur in adults | Frequently over 40 years but may occur in children |
| Symptoms | Polydipsia, polyphagia, polyuria | May be asymptomatic or symptomatic |
| Type of symptom onset | Often abrupt | Usually gradual (if symptomatic) |
| Physical habitus at time of onset | Usually thin | Usually obese |
| Family history of diabetes | Uncommon | Common |
| HLA type association | No | Yes |
| Islet cell antibodies | Frequently present at onset | Not present |
| Islet cell pathology | Insulitis at onset; Marked reduction in beta cells | Lesser reduction in beta cells |
| Stability of blood glucose levels | Wide fluctuations | Less marked fluctuations |
| Ketosis | Frequent | Uncommon |
| Vascular complications and neuropathy | Yes | Yes |
| Endogenous insulin | Negligible | Present in varying amounts |
| Insulin therapy | Essential | Required by some |

## 1.3 NON-INSULIN DEPENDENT DIABETES IN THE YOUNG

NIDDM in the young has probably been known to doctors for a very long time, but it seems to have evoked little interest as a field of study, and hence publication, until relatively recent times. Tattersall (1974) quotes an articles in the B.M.J. dating back to 1907, in which the occurrence of mild non-ketotic diabetes in youngsters in the tropics were reported. In a following communication (Tattersall, 1976) he stated that the first reference, historically, to what was labelled maturity onset diabetes in the young, may have been in Rollo's "An Account of Two Cases of Diabetes Mellitus" (1798) in which a father, two children and a grandchild with diabetes of a mild type were described. The problem, as Tattersall (1976) pointed out, is that reports antedating 1916 did not command blood sugar measurements; without blood glucose values, mild diabetes cannot be distinguished from renal glycosuria which may also be dominantly inherited. Graham (1921) and Joslin (1924) described patients diagnosed at 9, 11 and 20 years of age with unequivocal biochemical evidence of diabetes who could be satisfactorily controlled for at least 5 years by dietary measures alone. However, a paper by Cammidge (1928) requires special mention. Not only did he report cases of NIDDM in the young with strong familial backgrounds, but he also presented strong evidence for suggesting dominant inheritance in some cases. This report was destined to collect dust, despite the pioneering work of Cammidge in studying the inheritance of diabetes.

Nothing of consequence appeared in the literature until Fajans and Conn (1960) published the results of the response of mild diabetes to tolbutamide therapy in 14 relatively young patients. Their interest was clearly the response to therapy rather than the diabetic state. The only relevant clinical information given was that the patients

were non-obese, that the diabetes was asymptomatic and that their ages ranged from 11 to 35 years at the time when hyperglycaemia was diagnosed in response to glucose tolerance testing; the only family history mentioned was that there was a strong family history in 13 of the 14 patients. Yet, this publication became a key reference. It also seems as though the age of the oldest patient at the time of diagnosis (35 years) became a bench mark - the magical transition time from young to mature - even though Fajans himself was at pains to emphasise in his Banting memorial lecture (Fajans et al, 1978) that age of onset refers to age of recognition or diagnosis and not to the age of onset of the metabolic abnormality. Hence age of onset should not in itself be a criterion for classification. Be this as it may, from 1960 onwards publications on NIDDM in the young appeared with increasing frequency in the literature. (Campbell, 1960; Lister, 1966; Burkenholder et al, 1967; Johansen and Lundbaek, 1967; Paulsen et al, 1968; Sisk, 1968; Chiumello et al, 1969; Rosenbloom et al, 1972; Johansen, 1973; Tattersall, 1974; Thorell et al, 1975; Pond, 1975; Tulloch and Adamson, 1976; Dorner and Mohnike, 1976; Hager, 1977; Faber et al, 1978; Barbosa et al, 1978; Savage et al, 1979; Mutch and Stowers, 1980; Kobberling et al, 1980; Panzram and Adolph, 1981; Kesevan et al, 1981). This list is by no means exhaustive.

Today there can be no doubt as to the existence of NIDDM in the young. But what is far from clear is whether NIDDM in the young constitutes a heterogeneous or homogeneous entity; whether it differs in natural history from NIDDM in mature patients; whether it is biochemically distinct or identical with NIDDM in the mature; its prevalence in the diabetic population or in the population at large. As already pointed out, clinicians have probably been aware of NIDDM in the young for a long time, but somehow the accumulated literature has tended to focus

on circumstantial aspects of interest: criteria for patient selection have differed or were related to the purpose of study; methods, techniques and approaches differed; nomenclature differed. Thus, a rather confusing picture has emerged.

In the following sections an attempt will be made to define some of the unresolved issues.

### 1.3.1 Non-Insulin Dependent Diabetes in the Young with Dominant Inheritance. Maturity Onset Diabetes in the Young (Mody)

#### 1.3.1.1 Introduction

In his Banting memorial lecture Fajans stated that they used the term maturity-onset-diabetes in the young since 1964 (Fajans et al, 1978) having recognised that there was a strong familial association in the histories of those young patients with a mild form of diabetes. Quite apart from the report of Cammidge (1928) which has already been mentioned, it was a paper by Tattersall (1974) in which it was clearly established that a familial form of mild diabetes existed which was manifest at an early age and was dominantly inherited.

In the following year, while Tattersall was working with Fajans at Ann Arbor, the abbreviation of MODY (maturity onset diabetes in the young) was first used in publication (Tattersall and Fajans, 1975). In this publication they differentiated JOD from MODY and stressed once again the high familial aggregation of diabetes in the families of patients with MODY. Although they were careful to emphasise that to some extent the categorisation of a patient as having MODY was arbitrary, the term MODY came to be associated with a specific sub-type of diabetes in the minds of many workers.

Tattersall and Fajans (1975) used only two criteria for selection:

diagnosis of diabetes before the age of 25 years; normalisation of fasting hyperglycaemia, if present, for more than two years without the use of insulin. In fact, a retrospective diagnosis. However, as pointed out, subsequent workers broadened the criteria to include the demonstration of dominant inheritance and often the age of diagnosis was ignored. Basically, what Tattersall and Fajans (1975) studied, in present day terminology, were patients with non-insulin dependent diabetes, usually mild and non-ketotic, with an age of onset or diagnosis in childhood, adolescence or early adult life. Nevertheless, when other workers added the mode of inheritance as a selection criterion, the literature on non-insulin dependent diabetes in the young tended to polarise into the 'subtype' MODY as characterising a group with mild diabetes and dominant inheritance and those with non-insulin dependent diabetes in the young (NIDDY) in whom the mode of inheritance was not, or could not, be determined.

Quite clearly, these circumstances complicate both a review of the literature and the study of the disorder as well as the choice of designation. The sensible approach would be to use the generic term NIDDY for all those manifesting with non-insulin dependent diabetes in the young and qualify, with dominant inheritance, where the need arises. This designation will be followed.

#### 1.3.1.2 NIDDY with Dominant Inheritance

As already stated, Tattersall (1974) really placed NIDDY with a dominant mode of inheritance on the map. Characteristic clinical features were the patients' independence of insulin therapy, the co-existence of renal glycosuria in 2 of his 3 families, the rarity of complications and direct transmission through three or more

generations. His evidence in support of Mendelian dominant inheritance was based on a study of 3 families at King's College Hospital, London. In each case there was a strong family history of diabetes with at least 10 affected relatives. The phenotypical manifestations of diabetes in the 20 relatives, who were still alive, were similar to those in the propositii. Juvenile diabetes (IDDM), as presently defined, was never seen and significant weight loss was never a feature of the diabetes. Of the 23 patients with an age of onset before 30 years of age, 17 were females. Tattersall's (1974) reasons for proposing dominant inheritance were based on the following considerations: in each family there was direct transmission through at least 3 generations; almost every diabetic had a diabetic parent and the ratio of affected : unaffected children of diabetic parents was 1 : 1. In a further study of the inheritance of NIDDY carried out in Ann Arbor, Michigan on 26 propositii with NIDDY, 85% had a diabetic parent usually with a similar diabetic phenotype to that of the children (Tattersall and Fajans, 1975). Furthermore, 46% of families revealed direct vertical transmission of diabetes through 3 generations and 53% of siblings tested had 'latent' diabetes. They concluded that, although their findings were compatible with dominant inheritance of NIDDY, multifactorial inheritance could not be excluded conclusively. Further reports on NIDDY with dominant inheritance appeared subsequently. (Tulloch and Adamson, 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978; Faber et al, 1978; Kobberling et al, 1980; Panzram and Adolph, 1981). A low renal threshold for glucose was present in 2 of the 3 families described by Tattersall (1974) and 3 of the 5 families described by Kobberling et al (1980). Barbosa (1978) in studying his two kindreds with NIDDY recorded an association with HLA-A3 and BW15 in one of these two pedigrees. Other workers (Nelson and Pyke, 1976; Faber et al, 1978; Fajans et al, 1978; Panzram and

Adolph, 1981) could not confirm this association in their patients which would suggest some heterogeneity in inheritance. Cloutier and Fajans (1979) presented further evidence for heterogeneity in the inheritance of NIDDY in some families with NIDDY by finding that some members do not conform to the typical autosomal dominant pattern of inheritance.

These reports all stress the fact that the patients are non-insulin dependent and that there is strong evidence for autosomal dominant inheritance. But as Fajans et al (1978) already pointed out, there was evidence of heterogeneity, based on HLA type, insulin responses to glucose loading and incidence of vascular complications even within NIDDY patients with apparent autosomal dominant inheritance of the disorder. The concept that one is simply dealing with a dominantly inherited disorder, and all that it implies in terms of the one gene, one defect concept, is probably not tenable.

### 1.3.2. NIDDY excluding those with Dominant Inheritance

#### 1.3.2.1 Introduction

Having discussed reports on NIDDY patients in whom there seemed good evidence in support of dominant inheritance of the disorder, the evidence for the occurrence of NIDDY in patients in whom such a mode of inheritance was not demonstrated, will be examined. Any such discussion should be prefaced by the statement that many reports in which NIDDY was described without apparent dominant inheritance appeared before Tattersall (1974) put forward rather cogent evidence for this mode of inheritance. To have produced the evidence put forward by Tattersall (1974) required a single-minded purpose and rather special circumstances. Dominant inheritance in its pure form can easily be lost in subsequent

generations if the gene(s) is (are) diluted in a population free from the genotype, due to the process of genetic segregation at haploid division. The problem of tracing or recognising such a genotype when penetrance is incomplete or when a different genotype can give expression to a similar phenotypical manifestation is, of course, compounded immeasurably. Thus, as far as NIDDM is concerned, some familial aggregation has never been in doubt. These considerations should always be borne in mind when studying reports on NIDDY.

### 1.3.2.2 The Period Prior to 1974

Fajans et al (1973) drew attention to the fact that since their original report (Fajans and Conn, 1960) they had published a further four papers by 1966 in which attention was drawn to the occurrence of asymptomatic, latent or 'chemical' diabetes in children and young adults when such patients were submitted to glucose tolerance testing. Such patients may exhibit a non-progressive course of carbohydrate intolerance of the 'maturity onset-type' of diabetes. Since 1966, at least a further eight reports appeared which also indicated that asymptomatic diabetes can be discovered in young people when they were submitted to glucose tolerance testing (Lister, 1966; Burkeholder et al, 1967; Johansen and Lundbaek, 1967; Sisk, 1968; Paulsen et al, 1968; Chiumello et al, 1969; Kahn et al, 1969; Rosenbloom, 1970). This list of reports is not complete and in the same issue in which the proceedings of the conference on chemical diabetes was published, further papers appeared on the same subject. Suffice it to point out that the annotated publications indicate a general awareness of glucose intolerance of varying degrees in young people.

When the contents of these earlier publications are studied, it is clear that Lister (1966) and Johansen and Lundbaek (1967) clearly recognised

the occurrence of diabetes in the young which was different from the classical form of IDDM and resembled the diabetes found in older or mature-onset diabetics. Both publications were in the form of case reports and simply mentioned in passing the presence or absence of a family history. Burkeholder et al (1967) were mainly concerned with establishing whether siblings of diabetic children were intolerant to glucose and did not identify the type of diabetes they were dealing with in the diabetic children. Sisk (1968), although stating in the introduction that he confirmed the findings of Burkeholder et al (1967), was essentially concerned with establishing a one hour glucose tolerance test for genetic screening purposes and added nothing to an understanding of the nature of the diabetes he was dealing with.

Paulsen et al (1968) had a different purpose: they were studying obese children and incidentally subdivided the obese group into those with a family history of diabetes and those without. Fifteen (23%) of the 66 obese children studied had definitely abnormal tolerances; positive family histories were obtained in 9 of these 15 patients. In a further study of glucose tolerance in 42 unselected obese children, 12 had chemical diabetes (28%) (Martin and Martin, 1973). A positive family history was present in 8 of these 12 patients.

In a study of 41 children with normal weight (20 with a positive family history) and 38 obese children (16 with a positive family history), chemical diabetes was detected in 12 of these 79 children, six of these 12 children were obese, whilst a positive family history of diabetes was present in 11. (Chiumello et al, 1969). They concluded from their data that the occurrence of chemical diabetes was higher in children with a family history of diabetes.

An association between children with idiopathic hypoglycaemia and diabetes has been reported (Rosenbloom and Sherman, 1966). In a series

of 26 children with idiopathic hypoglycaemia there was a family history of diabetes in 50 percent of the patients. Three patients had diabetic glucose tolerance curves. (Kogut et al, 1969). The authors suggested that their data lent some support to the proposal of Rosenbloom and Sherman (1966) that the appearance of spontaneous hypoglycaemia in infancy may be an early manifestation of diabetes mellitus. Kahn et al (1969) once again pursued the genetic approach by studying the offspring of 80 conjugal diabetic parents, but gave no information on the type of diabetes the parents suffered and indicated that the ages of the offspring included children and adults.

At the Conference on Chemical Diabetes at Ponte Vedra Beach, Florida in December 1970, Drash (1973a) reported on his experience with 19 children who had close relatives with diabetes: 15 were asymptomatic siblings of insulin-dependent diabetics and on tolerance testing 6 were considered diabetic suspects and 2 to have 'chemical' diabetes. Drash did not give details on the other 4 children except that they had close relatives with diabetes; three proved to be 'chemical' diabetics. His main concern seems to have been the apparent hyperinsulinaemic response of the children related to diabetics during tolerance testing. He did, however, make the point that those with normal glucose tolerance curves showed greater hyperinsulinaemic responses than those with impaired glucose tolerance tests. Clearly Drash (1973a) was looking at insulin release from the aetiological point of view and not inheritance or in terms of differentiating diabetic type of the relatives. Pildes (1973) did recognise the differences in insulin response in children with ketoacidosis and those which were asymptomatic or had mild symptoms, but did not comment on inheritance. Colle and Belmonte (1973), on the basis of follow-up studies in 9 children with asymptomatic hyperglycaemia, came to the conclusion that their patients did not represent a homogenous group and had little to offer in terms of elucidating the natural history

of either JOD or mature-onset type of diabetes. The report by Fajans et al (1973) really added little to the understanding of diabetes in the young inasmuch as they dealt with asymptomatic, but so-called 'chemical' diabetes, as determined by tolerance testing. Drash (1973b) merely confirmed the work of Paulsen et al (1968) on obese children and noted that those with carbohydrate intolerance achieved lesser insulin responses than those without evidence of intolerance.

The annotated literature clearly reveals the confusion which existed by the beginning of the 1970's to any serious student of diabetes in the young. With few exceptions, the workers did not direct themselves to the study of diabetes per se in the children or young adults, but were concerned with side issues or finding some phenotypical manifestation of a diabetic genotype which could guide them in studying the natural history of the disease or the inheritance of the genotype. The hugely confusing issue of 'chemical' diabetes served to add to blurred vision and only with the wisdom of hindsight can it be gleaned that the patients were a mixture of IDDM and NIDDM. The same confusion arose in respect of parents, relatives or siblings and it seems that everybody was aware that diabetes shows familial aggregations and hence has something to do with inheritance although no satisfactory model of genetic transmission could be proposed. The evidence for the existence of NIDDY as an entity was not explicitly stated and hence its mode of inheritance only half-heartedly studied. Manifestly, the time was ripe for a purposefully directed study with clearly defined objectives which would give direction. Tattersall (1974) would oblige in due course.

### 1.3.2.3. The Period Following 1974

As discussed in Para. 1.3.1.2, Tattersall (1974) provided a full description of mild diabetes in the young which was dominantly inherited. This

paper proved to be a watershed in the study of NIDDY, particularly if read in conjunction with the follow-up paper in which the condition was clearly differentiated from IDDM in the young (Tattersall and Fajans, 1975). Henceforward workers defined their patients carefully and provided well documented family histories and, where possible, the mode of inheritance was deduced. Johansen and Gregersen (1977) published their findings in a family of 53 members in which they confirmed the existence of a syndrome of mild diabetes in the young which was dominantly inherited, but they already indicated that the syndrome was not necessarily genetically homogeneous. Much interest was raised when Leslie and Pyke (1978) reported on the chlorpropamide alcohol flush recorded in NIDDY with dominant inheritance and suggested that it would act as a genetic marker in this syndrome. Unfortunately this suggestion could not be confirmed (Kobberling et al, 1980) and may mean further heterogeneity in the syndrome. As already pointed out, Fajans et al (1978) drew attention to heterogeneity in the NIDDY group and their evidence was based on a number of considerations. Even Tattersall conceded that NIDDY is probably a heterogeneous syndrome although he pleaded for retention of the designation MODY as characterising the syndrome he described in 1974 (Johnston and Tattersall, 1981).

Mention should briefly be made of two developments which greatly aided a clearer understanding of diabetic type in the period under discussion. Studies on diabetic concordance in identical twins under the auspices of the British Diabetic Association (Tattersall and Pyke, 1972; Pyke and Nelson, 1976; and Pyke, 1977) revealed that while 47 of 53 (89%) of NIDDM identical twins were concordant, only 73 of 132 (55%) of IDDM pairs were concordant. Although there is criticism of this approach on various grounds, few would doubt today that the finding not only distinguished between NIDDM and IDDM as distinctive types, but defines NIDDM as unequivocally as inherited disorder. Yet, with the exception

of NIDDY with dominant inheritance, the mode of inheritance is undetermined. But support has certainly come for IDDM as a disorder based on environmental factors rather than inheritance of a diabetic genotype on the basis of HLA typing. Tatterall et al (1979) and Johnston and Tattersall (1981) have reviewed the frequency of haplotype in IDDM and the risk associated with specific types for developing IDDM. Inasmuch as it is believed that the gene loci coding for the various haplotypes relate to the gene loci coding for some immune responses, susceptability to certain infections (presumed to be viral) is expressed. This phenomenon may find expression in islet cell destruction, a characteristic feature in IDDM. HLA type frequency in NIDDM patients are not significantly different from the non-diabetic population and this appears to be true of NIDDY as well, although Barbosa et al (1978) did report a family in which the haplotype A3-BW15 segregates with the hyperglycaemic trait and they quoted occasional similar findings.

Before summarising the information on NIDDY, the South African contribution to NIDDY must be reviewed.

## 1.4 NIDDY IN SOUTH AFRICA

The South African literature has been deliberately left for separate consideration. Not because the contribution was trivial or inconsequential, but for the reason that the reports did not enter the main stream of consideration overseas. Indeed, had notice been taken of early published findings, much of the obfuscation in the literature of the 1960's could have been more directed and clearer. But perhaps too, it may have been a result of failure to follow up the earlier descriptions in South Africa.

Campbell (1960) certainly gave a very clear clinical description in an international journal: the age of onset was under 35 years; 85% were

overweight and three-quarters women; they had classical symptoms of 'acute' diabetes, yet presented with no more than a trace of ketones in the urine in the face of heavy glucosuria, raised blood glucose levels and being untreated for considerable periods of time. In his series, NIDDY constituted 16% of Indian diabetic patients; they were clearly different from the so called "J" and "K" types, who were thin and required insulin; Campbell could control these patients with sulphonylureas. He also indicated that this patients had a strong family history, were not dependent on insulin and, in fact, proposed the term insulin-independent diabetes in the young and stated that these people, as a group, were sufficiently unusual to qualify as a valid diabetic subgroup. Compared to the rather limited description which Fajans and Conn (1960) gave of what they were studying, this was indeed powerful direction. Cosnett (1960) in responding to Campbell's letter, supported his findings although he differed on the response which could be achieved with oral antidiabetic treatment. But he also found that the prevalence of this type of diabetic was much the same in his series (15%). It is not clear why these reports made no impact. Had it been noted there would have been much greater direction in the study of NIDDY in the 1960's.

Jackson (1978a) in his excellent review of the genetics of diabetes mellitus, makes two important points. Having detailed Tattersall's (1974) clinical findings, he states categorically that in Cape Coloured and Indian patients, whom he studied, the same pattern is not necessarily folllowed, particularly insofar as vascular complications are concerned. A further point of difference was that he could not demonstrate simple dominant inheritance because of the extreme frequency of diabetes in both ethnic groups, including the frequency of diabetes in both husband and wife. Quite clearly, as outlined in his review, once both husband and wife are diabetic, genetic analysis can fit both autosomal recessive

or dominant inheritance. In discussing the epidemiology of diabetes in South Africa, Jackson (1978b) again makes the point that NIDDY is common amongst the Indian population.

More recently, Asmal et al (1981) reported on NIDDY in 43 Indian and 9 Black patients. The criteria for labelling a patient NIDDY were clearly defined and clinically they confirmed Campbell's (1960) findings. The very high familial aggregation of diabetes in the families of the Indian patients were very noticeable; neuropathy, mainly peripheral neuropathy, was the commonest complication. But in line with the experience of Jackson (1978a), vascular complications were not uncommon. Furthermore, from two surveys of a total of 2420 Indian diabetics, they established that NIDDY constituted approximately 10% of the Indian diabetic population.

It is abundantly clear that the syndrome of NIDDM in the young was recognised in South Africa, particularly in the Indian population, before Tattersall (1974) brought some direction to studies overseas. The local clinical descriptions certainly defined a broad entity which could be distinguished from NIDDM. Unequivocal evidence of dominant inheritance in these patients have, however, not been established, but good reason for this omission exists.

## 1.5 THE CLINICAL FEATURES OF NIDDY IN SUMMARY

Present evidence establishes beyond all doubt that NIDDM in the young exists. This condition differs clearly from IDDM in the young in terms of insulin requirements, proneness to ketosis, HLA association and inheritance. As far as severity is concerned, there is quite a wide spectrum varying from mild non-progressive diabetes to severe diabetes. In respect of the prevalence of complications, which will be dealt with more fully in the following section, there is also a spectrum of severity.

A small group of patients with NIDDY has unequivocal evidence of dominant inheritance and, as a rule, the diabetic manifestations are very mild. However, even within this group, there is evidence of heterogeneity.

Evidence from South Africa, mainly in Indian patients, would suggest an unusual aggregation of diabetics in the families of NIDDY patients, but its precise mode of inheritance is undetermined. It would seem reasonable to think of this group of patients with NIDDY as a broad heterogeneous group at this stage, rather than defining specific sub-groups in terms of one or other phenotypical character. Mode of inheritance in populations with a high prevalence of diabetes will obviously be complex. Even if a genotype exists which is transmitted as an autosomal dominant characteristic, it is inconceivable that this genotype will not be mixed with other genotypes which predispose to diabetes in a community with a high prevalence of diabetes. And this is also true of phenotypical manifestations such as complications. At present, it is not known whether the genotype predisposing to diabetes is necessarily associated with a genotype predisposing to vascular complications.

What is less clearly established, is information on the endocrine status of patients with NIDDY and other biochemical parameters relating to the syndrome. In following chapters, these aspects will be dealt with.

## 1.6 VASCULAR COMPLICATIONS IN NON-INSULIN DEPENDENT DIABETES IN THE YOUNG

### 1.6.1 Introduction

Since the advent of insulin therapy and the control of the metabolic abnormalities associated with diabetes, vascular complications have emerged as the major cause of morbidity and mortality in this disorder.

The pathogenesis of these two major forms of vasculopathy (micro-angiopathy and macroangiopathy) remain unresolved but the consequences to the diabetic patients are indubitable. Microangiopathic changes have made diabetics 25 times more prone to blindness (retinopathy) and 17 times more prone to kidney disease (nephropathy). (Brownlee and Cahill, 1979). Accelerated atherogenesis in medium and large arteries (macroangiopathy) make coronary artery disease and stroke twice as common among diabetics and symptomatic peripheral arterial disease, three to four times more common. (Brownlee and Cerami, 1981). The average life expectancy of the diabetic patient is only two-thirds that of the general population (Crofford, 1975).

The obvious question is, therefore, whether patients with NIDDY are subject to the same vascular complications as the other diabetics and, if so, whether it is more or less severe in its manifestation in these patients.

### 1.6.2 Microangiopathy in Non-Insulin Dependent Diabetes in the Young

The earliest microangiopathic change is thickening of the capillary basement membrane. As the microangiopathic changes advance, capillary microaneurysms develop in the retina, endothelial cells and pericytes are lost and later new vessels and connective tissue form and extend into the vitreous fluid (retinitis proliferans) (McMillan, 1975; B.M.J. Editorial, 1977). In the kidney, the glomeruli become distorted with proliferation of the mesangial connective tissue. Kimmelstiel - Wilson nodular lesions form; there is sclerosis and narrowing of the afferent and efferent arterioles (Ireland, 1977).

#### 1.6.2.1 Retinopathy

The majority of workers reporting on retinopathy based their diagnosis

on fundal examination after mydriasis except for Johansen (1973), Fajans et al (1973) and Barbosa et al (1978) who also used flourscein angiography.

Tattersall (1974) recorded background retinopathy in 5 of 12 patients who were diagnosed before 30 years of age and at the time of examination had a mean duration of diabetes of 37 years; in 14 patients who had a mean duration of diabetes of 24 years, only 2 had background retinopathy (Tattersall, 1974). Pyke (1979), having collected more families with what they called MODY, reviewed these families and the original 3 families described by Tattersall (1974) from his department. He stated that many of these patients showed no diabetic retinopathy, several decades after the onset of diabetes and, when retinopathy did appear, it was usually mild with proliferative retinopathy being a rarity. Johansen and Gregersen (1977) reported background retinopathy in only 2 of 22 diabetic members in a family of NIDDY with dominant inheritance, despite a duration of diabetes of 2 to 44 years (mean of 13 years). In 3 other reports on NIDDY with autosomal dominant inheritance there was no evidence of any vascular complications (Tulloch and Adamson, 1976; Barbosa et al, 1978; Kobberling et al, 1980).

However, Fajans et al (1978) present further evidence for heterogeneity in NIDDY with autosomal dominant inheritance in regard to the occurrence of vascular complications. He and his collaborators found vascular complications in 24 of 69 patients (35%) from 6 pedigrees with 3 to 4 generations of diabetes. Retinopathy was present in 8 patients, 5 of whom had a duration of diabetes exceeding 15 years. Although they did not state how many of their patients with retinopathy had proliferative changes, the fact that blindness supervened in 3 patients would suggest that this was the sequel of proliferative changes (Cloutier and Fajans, 1979).

Reports on retinopathy in NIDDY, other than in those with autosomal dominant inheritance, have appeared (Soler et al, 1969; Johansen, 1973; Steel et al, 1976; Bigler and Adler, 1981; Asmal et al, 1981). Soler et al (1969) found background retinopathy in 3 patients aged 29, 31 and 35 years, respectively, at diagnosis; Johansen (1973) initially found evidence of background retinopathy in only 1 of 9 patients with NIDDY. However, after studying the vessels by flourescein angiography, he found microaneurysms in a further 4 patients, illustrating the greater sensitivity of flourescein angiography in demonstrating retinopathy. Steel et al (1976) found proliferative retinopathy in 5 patients between the ages of 34 and 39 years within 1 to 10 years following the diagnosis of diabetes, whilst Bigler and Adler (1981) described a 27 year old woman with proliferative retinopathy. In a preliminary report on NIDDY in Indians and Africans, retinopathy was demonstrated in 6 of 43 Indian patients and 2 of 9 African patients; no comment was made regarding proliferative changes (Asmal et al, 1981).

From these considerations it may be concluded that retinopathy is not as rare in NIDDY and that, in fact, it may manifest in the proliferative form.

### 1.6.2.2 Nephropathy

In most reports on NIDDY it would seem that the diagnosis of nephropathy was based on the presence of proteinuria and in many cases it is not clearly stated whether the proteinuria detected related to random urine specimens or 24 hour urine collections. Glomerular filtration rates (GFR) were assessed by creatinine clearances by 3 groups (Fajans et al, 1973; Johansen, 1973; Barbosa et al, 1978).

Nephropathy was not a feature in various reports on NIDDY with autosomal dominant inheritance (Tattersall, 1974; Tulloch and Adamson, 1976;

Barbosa et al, 1978; Johansen and Gregersen, 1977; Kobberling et al, 1980). The only report of nephropathy in NIDDY with autosomal dominant inheritance has been the single patient in the series of 6 such families described by Fajans et al (1978); however, no details on how nephropathy was diagnosed in this patient were given.

Nephropathy has been reported in NIDDY other than in those with autosomal dominant inheritance (Soler et al, 1969; Johansen, 1973; Steel et al, 1976; Bigler and Adler, 1981). Soler et al (1969) detected proteinuria in 1 of their 3 patients with NIDDY who had retinopathy (1.4.2.1). Of the 2 patients reported on by Steel et al (1976), the first patient died from renal failure four years after diagnosis and the second was uraemic, nine years after diagnosis. None of the NIDDY group of 9 patients, reported by Johansen (1973), had a reduced GFR or evidence of proteinuria (Albustix) except for 1 patient who manifested intermittent proteinuria. The clearest demonstration of nephropathy has been in a 27 year old woman with the nephrotic syndrome (Bigler and Adler, 1981). This patient had a creatinine clearance of 48,6 ml/min and a 24 hour urine protein of 5,3 grams; characteristic changes of Kimmelstiel-Wilson disease, including diffuse membrane thickening nodular lesions and capsular drop lesions were found in a renal biopsy. In 43 Indian patients with NIDDY only 3 had evidence of possible renal complications based on the presence of proteinuria (Albustix) and increased plasma creatinine levels (Asmal et al, 1981).

A major problem in the diagnosis of diabetic nephropathy in the absence of histological evidence based on renal biopsy, is that the renal disease demonstrated by non-invasive techniques could be incidental. However, in the reports cited above, nephropathy was invariably associated with retinopathy. Hence such combined evidence

may be accepted as a practical approach to the diagnosis of diabetic nephropathy (Barnett et al, 1981a; Ireland, 1977). On this basis, it would seem as though nephropathy is rare in NIDDY and most unusual in those patients with autosomal dominant inheritance.

### 1.6.3 Macroangiopathy in Non-Insulin Dependent Diabetes in the Young

No evidence of macrovascular disease was found in the studies on NIDDY reported by Tulloch and Adamson (1976), Barbosa et al (1978) and Kobberling (1980). In the original 3 families reported by Tattersall (1974), 4 patients had had myocardial infarction and one underwent an amputation. Of the 22 members of a NIDDY family with autosomal dominant inheritance, only one had a myocardial infarction and a further patient had peripheral atherosclerosis (Johansen and Gregersen, 1977).

However, Fajans et al (1978) recorded clinically manifest macroangiopathy in 16 of 69 patients in 6 NIDDY families. The prevalence of macroangiopathy (16/69) was more frequent than that of microangiopathy (9/69) (Fajans et al, 1978). Of the 16 patients with macrovascular disease, the duration of diabetes was in excess of 15 years in 12 patients. All 9 patients with peripheral vascular disease and 6 of the 9 patients with myocardial infarction had durations exceeding 15 years; generalised severe atherosclerosis was present in 2 patients. There are no reports on macrovascular disease in Indian NIDDY from South Africa except for the view held by Jackson (1978a) that vascular complications are not uncommon in South African Indian patients with NIDDY.

### 1.6.4 The Chlorpropamide Alcohol Flush and Vascular Complications

Pyke (1979) produced evidence that diabetics who exhibit the phenomena of chlorpropamide alcohol flushing (CPAF) have a lesser chance of developing vascular complications when compared to those diabetics who

do not flush. This observation stems from his findings of positive CPAF in 90% of his group of NIDDY with autosomal dominant inheritance; his patients with NIDDY were relatively free from vascular complications. The finding of a lesser susceptibility to vascular complications in diabetics who are CPAF positive was also exhibited by NIDDM with a mature onset (Barnett and Pyke, 1980; Barnett et al, 1981a; Barnett et al, 1981b). However, Kobberling et al (1980) found only 29% of his group of 14 NIDDY patients without vascular complications to have a positive CPAF test. Panzram and Adolph (1981) recorded a positive CPAF test in only 20% of 40 NIDDY patients, but no mention was made about the number of patients with vascular complications. In 3 families with NIDDY with a 4 generation vertical transmission of diabetes, the CPAF test was positive in about two-thirds of affected members; in some of these families, members with severe vascular disease were found whilst other members of younger generations had a positive CPAF test (Fajans, 1981). Fajans has concluded that a positive response to the CPAF test is unlikely to be a reliable genetic marker for the absence of vascular disease (Fajans, 1981).

### 1.6.5 Neuropathy in Non-Insulin Dependent Diabetes in the Young

Diabetic neuropathy is probably the most common of the chronic complications of diabetes (Ellenberg, 1975). Recent studies reveal that, whereas symptomatic distal symmetrical polyneuropathy is present in roughly 25% of patients with diabetes, electrophysiological evidence of abnormal peripheral nerve function can be obtained in nearly all diabetic subjects (Ward et al, 1971; Campbell et al, 1976; Fraser et al, 1977). There is, however, much controversy as to the role of metabolic aberrations and vascular factors in the genesis of neuropathy in diabetics (Ellenberg, 1970; Thomas and Ward, 1975; Jackson et al, 1978; Ward, 1977; Clarke et al, 1979; Faerman et al, 1980).

The concept that diabetic neuropathy may be caused by a specific angiopathy was first introduced in the 1950's by Fagerberg (1959). Subsequent studies have not supported a relationship between thickening and hyalinization of the neural arterioles and the severity of diabetic polyneuropathy (Chopra et al, 1969; Greenbaum, 1964; Thomas and Lascelles, 1966). The major objection to the vascular theory of diabetic nerve damage is based on the fact that the clinical manifestations of diabetic neuropathy are frequently reversible (Jackson et al, 1978). At present, therefore, the direct participation of vascular abnormalities in the pathogenesis of diabetic polyneuropathy remains speculative (Clements, 1979). Most workers, when discussing vascular complications, make no mention of neuropathy (Fajans et al, 1978; Bennett, 1979; Danowski et al, 1980; Brownlee and Cerami, 1981; Ditzel and McMillan, 1981).

The observations that abnormal peripheral nerve function consistently follows the onset of hyperglycaemia in secondary diabetes, in both man and rodents, strongly support the notion that the polyneuropathy is secondary to the metabolic disorder (Preston, 1967; Gabbay, 1973; Greene et al, 1975). Decreased motor and sensory nerve conduction velocities are consistently observed in newly diagnosed hyperglycaemic diabetic subjects; after the institution of therapy, there is a rapid improvement in nerve function, but it does not return to normal (Ward et al, 1971; Campbell et al, 1976; Fraser et al, 1977). The majority of studies have concluded that polyneuropathy is much more common in diabetic patients with poor control (Graf et al, 1979; Pirart, 1978). Although defects in the synthesis of myelin lipids and decreases in the incorporation of radioactive leucine into myelin-associated proteins have been demonstrated in experimental diabetes, the relationship between such changes and the development of polyneuropathy remains enigmatic (Clements, 1979).

A further metabolic mechanism in the pathogenesis of neuropathy is an increase in the intracellular polyol pathway activity (Ward et al, 1972; Gabbay, 1973). The concentrations of glucose, sorbitol and fructose are markedly increased in the sciatic nerve of the rat with experimental diabetes; after institution of insulin therapy, a rapid decrease in the nerve content of the intermediates of the polyol pathway is consistenly observed (Ward et al, 1972; Gabbay, 1973; Greene et al, 1975). Although sorbitol and fructose accumulate in very large amounts in the nerves of diabetic animals, their exact role in the pathogenesis of nerve damage is not known (Jackson et al, 1978; Gabbay, 1977).

Another potential factor in the pathogenesis of neuropathy is the reduction of myoinositol, which has an important role in lipid synthesis and is essential for the normal structural integrity of peripheral nerves (Gabbay, 1973; Ward et al, 1972; Greene et al, 1975). Chronic depletion of myoinositol occurs in uncontrolled diabetics, due to increased urinary excretion; however, this trend is reversed as control is established (Daughday and Larner, 1954). In support of a role for myoinositol depletion is the observation that motor conduction velocities are increased in diabetic animals when given a dietary supplement (Greene et al, 1975). Although these alterations in the content of myoinositol have been demonstrated in the sciatic nerves of rats with severe diabetes, it should be mentioned that they have not been observed in the nerves of animals with lesser degrees of hyperglycaemia; furthermore, the improved nerve conduction velocity observed following oral myoinositol supplementation seems to apply to the severely diabetic rat and not those mildly diabetic (Greene et al, 1975; Jeffereys et al, 1978; Palmano et al, 1977). In man, very little information is available to indicate that similar alterations in myoinositol contribute to

the pathogenesis of diabetic polyneuropathy (Clements, 1979).

A review of the literature reveals that in most reports on NIDDY, where neuropathy was sought, no evidence was forthcoming (Johansen, 1973; Tulloch and Adamson, 1976; Barbosa et al, 1978; Kobberling et al, 1980). Neuropathy was recorded in only 1 of the 23 patients in Tattersall's series (1974) and the only other report of neuropathy was a 29 year old male, who had nephropathy and retinopathy (Soler et al, 1969). A different situation seems to obtain in Indian patients with NIDDY: Thandroyen et al (1980) reported peripheral neuropathy in 9 patients and autonomic neuropathy in 3 from a group of 25 patients with NIDDY. Asmal et al (1981) also recorded peripheral neuropathy in 31 percent of 43 Indian patients with NIDDY.

In this study, the complication of neuropathy will be recorded on the basis of clinical finding, but no specific studies will be directed at elucidating the aetiology.

# CHAPTER 2

# HORMONAL STUDIES IN DIABETES MELLITUS WITH SPECIAL REFERENCE TO NON-INSULIN DEPENDENT DIABETES IN THE YOUNG

"When you can measure what you are speaking about and express it in numbers, you know something about it, but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind." (Lord Kelvin)

## 2.1 INSULIN AND DIABETES

Evidence that diabetes mellitus might be of pancreatic origin was furnished in 1889 by von Mering and Minkowski who demonstrated that total pancreatectomy in dogs was followed by hyperglycaemia, glycosuria, ketosis and death. Following a prolonged search by numerous workers for a pancreatic anti-diabetic factor, a substance was extracted from the pancreas which, when administered to a diabetic dog, resulted in a significant reduction in the blood glucose levels (Banting and Best, 1922). This epochal discovery of a pancreatic extract with hypoglycaemic properties (insulin) by Banting and Best had a tremendous impact and the view that diabetes was entirely due to a deficiency of insulin dominated thinking about the aetiology of diabetes thereafter (Reaven and Olefsky, 1978a).

However, Himsworth (1936), contrary to this general consensus, proposed that diabetes could be differentiated into insulin-sensitive and insulin-insensitive types. This notion received support from the early attempts to estimate circulating insulin levels by use of bioassay.

### 2.1.1 Bioassay for Insulin

Results of bioassays of insulin indicated that many patients with non-

ketotic diabetes appeared to have normal or increased plasma insulin-like activity (ILA) (Bornstein and Lawrence, 1951; Vallance-Owen et al, 1955; Steinke et al, 1961; Samaan and Fraser, 1963). The earliest bioassays relied on the effects of serum or serum extracts on blood glucose levels of animals rendered more insulin-sensitive by adrenalectomy and hypophysectomy (Bornstein, 1950). Later assays depended upon the metabolic effects produced by insulin on suitable isolated tissues; the biological systems most commonly used were glucose uptake by rat epididymal fat pad or isolated rat diaphragm and the rate of oxidation of $^{14}C$ labelled glucose (Vallance-Owen and Hurlock, 1954; Martin et al, 1958; Renold et al, 1960). There were marked differences in the insulin-like activity (ILA) measured by different bioassay procedures and levels obtained using the fat pad technique were considerably higher than those obtained using the rat diaphragm assay (Berson and Yalow, 1966). Furthermore, the bioassays were tedious to perform, had relatively poor sensitivity, lacked precision and were non-specific (Soeldner, 1971; Bondy and Felig, 1974).

This uncertainty as to the true levels of plasma insulin in patients with diabetes ended abruptly in 1960 when Yalow and Berson (1960a) published their study in which the plasma insulin levels were quantitated by radioimmunoassay. In this work they described a method for measuring insulin that combined specificity with a degree of sensitivity needed to measure the minute concentrations of insulin in the circulation. Moreover, the immunoreactive insulin levels (IRI) rose appropriately after a glucose load, was absent after pancreatectomy and was virtually absent from the plasma of patients with IDDM whose pancreases were known to contain no extractable insulin. Although it is known now that certain constituents which are not biologically active insulin are measured by this technique, there is general consensus on the view expressed by Bondy and Felig (1974) that immunoassay of insulin is the

most specific and reliable indicator of biologically active insulin. In the following sections some of the qualifications in the use of immunoreactive insulin levels will be considered.

### 2.1.2 Biosynthesis of Insulin

Insulin is elaborated initially by the Beta cells of the endocrine pancreas as pre-proinsulin, a larger polypeptide precursor (11,500 daltons) which is rapidly converted to proinsulin (9,000 daltons) in ribosomes associated with the rough endoplasmic reticulum (Steiner, 1977; Rubenstein, 1981). Proinsulin consists of a singly polypeptide chain ranging in length from 78 (dog) to 86 (human) amino acid residues. The variation in the length of the mammalian proteins occur only in the connecting polypeptide portion which links the carboxy-terminus of the insulin B-chain to the amino terminus of the A-chain. The known mammalian proinsulins have pairs of basic residues at either end of the connecting peptide which link it to the insulin chains (Steiner, 1977). After synthesis, proinsulin is transported by an energy requiring process to the Golgi apparatus (Rubenstein, 1981). Following its transport to the Golgi apparatus, proinsulin is condensed into membrane enclosed granules. Conversion of proinsulin to insulin and the C-peptide entails an excision of the basic residues which link the connecting peptide to the A- and B-chains. This conversion reaction is initiated in the Golgi apparatus and continues for many hours within the granules as they collect and mature in the cytosol (Rubenstein, 1981). Following appropriate stimulation, the granules migrate to the plasma membrane whence they release, by a process of emiocytosis, insulin and C-peptide in equimolar concentration together 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

TABLE 2.1

THE METABOLIC EFFECTS OF INSULIN

| | | |
|---|---|---|
| A) | LIVER | |
| | ANTI-CATABOLIC | ↓ GLYCOGENOLYSIS |
| | | ↓ GLUCONEOGENESIS |
| | | ↓ KETOGENESIS |
| | ANABOLIC | ↑ GLYCOGEN SYNTHESIS |
| | | ↑ FATTY ACID SYNTHESIS |
| B) | MUSCLE | |
| | ANTI-CATABOLIC | ↓ PROTEIN CATABOLISM |
| | | ↓ AMINO ACID OUTPUT |
| | ANABOLIC | ↑ AMINO ACID UPTAKE |
| | | ↑ PROTEIN SYNTHESIS |
| | | ↑ GLYCOGEN SYNTHESIS |
| C) | ADIPOSE TISSUE | |
| | ANTI-CATABOLIC | ↓ LIPOLYSIS |
| | ANABOLIC | ↑ GLYCEROL SYNTHESIS |
| | | ↑ FATTY ACID SYNTHESIS |

↑ denotes stimulation

↓ denotes inhibition

system and appears to be dependent on intracellular shifts of calcium ions (Malaisse et al, 1975).

### 2.1.3 Metabolic Effects of Insulin

Insulin is rapidly removed from the portal circulation by the liver which clears about 40 to 60% of the hormone presented to it in a single passage (Kaden et al, 1971; Felig and Bondy, 1974). Thus, although significant quantitative differences occur between peripheral and portal blood levels, insulin levels in the 2 samples correlate well with each other (Horwitz and Rubenstein, 1979). The first step in the action of insulin is binding to a glycoprotein receptor on the surface membrane of the cell (Kahn, 1980). After this binding the insulin receptor complex is formed and one or more signals are generated: regardless of its precise physico-chemical nature this signal(s) interacts with a variety of effector units that mediate the entire host of biologic actions of insulin (Olefsky, 1981). The actions of insulin involves principally the three major metabolic fuels, carbohydrate, protein and fat and occurs in three principal tissues, liver, muscle and adipose tissue. In each of these tissues there are anti-catabolic as well as anabolic effects which reinforce each other (Sherwin and Felig, 1981). The metabolic effects of insulin are schematically summarised in Table 2.1 (Sherwin and Felig, 1981). Minimal deficiency of insulin results in diminshed ability to increase effectively the storage reservoir of body fuels because of inadequate disposal of ingested food (e.g. glucose intolerance). A major deficiency of insulin, by contrast, will result in both inadequate fuel storage in the fed state and an excessive mobilisation or production of endogenous metabolic fuels in the fasted state (e.g. fasting hyperglycaemia, increased fatty acid levels and hyperaminoacidaemia); insulin deficiency in its most severe form, ketoacidosis, results in overproduction of

glucose and marked acceleration of all catabolic processes, for example, lipolysis and proteolysis.

### 2.1.4 Insulin Secretion in Diabetes

The dramatic effect of injected insulin (Banting and Best, 1922) on lowering the blood glucose levels in patients with hyperglycaemia led to the belief that hyperglycaemia and diabetes mellitus were the expression of insulin deficiency.

Himsworth (1936) presented data indicating that patients with diabetes could be divided into those who were insulin-sensitive and those who were insulin-insensitive. He continued to publish evidence pointing to a complex causality of the diabetic syndrome and stated: "We should accustom ourselves to the idea that a primary deficiency of insulin is only one, and then not the commonest cause of the diabetic syndrome." (Himsworth, 1949).

However, it was not until Yalow and Berson (1960a) published their epochal paper, in which the radioimmunoassay of insulin was described, that it became unequivocally clear that diabetes mellitus was not necessarily associated with an absolute insulin deficiency. These pioneering studies (Yalow and Berson, 1960a and 1960b) revealed that in mild maturity onset diabetes the insulin secretion in response to oral glucose loading was initially delayed; at 30 minutes plasma insulin levels were lower than in controls but plasma insulin concentrations continued to increase attaining values at 2 hours which were significantly greater than that in reference subjects. They demonstrated that the mean plasma insulin response to oral glucose was at least as great in their patients as that in normal subjects. Since the glucose levels were much higher in the diabetic patients they interpreted this phenomenon as evidence of insulin-insensitivity. As a result of these

observations, Yalow and Berson (1960a and 1960b) suggested that insulin deficiency could not be the sole cause of diabetes in man.

There is fairly general agreement that sluggish or delayed insulin secretion in response to glucose is found in mild diabetics (Yalow and Berson, 1960a and 1960b; Perley and Kipnis, 1966; Seltzer et al, 1967; Cerasi and Luft, 1967).

A very large number of papers have been published which confirms that many diabetics are not suffering from an absolute deficiency of insulin; definite patterns have been established in which the plasma insulin response is directly related to the degree of hyperglycaemia. (Hales and Randle, 1963; Berson and Yalow, 1965; Buchanan and McKiddle, 1967; Reaven and Miller, 1968; Chiles and Tzagouris, 1970; Savage et al, 1975; Reaven et al, 1976). Thus, most patients with the earliest discernible degree of glucose intolerance have insulin levels, in response to oral glucose, that are somewhat higher in absolute terms than that of normal subjects and increasing degrees of hyperglycaemia tend to be associated with appropriate increases in the insulin response. However, as unequivocal fasting hyperglycaemia supervenes, the plasma insulin response to oral glucose begins to fall and most patients with significant fasting hyperglycaemia ( $\geq$ 8,3 mmol/$\ell$) have an attenuated and decreased insulin response during a standard oral glucose challenge.

#### 2.1.4.1 Obesity and Insulin Secretion

The importance of obesity in modulating the insulin response has been realised since 1963 (Karam et al, 1963; Bagdade et al, 1967). Karam et al (1963) pointed out that an excessive insulin response to oral glucose was frequently seen in obese patients. Bagdade et al (1967) demonstrated that both obese diabetics and non-diabetics had greater

insulin responses than their non-obese counterparts respectively; furthermore, the insulin response in diabetics (obese and non-obese) were impaired when compared to weight-matched controls (Bagdade et al, 1967). When Karam and his associates (1965) extended their studies to include diabetics, they found that obese diabetics had increased insulin responses whereas the thin patients had decreased responses. On the basis of these results they concluded that the plasma insulin response was lower in patients with diabetes and that the increased levels of plasma insulin previously reported in diabetic patients were secondary to obesity. However, although they matched diabetics with controls on the basis of weight, they did not match them on the basis of degree of hyperglycaemia. Since the thin diabetics were significantly more hyperglycaemic than the obese diabetics, their diminished insulin response could simply reflect the fact that patients with severe diabetes, thin or obese, secrete less insulin than do mild diabetics. Indeed, most studies indicate that when normal subjects and diabetics are matched for weight and degree of glucose intolerance, patients with mild diabetes, as a group, have insulin levels greater than, or equal to, controls. However, hypoinsulinaemia is seen when significant fasting hyperglycaemia supervenes (Berson and Yalow, 1965; Buchanan and McKiddle, 1967; Reaven and Miller, 1968; Chiles and Tzagournis, 1970; Savage et al, 1975). Furthermore, although obese subjects tend to have a greater insulin response to oral glucose than non-obese subjects, not all obese subjects exhibit hyperinsulinism (Reaven and Olefsky, 1978a); glucose intolerance and insulin resistance can occur in NIDDM in the absence of obesity (Ginsberg et al, 1974; Reaven et al, 1976; Davis et al, 1979; Harano et al, 1978; Defronzo et al, 1979). The evidence is, however, clear that obesity is an important metabolic variable which can significantly influence both insulin secretion and resistance, but its importance should not be overestimated.

### 2.1.4.2 Insulin Deficiency and Insulin Resistance

It is likely that insulin resistance is the primary defect in chemical diabetes (impaired glucose tolerance) and may as well be a secondary manifestation in subjects with severe insulin deficiency (Reaven et al, 1976; Reaven and Olefsky, 1977). Considerable heterogeneity exists in the plasma insulin response to oral glucose in both normal controls and patients with NIDDM (Reaven and Olefsky, 1977; Fajans et al, 1974). Glucose tolerance can deteriorate as a result of an increase in insulin resistance and/or a decrease in insulin secretion (Reaven, 1980). It also appears that insulin resistance can culminate in hypoinsulinaemia and vice versa. The difficulty in unravelling the relative roles that primary defects in either insulin resistance, or insulin secretion, may play in the genesis of NIDDM is further compounded by the impact of obesity on both variables. The view expressed by Reaven (1980) is most acceptable at the present time: "Insulin independent diabetes (NIDDM) comprise a metabolically heterogeneous population, with an almost infinite combination of changes in tissue insulin sensitivity and insulin secretory response."

### 2.1.4.3 Proinsulin Secretion

The relative concentration of proinsulin to total immunoreactive insulin should be mentioned. In studies using human insulin standards for the measurement of both proinsulin and insulin, PLC (proinsulin-like components) comprise 15% of the total immunoreactive insulin concentration (range 0 - 22%) (Rubenstein et al, 1977). Obese patients with hyperinsulinism have raised fasting concentrations of proinsulin and a greater absolute increase in proinsulin after glucose administration than subjects of normal weight. However, the high levels are matched by increased insulin concentration. Hence the relative proportions of

the two polypeptides are generally in the same range as that observed in healthy subjects. (Gorden and Roth, 1969; Rubenstein, 1979). Gorden et al (1974) found that patients with severe glucose intolerance and associated hypoinsulinaemia have higher proportions of their total immunoreactive insulin in the proinsulin-like form and interpreted this observation as a reflection on the functional integrity (maturation) of the B-cell granule.

#### 2.1.4.4 Insulin Secretion in IDDM

Patients with IDDM and ketosis show a pattern of insulin response characterised by abnormally low values throughout the duration of testing (Ehrlich and Bambers, 1964; Hales and Randle, 1963; Parker et al, 1968). The insulin secretory pattern in IDDM is an absolute insulin deficiency resulting from severe Beta cell dysfunction.

### 2.1.5 Insulin Secretion in Non-Insulin Dependent Diabetes in the Young

The most comprehensive studies on insulin secretion in NIDDY emanates from Ann Arbor, Michigan where Fajans and associates have described the insulinaemic response to oral glucose in great detail (Fajans et al, 1969; Fajans et al, 1974; Fajans, 1981; Fajans et al, 1976; Fajans et al, 1978; Fajans et al, 1979). The majority of patients in their series have a delayed and subnormal insulin response to oral glucose although there was a wide spectrum of insulin responses which their patients displayed. When 25 NIDDY patients, aged 9 to 17 years at diagnosis, were compared to 125 control subjects, their plasma glucose levels were higher throughout the 3 hour period of testing and the insulin response was significantly delayed and subnormal. A comparison of mean plasma glucose levels in 30 NIDDY patients, aged 18 to 25 years at diagnosis, once again revealed significantly higher plasma glucose

levels (Fajans et al, 1974). For all 30 NIDDY patients the mean level of plasma insulin was significantly lower at 30 minutes after ingestion of glucose when compared to control values. However, when the sum of insulin increments during the glucose tolerance test were considered, 6 had responses which were greater than the mean plus one standard deviation (X + 1 S D) of the sum of increments of the control subjects; the remaining 24 patients had subnormal values. In continuing their studies on a total number of 68 non-obese diabetics they found that 21 patients had increments in plasma insulin response to glucose which was less than one standard deviation below the mean of normal control subjects; these patients were designated as "low insulin responders". On the other hand, 13 patients had increments exceeding the X + 1 S D of control values; these were designated "high insulin responders". The glucose tolerance of these two groups only showed significant differences at $2\frac{1}{2}$ and 3 hours. They further demonstrated familial aggregation of the type of insulin responses. As stated at the outset, most diabetic members of NIDDY families had low insulin responses or responses below the mean of the controls. No family has members with low and high insulin responses appearing together. Progression to insulin-requiring diabetes only occurred in individuals who had low insulin responses. To date (1981) they remarked that none of their non-obese NIDDY patients with high insulin responses had progressed to insulin-requiring diabetes (follow-up of up to 20 years). They concluded that a "low insulin response" was a more reliable prognostic indicator of decompensation to the requirement of insulin than the degree of carbohydrate intolerance. However, not all "low responders" progressed to insulin dependence as is evidenced by their follow-up of patients for up to 20 years.

Prolonged prospective longitudinal studies have indicated that, when glucose intolerance improved in "low insulin responders", insulin

responses increased significantly, suggesting that greatly decreased insulin responses appear to be the determinant, in part at least, of the abnormal carbohydrate tolerance. On the other hand, when glucose tolerance improved in those patients with high insulin responses, the plasma levels of insulin decreased concomitantly. This suggests that in these latter patients hyperinsulinaemia is secondary or compensatory to factors that cause glucose intolerance. The findings, outlined above, were interpreted as evidence for heterogeneity of insulin responses in NIDDY.

Delayed and attenuated insulin responses in the majority of patients with NIDDY have been corroborated by numerous workers (Chiumello et al, 1969; Thorell et al, 1975; Faber et al, 1978; Barbosa et al, 1978). Although most of the patients reported by Barbosa et al (1978) had hypoinsulinaemic responses, 12% had hyperinsulinism by the criteria of Fajans et al (1974). The majority of Barbosa's patients with hyperinsulinism (75%) were not obese (Barbosa et al, 1978).

Children with chemical diabetes (excluding those with obesity and attendant hyperinsulinism) demonstrated normal or increased insulin responses to glucose ingestion (Rosenbloom, 1970; Balsam et al, 1973; Drash, 1973; Pildes, 1973; Jackson et al, 1973; Rosenbloom, 1973).

It is apparent that patients with NIDDY can exhibit hyperinsulinaemic or hypoinsulinaemic responses to a glucose load. The precise mechanism of the secretory defect in the hypoinsulinaemic patients remains to be clarified; however, in NIDDM, increased adrenergic tone (Robertson et al, 1976) and increased prostaglandin synthesis (Robertson and Chen, 1977) have been postulated to play a role in the insulin secretory defect. Others have proposed that a decreased Beta cell replication rate or early senescence of Beta cells may occur (Rowe et al, 1977). The basis of the insulin secretory defect in hypoinsulinaemic NIDDY

remains to be elucidated. In NIDDY with hyperinsulinaemic responses, a state of insulin resistance obtains and receptor and post receptor defects could be considered. It would also seem that the heterogeneity in insulin secretory patterns suggested by Reaven (1980) for NIDDM and certainly found by Fajans and his co-workers, are relevant.

## 2.2 GLUCAGON AND DIABETES

### 2.2.1 Introduction

Glucagon is a 29 amino acid linear polypeptide with a molecular weight of 3,485 daltons. It originates primarily from the alpha cell of the endocrine pancreas although a significant amount comes from alpha ($\alpha$) cells in the stomach and other parts of the gastro-intestinal tract.

Glucagon is synthesised in the alpha cells from a larger precursor molecule 18,000 - 19,000 daltons (Tager, 1981; Lund et al, 1980). This pre-prohormone is converted to glucagon through a series of proteolytic cleavages (Patzelt et al, 1979; Hellerstrom et al, 1974; Noe and Bauer, 1978). Although the function of the peptides derived from the gut, GLI (glucagon-like immunoreactivity) or enteroglucagon is still unknown, certain of the smaller moeties are reported to bind to hepatic glucagon receptors and to have biological activities similar to, but less potent than, that of glucagon (Holst, 1975; Sasaki et al, 1975).

Together with its major antagonist, insulin, glucagon has a central role in mediating nutrient balance; the insulin/glucagon ratio determines the anabolic-catabolic setting of the organism (Unger, 1971). Pancreatic glucagon's prime physiological role is as a substrate provider. The major site of action is the liver where its stimulatory effect on glycogenolysis is mediated via cyclic AMP (Sutherland et al, 1968;

Pohl et al, 1969) but at the same time hepatic gluconeogenesis is also stimulated by facilitation of hepatic uptake of gluconeogenic amino acids (Cahill, 1970; Felig et al, 1971). In adipose tissue glucagon stimulates lipolysis, increasing the supply of fatty acids for hepatic ketogenesis (Foa, 1975) and hence glucagon plays a role in ketotic states.

Plasma concentrations of immunoreactive glucagon (IRG) reported in the literature have varied widely mainly because of the variable cross-reactivity of different antisera to extra-pancreatic GLI (Glucagon-like immunoreactivity). (Harris et al, 1979). A number of investigators have reported on the presence of 4 separate molecular forms of glucagon on chromatography of plasma taken from healthy individuals; big plasma glucagon (60,000 daltons), proglucagon (9,000 daltons), true glucagon (3,500 daltons) and a small peak which is immunoreactive (2,000 daltons) (Valverde et al, 1974; Weir et al, 1975; Kuku et al, 1976). Data on the molecular pattern of plasma IRG in diabetes suggest that hyper-glucagonaemia is associated with increased biological activity, since the increase is mainly in the 3,500 dalton component.

### 2.2.2 Role of Glucagon in Diabetes

Plasma glucagon (IRG) levels are usually substantially increased in ketoacidosis, hyperosmolar non-ketotic coma and poorly controlled diabetes mellitus (Muller et al, 1973; Lindsey et al, 1974; Gerich 1977). In mild or moderate diabetes IRG levels may be normal or moderately increased (Lefebvre and Luyckx, 1979). Other abnormalities demonstrated in diabetics include reduced inhibition of IRG levels by oral or intravenous glucose, even occasionally a paradoxical rise in IRG after oral glucose and an exaggerated glucagon response to intra-venous arginine infusion or to a large protein meal (Wise et al, 1973;

Buchanan and McCarroll, 1972; Sieno et al, 1978, Muller et al, 1970).

When physiological levels of glucagon were infused in patients with IDDM in whom the secretion of endogenous insulin, glucagon and growth hormone were suppressed by somatostatin, blood glucose, ketones and fatty acid levels increased. These findings confirm the expected effects of the hormone in the absence of a normal insulin response (Gerich et al, 1976).

However, Sherwin et al (1976) reported that hyperglucagonaemia produced by infusing glucagon premixed in a solution containing the patient's own whole blood did not aggravate the hyperglycaemia or hyperketonaemia of IDDM. In a subsequent study on diabetics, in whom glucagon was infused at the same rate, but without blood, the metabolic state did deteriorate as manifested by increased hyperglycaemia, marked glycosuria and ketonuria in the face of adequate insulin levels (Raskin and Unger, 1977; Raskin and Unger, 1978). The apparent discrepancy in the two studies could be explained in part by the fact that in Sherwin's study (1976) the biological activity of the infused glucagon was destroyed when premixed with whole blood (Unger et al, 1976).

On the other hand, there is little doubt today that glucagon excess, when present, exacerbates the metabolic abnormalities seen in diabetics with insulin insufficiency (Pek, 1977; Unger and Orci, 1981).

Lefebvre and Luyckx (1979) in their review conclude that: "There is thus general agreement that plasma glucagon levels are high or relatively high in diabetes mellitus". In IDDM the relative hyperglucagonaemia is secondary to an absence or paucity of β-cells since the normal inhibitory influence of insulin on α-cells is removed

(Dobbs et al, 1975; Unger and Orci, 1975; Unger, 1978). The mechanism for the hyperglucagonaemia in NIDDM is unknown, but is probably related to resistance of the α-cell to insulin (Unger, 1981).

Consensus seems to prevail on the view that the hyperglucagonaemia, present in diabetes, is an adaptation to the diabetic state and not a causal factor in the development of diabetes (Lawrence and Abraira, 1981).

### 2.2.3 Glucagon in Non-Insulin Dependent Diabetes in the Young

The only report to date on glucagon levels in NIDDY has been that of Barbosa et al (1978). They found that the glucagon levels were lower in NIDDY in the fasting state when compared to controls, but this difference was not statistically significant. In response to oral glucose there was adequate suppression of plasma glucagon which was of the same magnitude as in controls (Barbosa et al, 1978). They concluded that α-cell function was normal in their NIDDY patients.

## 2.3 GROWTH HORMONE AND DIABETES

### 2.3.1 Growth Hormone as a Diabetogenic Agent

Growth hormone (GH) is potentially diabetogenic by virtue of the fact that it increases hepatic glucose output (gluconeogenesis) and exerts a peripheral anti-insulin effect (Ganong, 1979). Furthermore, growth hormone stimulates overall protein synthesis and has a lipolytic action on adipose tissue in the intact animal (Grodsky, 1977).

A possible association between human growth hormone (HGH) and the development of diabetes in man was originally suggested by the clinical observation that impaired glucose tolerance occurred in acromegaly

(Coggleshall and Root, 1940). The potential of growth hormone as a diabetogenic agent was supported from the studies of Houssay and Biasotti (1930) and Young (1939). Houssay and Biasotti demonstrated a role for the hypophysis in experimental diabetes in animals: they found that the severity of diabetes decreased following extirpation of the hypophysis and increased again after administration of extracts of its pars distalis (Houssay and Biasotti, 1930). Young (1937) was able to produce diabetes in intact dogs by administration of crude anterior pituitary extracts and suggested that the pituitary was involved in the initiation of the disease. Cotes et al (1949) and Houssay and Anderson (1949) independently demonstrated that GH was the active substance in pituitary extracts which induced diabetes in animals. Further support for the diabetogenic potential of GH came when it was shown that human GH (HGH) induced temporary diabetes in hypophysectomized non-diabetic patients, caused deterioration of the metabolic state in the diabetic and an impairment of intravenous glucose tolerance in normal subjects (Ikkos and Luft, 1960a, 1960b, Ikkos et al, 1962).

These historical studies apart, much circumstantial and direct evidence exists that GH is not aetiologically important in primary diabetes. Acromegaly is a classical example: most acromegalics produce large amounts of GH for many years yet only 12% have clinical diabetes and a further 25% have impaired glucose tolerance (Besser, 1976). It has also been shown that acromegalics with diabetes have a family history of diabetes more often than those without diabetes (Fraser, 1960). Furthermore, impaired glucose tolerance has been demonstrated in patients with isolated GH deficiency (Merimee et al, 1970).

### 2.3.2 Growth Hormone Levels in Diabetes

Several reports exist in which abnormal levels of HGH were found under a variety of conditions (fasting, stimulation with arginine, exercise and glucagon) in IDDM (Drash et al, 1968; Johansen and Hansen, 1971; Yde, 1970; Molnar et al, 1972; Sperling et al, 1973; Vigneri et al, 1976). Abnormally high levels of GH have also been reported in patients with NIDDM (Yde, 1970; Hansen, 1973; Kjeldsen et al, 1975; Vigneri et al, 1976). In NIDDM the abnormal secretion of HGH seems to be confined to the non-obese patients (Hansen, 1973; Baird et al, 1973; Kjeldsen et al, 1975). In obese patients with NIDDM, low HGH levels are usually found which are usually not significantly different from the values found in obese non-diabetics (Yalow et al, 1965; Burday et al, 1968; Sabeh et al, 1969; Baird et al, 1973; Merimee et al, 1979). Support for the claim that the abnormal HGH responses are secondary to the metabolic state and not a primary event in the genesis of idiopathic diabetes came from many studies which have shown that the abnormalities in HGH secretion can be restored to normal after institution of strict metabolic control (Johansen and Hansen, 1971; Hansen, 1971; Baird et al, 1973; Corrall et al, 1974; Hansen, 1974; Vigneri et al, 1976). In mild and stable diabetics HGH levels are normal and can be suppressed adequately in response to oral glucose (Hunter et al, 1966; Hagen and Ajlouni, 1975; Merimee, 1979).

Although low levels of somatomedins have been demonstrated in the serum of diabetic animals and patients, their relative importance to the diabetic state remains to be elucidated. (Phillips and Young, 1976; Baxter et al, 1979; Winter et al, 1979).

### 2.3.3 Growth Hormone and Vascular Disease in Diabetes

HGH has however been incriminated in the genesis of microvascular disease (Lundbaek et al, 1970). Support for this thesis came from many reports showing increased growth hormone levels and responses to various challenges in diabetics with retinopathy as compared to those without this complication (Beaumont et al, 1971; Passa et al, 1974; Knopf et al, 1972; Lundbaek, 1976; Larkins et al, 1978). Furthermore, progression of severe retinopathy has been ameliorated by hypophysectomy and it has been shown that between 60 to 80% of patients with proliferative retinopathy stabilise after hyphophysectomy (Goldberg and Fine, 1969; Bradley and Ramos, 1971; Kohner et al, 1976). In a group of glucose-intolerant, HGH-deficient dwarfs, matched according to sex, age, duration of diabetes with non-growth hormone deficient diabetics, no retinopathy was observed over a 10 year follow-up period suggesting that increased growth hormone may be a contributing factor in the causation of diabetic microangiopathy. (Merimee et al, 1969; 1970; Merimee, 1978). However, recently a HGH-deficient diabetic with severe nephropathy and retinopathy has been reported, a finding manifestly at variance with a primary role of HGH in the aetiology of microangiopathy (Rabin et al, 1979). The fact that acromegalics with, and without, glucose intolerance are relatively free from microvascular disease is further evidence against a primary role for HGH in the aetiology of microangiopathy (Flier and Roth, 1979; Brownlee and Cerami, 1981; Kozak and Cooppan, 1982).

In summary, it would seem that abnormal HGH secretion, when present in diabetics, appears to be secondary to the loss of metabolic control and if a role has to be assigned to HGH in the genesis of the microvascular complications, this would seem at best permissive.

### 2.3.4 Growth Hormone Secretion in Non-Insulin Dependent Diabetes in the Young

The only reports on HGH in NIDDY which have been published to date, appear to be the studies of Drash (1973) and Barbosa et al (1978). Drash (1973) showed that the HGH response to oral glucose in a group of 12 patients with NIDDY was no different from normal: an initial prompt suppression of HGH was followed by a rebound rise.

Barbosa et al (1978) found normal fasting HGH levels in his patients. However, neither the NIDDY group nor the controls showed HGH suppression after oral glucose. There was a significant rebound rise in the HGH response in both NIDDY and controls over basal values, but the rebound rise in NIDDY was no different from that of the controls. These results were interpreted as reflecting the mild degree of hyperglycaemia (Barbosa et al, 1978).

Hence HGH secretion in patients with NIDDY do not appear to be disturbed.

## 2.4 CORTISOL AND DIABETES

### 2.4.1 Cortisol as a Diabetogenic Agent

Cortisol is potentially diabetogenic by virtue of its actions on protein, fat and carbohydrate metabolism. Apart from stimulation of protein catabolism and lipolysis, cortisol also increases glucose production (gluconeogenesis) and inhibits peripheral glucose utilisation (Fain et al, 1963; Baxter and Forsham, 1972; Grodsky, 1977).

The concept that cortisol may participate in the diabetic state is not new. As early as 1926, a diabetic in whom insulin requirements decreased rapidly with the onset of adrenal insufficiency was described

(Unverricht, 1926). Numerous reports since then confirmed the striking feature of increased insulin sensitivity in diabetics who develop Addison's disease (Kozak, 1971).

About 75% of patients with Cushing's syndrome have impaired glucose tolerance but overt clinical diabetes is only present in 25% of patients (Boshell and Chandalia, 1970; Williams and Porte, 1974; Kozak and Cooppan, 1982). Most patients have hyperinsulinism, both basal and stimulated, irrespective of whether glucose tolerance is disturbed (Modigliani et al, 1970; Klink and Estrich, 1964). Clinically, the diabetes tends to be mild, stable and relatively resistant to insulin; ketoacidosis rarely supervenes and, when it does, it is usually mild (Williams and Porte, 1974). Despite some cases of prolonged glucose intolerance in Cushing's syndrome, specific late vascular complications are rarely observed (Flier and Roth, 1979). With treatment of the primary lesion in Cushing's syndrome, the abnormal glucose tolerance reverts back to normal in the majority of patients (Soffer et al, 1961; Bondy and Felig, 1974).

In patients treated for long periods with therapeutic doses of steroids, diabetes developed in less than 1% and then usually in those with a family history of diabetes; remission of the diabetes occurred in the majority on cessation of therapy (Bookman et al, 1953; Sperling, 1977).

### 2.4.2 Adrenocortical Activity in Diabetes

In primary diabetes most workers found normal values for urinary 17-hydroxycorticosteroids, 17-oxogenic steroids and plasma corticosteroid levels in both NIDDM and IDDM, irrespective of the presence of vascular complications (Jakobson, 1958; Rifkin et al, 1958; Dancaster and Jackson, 1963; Szucs and Csapo, 1964; Serio et al, 1968; Asfeldt, 1969; Asfeldt, 1972; Loreti et al, 1974). More recent studies have

confirmed these earlier observations (Sperling, 1977) although the stress situations of hypoglycaemia and ketoacidosis do result in abnormal adrenal steroid profiles in diabetics (Bondy and Felig, 1974; Sperling, 1977). However, other workers claimed increased adrenocortical activity in diabetics in the absence of ketoacidosis or hypoglycaemia (Goth et al, 1956; Lentle and Thomas, 1964; Winkler et al, 1973; Tai and Lee, 1975). Goth et al (1956) found increased urinary corticosteroid excretion and plasma 17 hydroxy-corticosteroids (17 OHCS) in patients with relatively high levels of glucose and suffering from IDDM; by contrast, in a group of well-regulated patients with IDDM and relatively low blood glucose levels, urinary excretion of corticosteroids and fasting 17 OHCS levels were normal. Lentle and Thomas (1964) reported higher plasma cortico-steroid levels in IDDM and NIDDM, the values being still higher in patients with vascular complications; similar increases in cortisol levels were observed by Tai and Lee (1975), when they studied poorly controlled diabetics and physiological increases in cortisol levels have been shown to accentuate the hyperglycaemia in diabetics, even in the face of insulin therapy (Shamoon et al, 1980).

The apparent conflict in findings over the years should be ascribed in part to different methodologies and assessments used and the consensus at the present time is that hyperactivity of the adrenal cortex observed in some studies is secondary to the stress of metabolic decompensation in diabetics who are not well controlled (Asfeldt, 1972; Tai and Lee, 1975; Sperling, 1977).

### 2.4.3 Cortisol and Diabetic Vasculopathy

As already pointed out, increased cortisol levels have been reported by some workers in diabetics with vascular complications (Lentle and Thomas, 1964; Tai and Lee, 1975). In elegant studies for the time,

Lentle and Thomas (1964) showed that cortisol secretion rates and urinary corticosteroid excretion were increased in diabetics with complications when compared to controls. Additional evidence which suggested a role for cortisol in the pathogenesis of diabetic vascular disease include the finding that the adrenal glands of diabetics with retinopathy weigh more than those without retinopathy (Becker, 1952); bilateral adrenalectomy may benefit patients with retinopathy (Malins, 1962) and, of course, steroid therapy in diabetics who underwent adrenalectomy is known to aggravate retinopathy, if present (Ehlers, 1953; Jackson, 1955). However, although these studies would tend to support a role for cortisol in diabetic vascular disease, the natural model of excess glucocorticoid secretion afforded by Cushingoid man argues against an important role for cortisol in the genesis of diabetic vasculopathy: less than 5% of patients with Cushing's syndrome present with diabetic retinopathy (Williams and Porte, 1974). Manifestly, this whole area requires more careful study. The pathogenesis of vascular complications is very much a function of time, whereas cortisol responses is an acute phenomenon, a response to many and varied provocations, some sustained, others short-lived.

### 2.4.4 Adrenocortical Status in Non-Insulin Dependent Diabetes in the Young

No group of workers have given any attention in published form to the adrenocortical status of patients with NIDDY and it is clearly necessary that such investigations be undertaken.

## 2.5 CATECHOLAMINES AND DIABETES

### 2.5.1 The Diabetogenic Potential of Catecholamines

Adrenaline and, to a lesser extent, noradrenaline, activate phosphorylase

in liver and skeletal muscle, resulting in increase glycogenolysis; gluconeogenesis is enhanced in the liver and glucose uptake by muscle is decreased (Grodsky, 1977; Young and Landsberg, 1977; Alberti and Nattrass, 1977; Flier and Roth, 1979). In adipose tissue, adrenaline and noradrenaline have almost equal potency in stimulating hormone-sensitive lipase (Grodsky, 1977; Alberti and Nattrass, 1977). These glycogenolytic, gluconeogenic and lipolytic actions are mediated via beta receptor stimulation (Ganong, 1979). However, the effect of adrenaline and noradrenaline on the endocrine pancreas is predominantly via alpha receptor stimulation and ensuing inhibition of insulin secretion (Sperling, 1977; Ganong, 1979). The above effects of catecholamines qualify them as insulin antagonists and potentially diabetogenic agents.

In patients with phaeochromocytomas, fasting plasma glucose is usually in the normal range, despite the increased secretion of catecholamines (Spergel et al, 1968; Vance et al, 1969). However, when glucose intolerance and overt diabetes do occur, it is usually mild and anti-diabetic therapy is rarely required (Bondy and Felig, 1974; Engelman, 1977; Flier and Roth, 1979). Diabetic complications are not a feature and the glucose intolerance usually reverts back to normal with successful removal of the tumour (Engelman, 1977; Flier and Roth, 1979).

### 2.5.2 Plasma Catecholamines in Diabetes

Normal levels of catecholamines have been reported in diabetics who are not poorly controlled (Christensen, 1972; Cryer et al, 1978; Christensen, 1979). The most comprehensive study on catecholamine levels in diabetics to date has emanated from Missouri (Cryer et al, 1978). In this study they found that the mean fasting and posturally

stimulated catecholamine levels of 100 diabetics (66 IDDM, 34 NIDDM) was not significantly different from controls; the data in this study was interpreted as evidence that alterations in sympathetic neural and adrenomedullary net secretory activity are not features of diabetes mellitus per se (Cryer et al, 1978). The finding of increased plasma catecholamines in poorly controlled diabetics, especially in those with ketoacidosis, is hardly surprising since catecholamines have been invoked as the classical stress hormones and these patients are burdened by the stress of metabolic decompensation (Christensen, 1974; Sperling, 1977; Tamborlane et al, 1979). Furthermore, it has been shown that infusion of catecholamines in IDDM, in the face of an ongoing insulin infusion, aggravates the hyperglycaemia substantially (Shamoon et al, 1980). This is understandable since these hormones have diabetogenic properties.

The consensus of available data would suggest that derangements in catecholamine metabolism are secondary to poor control, rather than primary events in the pathogenesis of diabetes; however, when increased levels are present, it would tend to aggravate the diabetic state (Christensen, 1979).

# CHAPTER 3

# LIPIDS AND LIPOPROTEINS IN DIABETES MELLITUS

## 3.1 INTRODUCTION

Recent years have seen accelerating progress in our understanding of lipoprotein structure and metabolism. Germane to any discussion of lipid aberrations is an outline on the classification of lipoproteins and lipoprotein metabolism. Accordingly, before examining the aberrations in lipids and lipoproteins in diabetes, the most recent classification and the newer aspects concerning lipoprotein metabolism will be presented.

## 3.2 CLASSIFICATION OF LIPOPROTEINS

The insolubility of lipids in aqueous media requires some carrier mechanism for transport in blood and this function is fulfilled by the apoproteins. These lipid and apoprotein macromolecules are termed lipoproteins. Various classes of lipoproteins can be separated in serum from one another according to their size (gel filtration), floatation density (ultracentrifugation), net surface charge (electrophoresis) or other physico-chemical properties (precipitation and absorption techniques). These physico-chemical properties permit lipoproteins to be classified into 4 major classes: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The chief characteristics of these 4 classes are shown in Table 3.1 (Wilson and Brown, 1978; Lewis, 1979; Thompson, 1979). It is obvious that the different lipoprotein classes contain varying amounts of cholesterol, cholesterol esters, triglycerides, phospholipids and apoproteins. Approximately 60 percent of the plasma cholesterol resides in the LDL fraction whilst at least

TABLE 3.1

CHARACTERISTICS OF PLASMA LIPOPROTEINS

| | CHYLOMICRONS | VLDL | LDL | HDL |
|---|---|---|---|---|
| Density range | < 0,95 | 0.95-1.006 | 1.006-1.063 | 1.063-1.21 |
| Electrophoresis | immobile | pre-beta | beta | alpha |
| Lipid (%) | 99 | 90 | 75 | 50 |
| Cholesterol (%) | 5 | 15 | 45 | 20 |
| Triglycerides (%) | 90 | 60 | 10 | 3 |
| Phospholipids (%) | 4 | 15 | 20 | 27 |
| Protein (%) | 1 | 10 | 25 | 50 |
| Major Apoproteins | A, B, C | B, C, E | B | A |

70 percent of the fasting plasma triglycerides is present in the VLDL class (Ganda, 1982). Chylomicrons consist largely of absorbed dietary triglycerides in combination with phospholipids, cholesterol and apoproteins and are usually absent in the fasting state. VLDL which comprises mainly of endogenous triglycerides is synthesised in the liver and to a lesser extent in the small intestine (West and Shaw, 1981). With regard to LDL it appears that most of the LDL is derived as a product of VLDL metabolism but there is also evidence for direct secretion of LDL from the liver (Steinberg, 1981). The bulk of the HDL mass appears to arise from the interaction of nascent HDL secreted by the liver and intestine and lipids and proteins released during catabolism of triglyceride rich lipoproteins (Krauss, 1982). HDL can be subdivided into two subclasses, $HDL_2$ (density range 1.063 - 1.12) and $HDL_3$ (density range 1.12 - 1.21)(Anderson et al, 1978). In general, the concentration of HDL reflects the concentration of $HDL_2$ but not $HDL_3$ (Rifkind, 1981).

Whilst the major apoproteins of VLDL are apoproteins B, C and E, the major apoprotein of LDL is Apoprotein-B (Apo B) and Apoprotein A-1 is the major apoprotein of HDL. In addition to their roles in lipid transport, these apoproteins have additional functions in lipoprotein metabolism (Levy, 1981). The C-II fraction of Apoprotein C is an essential cofactor for the enzyme, lipoprotein lipase (LPL), whilst Apo A-1 activates the enzyme, lecithin cholesterol acyl transferase (LCAT) which is responsible for the esterification of cholesterol in plasma (Fielding et al, 1972; Eisenberg, 1979). In addition, Apo-B is important for the recognition of LDL by its cellular receptor (Fredrickson et al, 1978).

## 3.3 LIPOPROTEIN METABOLISM

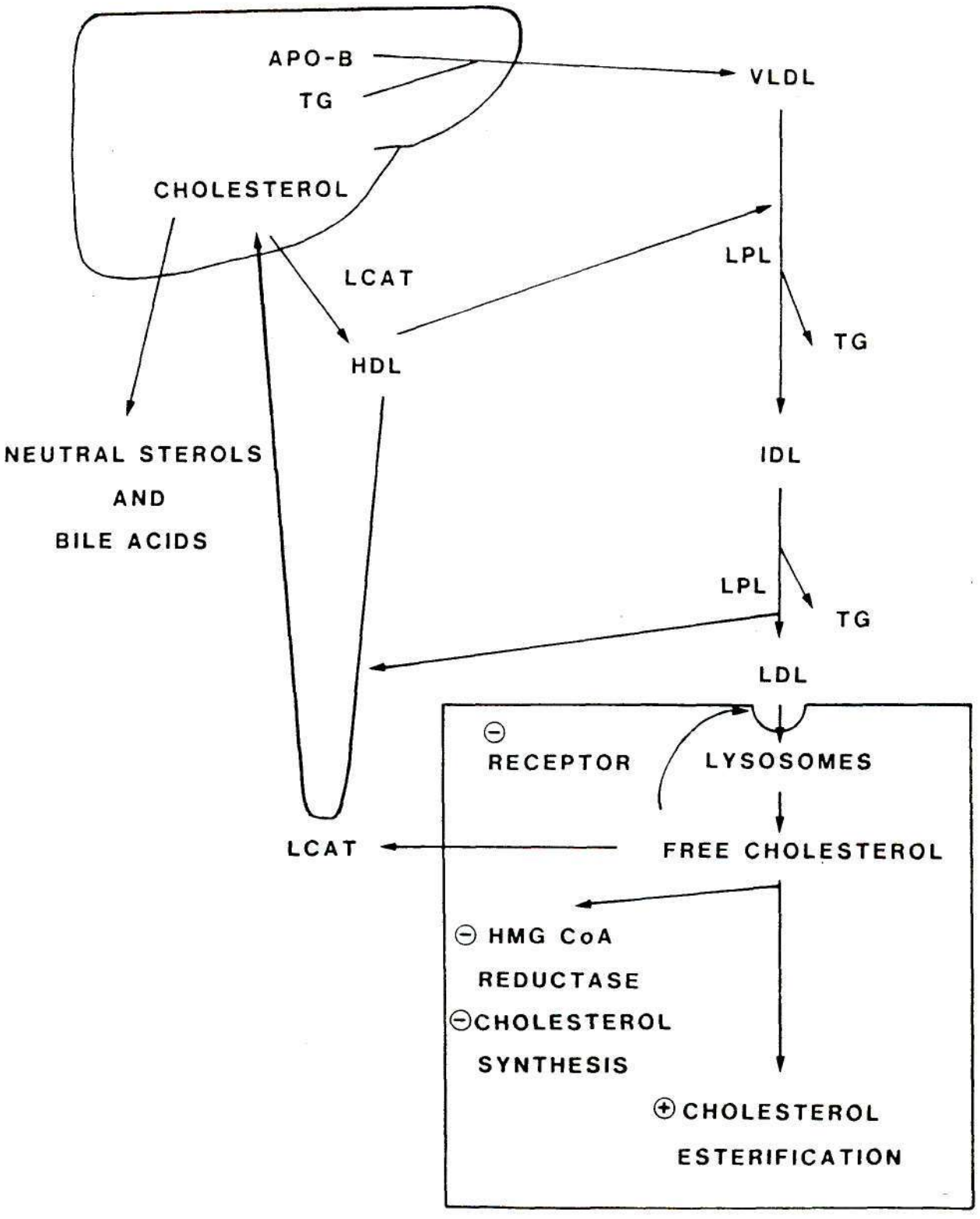
The intestine produces lipoproteins from dietary triglycerides and

cholesterol. Ingested triglycerides are hydrolysed to fatty acids and monoglycerides in the small intestine. These are taken up by mucosal cells, resynthesised into triglycerides and incorporated along with cholesterol into large lipoproteins called chylomicrons. After entering the bloodstream via the thoracic duct, the chylomicrons acquire Apo C and Apo E from HDL. (Havel, 1978; Fredrickson et al, 1978). The chylomicrons are cleared in the periphery at sites in the capillary beds of adipose tissue and muscle by lipoprotein lipase (LPL) which is activated by Apo C 11 (Havel et al, 1982) and the resultant fatty acids and glycerol enter the adipose tissue and muscle for energy storage or utilization (Miller, 1979). As the triglycerides are removed from chylomicrons, much of the Apo C, Apo A and surface lipids (cholesterol ester and phospholipids) are transferred to HDL (Havel, 1982). The remaining chylomicron "remnant" is then taken up by the liver where the lipid and protein constituents undergo lysosomal catabolism (Havel, 1982).

VLDL secreted by the liver and, to a minor extent, by the intestine carries endogenous lipid. VLDL-triglycerides are synthesised by esterification of fatty acids with glycerophosphate. The derivation of the other VLDL lipids is less well understood. The lipids destined for transport are packaged together with specific apoproteins (mainly Apo B) and secreted into the circulation. A scheme for the metabolism of VLDL is presented in Figure 3.1. The first phase of VLDL metabolism by LPL to yield IDL (intermediate density lipoprotein) is similar to the metabolism of chylomicrons to yield the chylomicron remnant. IDL which has a density range of 1.006 - 1.019 has two possible fates in man: it can be degraded further to LDL or it can be removed by the liver like the chylomicron remnant (Grundy, 1982). In normal man most IDL is converted to LDL (Smith et al, 1978; Eisenberg, 1979; Grundy, 1982). With the delipidation process, the VLDL particle experiences a

FIGURE 3.1

SCHEMATIC REPRESENTATION OF LIPOPROTEIN METABOLISM



steady relative increase in protein, phospholipid and cholesterol content (Levy, 1981). Furthermore, the precursor-product relationship of the Apo B in VLDL and LDL in this unidirectional conversion of VLDL to LDL has been established in man (Sigurdsson et al, 1975; Reardon et al, 1978). The removal of LDL from the circulation occurs largely by a receptor mediated process in peripheral tissues (Goldstein and Brown, 1975; Brown and Goldstein, 1976). Binding of LDL to its receptor is essentially irreversible resulting in the internalisation of LDL by endocytosis. After lysosomal degradation of the LDL, the free cholesterol within the cell exerts 3 major effects: it inhibits cellular cholesterol synthesis through repression of the activity of 3 hydroxyl-3 methylglutaryl coenzyme-A reductase (HMG Co A reductase); it enhances cholesterol esterification by activation of microsomal fatty acyl-Co A cholesterol acyltransferase and finally it suppresses LDL receptor synthesis thereby reducing further LDL uptake. In addition to this receptor mediated process, degradation of LDL also occurs through a receptor-independent scavenger mechanism (Havel et al, 1980).

Although many peripheral cells can take up and degrade LDL, they do not have the capacity to catabolize cholesterol which must be returned to the liver for excretion (Levy, 1981). The transport of cholesterol derived from LDL uptake and endogenous synthesis from peripheral tissues to the liver for catabolism and excretion was suggested by Glomset (1970) to be a function of HDL acting in concert with LCAT. Glomset's suggestion was prompted by his finding that HDL promotes an efflux of cholesterol from cultured peripheral cells (Glomset, 1970). It is presently believed that the redundant free cholesterol from the periphery enters HDL where it is esterified by LCAT which is complexed with the HDL; thereafter the esterified cholesterol is transported to the liver for excretion as bile acids or neutral sterols (Miller, 1980;

Fredrickson et al, 1978; Chait and Brunzell, 1981; Grundy, 1982). Although final proof of this concept is awaited, this remains the most likely mechanism of centripetal or reverse cholesterol transport.

## 3.4 LIPID AND LIPOPROTEIN ABERRATIONS IN DIABETES

### 3.4.1 Introduction

Interest in lipids and lipoproteins in diabetes stems primarily from the observation that diabetics have an increased propensity to accelerated atherogenesis with its attendant increased morbidity and mortality; hyperlipoproteinaemia is one of the major determinants of these atherosclerotic sequalae (Santen et al, 1972; Bierman and Brunzell, 1978; West, 1978; Kannel and McGee, 1979; Ganda, 1980). The state of the art concerning diabetes and hyperlipoproteinaemia is succintly summarised by the Joint Working Party on the Prevention of Coronary Artery Disease (1976): "There is much evidence of the increased frequency of hyperlipidaemia in the clinical diabetic, often associated with obesity and usually manifest by raised triglyceride levels and occasionally with hypercholesterolaemia. Elevation of both these lipids in non-diabetics is associated with increased future risk of coronary artery disease, but there has been little examination of such a predictive role in diabetics. Nor is it known whether a given degree of hyperlipidaemia constitutes a greater risk to the diabetic than to the non-diabetic. However, diabetics with arterial disease have higher serum lipids than those without."

The association of lactescent serum with diabetes was first noted in 1799 by Mariet and in the pre-insulin era lactescent serum was observed commonly in diabetic subjects (Stout et al, 1975). The frequency of hyperlipidaemia reported in diabetes has varied considerably among

the numerous series published since Blix (1926) recognised florid lipaemia in about 3 percent of untreated diabetes. In a recent review Ganda (1982) points out that the prevalence of hyperlipidaemia ranges from 20 to 70 percent in various series. In most studies the commonest lipid abnormality is an increased fasting plasma triglyceride level but increased plasma cholesterol levels have also been found in diabetics (Lowy and Barach, 1958; New et al, 1963; Albrink et al, 1963; Wilson et al, 1970; Santen et al, 1972; Hayes et al, 1972; Garcia et al, 1974; Kaufman et al, 1975; Steiner et al, 1978; Howard et al, 1978; Simpson et al, 1979).

### 3.4.2 Hypertriglyceridaemia and Diabetes

Severe hypertriglyceridaemia which may be accompanied by eruptive xanthomas and lipaemia retinalis was recognised commonly in the pre-insulin era especially in conjunction with ketoacidosis; it is infrequent today because patients with untreated IDDM are not often encountered (Shafir, 1975).

Since insulin plays a critical role in the production and clearance of triglycerides, hypertriglyceridaemia in diabetes can result theoretically from increased production and/or decreased utilization of triglycerides (Nikkila et al, 1977; Bierman and Brunzell, 1978; Ganda, 1980). Very poorly controlled diabetics, especially those who are ketotic, have the severest form of hypertriglyceridaemia which is a combination of chylomicronaemia and increased secretion of VLDL; the chylomicronaemia clears with adequate control of diabetes most often leaving a residual increase in VLDL. The predominant defect in these patients is considered to be an impaired clearance of triglycerides by lipoprotein lipase (LPL) (Blix, 1926; Bagdade et al, 1967a; Stout et al, 1975). In less severe IDDM the increased triglyceride levels are thought to

be due to a decreased activity of lipoprotein lipase (Nikkila and Kekki, 1973; Steiner et al, 1975; Nikkila et al, 1977; Brownlee and Cerami, 1981). By contrast, there is little evidence for increased VLDL-triglyceride production in IDDM which is well controlled (Nikkila and Kekki, 1973; Nikkila et al, 1977; Brownlee and Cerami, 1981).

In NIDDM the position in respect of hypertriglyceridaemia is more complicated. It is well recognised that hyperinsulinism and hypertriglyceridaemia are associated and that in the presence of increased insulin levels, hepatic VLDL-triglyceride production is increased (Topping and Mayes, 1972; Olefsky et al, 1974; Vranic et al, 1980). Obesity with its attendant hyperinsulinism could thus promote increased VLDL-triglyceride synthesis (Olefsky et al, 1974; Steiner, 1981). In NIDDM which is associated with a high prevalence of obesity and often hyperinsulinism, an important mechanism in generating hypertriglyceridaemia is increased VLDL-triglyceride secretion (Nikkila and Kekki, 1973; Shafir, 1975; Nikkila et al, 1977; Reaven and Greenfield, 1981; Kissebah et al, 1982). However, there is also evidence of impaired removal of triglycerides as a result of decreased lipoprotein lipase activity (Pykalisto et al, 1975; Nikkila et al, 1977; Taylor et al, 1979; Taskinen and Nikkila, 1979).

Thus, while in IDDM decreased clearance of triglycerides as a result of decreased LPL activity is the major factor in the generation of hypertriglyceridaemia, in NIDDM both increased production and decreased clearance have been incriminated.

### 3.4.3 Hypercholesterolaemia and Diabetes

Increased plasma cholesterol levels have been reported by some workers in diabetic patients (3.4.1). The increased plasma cholesterol is

commonly associated with an increased triglyceride level and reflects an increase in VLDL secretion. However, when the increased cholesterol level is the only abnormality it suggests an increase in LDL cholesterol (Bierman and Brunzell, 1978; Brownlee and Cerami, 1981). The mechanisms governing the increased cholesterol levels in those cases of diabetics in which it has been reported are less well investigated. Cholesterol absorption and sterol balance studies in diabetics have suggested that normolipaemic diabetics have a mild increase in cholesterol synthesis and a slight decrease in cholesterol degradation (Bennion and Grundy, 1977; Palumbo et al, 1978). Others have failed to report any significant effect of diabetes on sterol balance (Saudek and Brach, 1978) and LDL receptor activity as assessed by binding and degradation of $^{125}$I-LDL did not differ in diabetics when compared to controls (Chait et al, 1979). The concentration of the major apoprotein of LDL (Apo B) was no different in normolipaemic diabetics and controls (Schonfeld et al, 1974a).

Thus it is apparent that elevated serum cholesterol levels do occur in diabetics and are usually due to increased VLDL levels since most of these patients have increased triglyceride levels also; however, increased cholesterol levels with normal triglyceride levels can occur and is usually due to an increase in LDL.

### 3.4.4 High Density Lipoproteins and Diabetes

Major interest has recently focussed on high density lipoproteins (HDL) in diabetics because of the independent inverse correlation between HDL-cholesterol and atherosclerotic disease (Miller and Miller, 1975; Rhoads et al, 1976; Castelli et al, 1977; Miller et al, 1977; Goldbourt and Medalie, 1979; Kannel et al, 1979). The accumulated data in human diabetes has shown that HDL-cholesterol levels in

diabetic patients may be low, normal or high depending mainly on the type of diabetes and the treatment offered (Ganda, 1980; Nikkila, 1981). Variations in total HDL-cholesterol levels mainly reflect the $HDL_2$ subfraction which is the component most closely related to atherosclerotic vascular disease (Hammett et al, 1979; Miller, 1980; Rifkind, 1981). However, it should be pointed out that while the recent upsurge in interest in HDL was initiated by Miller and Miller (1975), low levels of HDL-cholesterol had been demonstrated in diabetics over 30 years ago (Bar et al, 1951).

### 3.4.4.1 High Density Lipoproteins in IDDM

In newly detected untreated IDDM, HDL-cholesterol levels are consistently decreased but rise slowly after initiation of insulin therapy (Nikkila, 1981). However, HDL-cholesterol levels in patients with IDDM on conventional insulin therapy are either normal or increased (Calvert et al, 1978; Nikkila and Hormilla, 1978; Mattock et al, 1979; Durrington, 1980; Sosenko et al, 1980; Eckel et al, 1981a; Lisch and Sailer, 1981; Nikkila, 1981). Many studies have shown that HDL-cholesterol levels seem to be largely independent of the degree of diabetic control (Kennedy et al, 1978; Elkeles et al, 1978; Mattock et al, 1979; Durrington, 1980; Sosenko et al, 1980). However, a significant association between HDL-cholesterol and either glucose or glycosylated haemoglobin ($HbA_1$) levels have been reported in a few studies; most of these studies have reported a significant negative correlation (Calvert et al, 1978; Lopes-Virella et al, 1977a; Lisch and Sailer, 1981); whilst a positive correlation between $HbA_1$ and HDL-cholesterol has been reported in IDDM (Klujber et al, 1979). These findings would suggest that the relationship is probably indirect, being accounted for by other factors, e.g. insulin dosage (Nikkila, 1981).

It is likely that the increased HDL-cholesterol levels in IDDM are related to the peripheral hyperinsulinism produced by the insulin therapy; the thesis being that the exogenous hyperinsulinaemia stimulates lipoprotein lipase which in turn results in accelerated turnover of triglyceride rich lipoproteins with subsequent increase in intravascular HDL formation (Nikkila, 1981). The fact that there is a positive correlation between HDL-cholesterol and lipoprotein lipase in IDDM lends further support to this theory (Nikkila and Hormilla, 1978).

### 3.4.4.2 High Density Lipoproteins in NIDDM

In contrast to IDDM, the mean HDL-cholesterol level reported in NIDDM has been similar to or lower than the corresponding value in a non-diabetic population (Lopes-Virella et al, 1977a; Calvert et al, 1978; Howard et al, 1978; Kennedy et al, 1979; Mattock et al, 1979; Durrington et al, 1980; Lisch and Sailer, 1981; Taylor et al, 1981). Since patients with NIDDM exhibit a high prevalence of obesity and hypertriglyceridaemia and plasma HDL-cholesterol levels are inversely related to both relative body mass and serum triglycerides, it is important to separate the effects of diabetes from those of obesity and hypertriglyceridaemia (Gleuck et al, 1976; Shaeffer et al, 1978; Kennedy et al, 1978; Miller, 1980; Glomset, 1980; Nikkila, 1981). Although the inverse correlation between HDL-cholesterol and triglycerides has been corroborated by numerous investigators, some have failed to demonstrate the inverse correlation between HDL-cholesterol and obesity (Lopes-Virella et al, 1977a; Howard et al, 1978; Mattock et al, 1979; Taylor et al, 1981; Taskinen et al, 1982). Schonfeld et al (1974) observed decreased HDL-cholesterol levels in diabetics with and without hypertriglyceridaemia. A well controlled study on HLD levels in NIDDM was recently reported (Taylor et al, 1981).

Patients and controls were carefully matched for age, sex, obesity, cigarette smoking and alcohol consumption; while the mean triglyceride levels in the two groups were not different, the HDL-cholesterol levels were significantly lower in the NIDDM group. On dividing the diabetics and controls into three subgroups depending on the fasting triglyceride levels ( <1,5; 1,5 - 3; > 3 mmol/ℓ), they showed that although there was no significant difference in the triglyceride levels between diabetics and controls in each subgroup, HDL-cholesterol levels were significantly lower in the diabetics. Apo A-1 concentrations were, however, the same in the diabetic and control groups, which did not confirm a previous report of decreased HDL-cholesterol levels and Apo A-1 concentrations in NIDDM (Goldberg and Rubenstein, 1980).

A further problem in studying HDL-cholesterol levels in NIDDM is that therapy might influence levels. Low levels have been reported in diabetics treated with sulphonylureas but not in those controlled by diet (Calvert et al, 1978; Kennedy et al, 1978; Baron et al, 1978; Lisch and Sailer, 1981). In contrast to these findings, a significant increase in HDL-cholesterol has been reported after one year of sulphonylurea therapy (Paisley et al, 1978). The issue of the possible effects of sulphonylureas on HDL-cholesterol requires further prospective studies for clarification (Nikkila, 1981).

In addition, the relationship between HDL-cholesterol levels and the glycaemic status in NIDDM is also conflicting. Whereas some reports of a significant inverse correlation between HDL-cholesterol and either the plasma glucose or glycosylated haemoglobin ($HbA_1$) levels have appeared (Lopes-Virella et al, 1977a; Calvert et al, 1978; Lisch and Sailer, 1981; Taskinen et al, 1982), most studies have failed to demonstrate such an inverse relationship (Kennedy et al, 1978; Yudkin et al, 1978; Mattock et al, 1979; Sommariva et al,

1980; Aleyassine et al, 1980; Taylor et al, 1981; Rendel et al, 1982).

The underlying mechanisms resulting in the changes in HDL in NIDDM are not resolved (Nikkila, 1981). It seems likely that the changes in HDL are related to the obesity, hypertriglyceridaemia and insulin resistance which commonly accompany patients with NIDDM. Insulin resistance in the periphery results in subnormal lipoprotein lipase activity (Taylor et al, 1979; Taskinen et al, 1982) with consequential decrease in HDL-cholesterol levels (Nikkila, 1981; Taylor et al, 1981). Supporting this proposition is the finding that HDL-cholesterol and lipoprotein lipase activity are positively related (Taskinen et al, 1982).

### 3.4.5 Lipids and Vascular Disease in Diabetes

As in the non-diabetic, hyperlipidaemia is a major risk factor in the genesis of accelerated atherogenesis (macroangiopathy) in the diabetic (Steiner, 1978; Bierman and Brunzell, 1978; Ganda, 1980; Alberti and Press, 1982). Increased lipid levels have been found in diabetics with macrovascular disease when compared to both diabetics without macrovascular disease and non-diabetic controls. Whilst hypercholesterolaemia has been reported in patients with macrovascular disease, hypertriglyceridaemia is the more frequent finding (Lowy and Barach, 1958; Albrink et al, 1963; New et al, 1963; Ahuja et al, 1969; Santen et al, 1972; Reckless et al, 1978; Beach et al, 1979; Brownlee and Cerami, 1982). More recent studies have also shown that diabetics with macrovascular disease have lower levels of HDL-cholesterol and higher levels of LDL-cholesterol (Lopes-Virella et al, 1977b; Reckless et al, 1978; Beach et al, 1979). The consensus would thus favour a role for lipid aberrations in the genesis of macrovascular disease.

When the data on lipid and lipoprotein levels in diabetics with microvascular disease is examined a different situation seems to obtain. Numerous studies have failed to show a significant relationship between lipid and lipoproteins with microvascular disease (Lowy and Barach, 1958; Albrink et al, 1963; New et al, 1963; Bergquist, 1970; Elkeles et al, 1971). However, increased lipid levels manifesting primarily as hypertriglyceridaemia have been found in diabetics with microangiopathy (Yoshida et al, 1967; Ahuja et al, 1969; Bhan et al, 1971; Hart et al, 1971; Martin and Warne, 1975; Kissebah et al, 1975). In a recent study increased levels of LDL-cholesterol have been found in diabetics with retinopathy; HDL-cholesterol and triglyceride levels were not disturbed (Dornan et al, 1982). Judging from the accumulated data one would concur with Kohner (1977) who stated that hyperlipidaemia is not considered to play a major role in the pathogenesis of diabetic microangiopathy. Obviously more detailed studies, especially the estimation of lipoproteins, would be required to establish the relationship between lipid aberrations and diabetic microvascular disease.

### 3.4.6 Lipids and Lipoproteins in Non Insulin Dependent Diabetes in the Young

It would appear that no group of workers have explored the lipid and lipoprotein status of patients with NIDDY with and without vascular disease and it is clearly necessary that such investigations be undertaken.

# CHAPTER 4

# CLINICAL FEATURES OF INDIAN PATIENTS WITH NON-INSULIN DEPENDENT DIABETES IN THE YOUNG

## 4.1 PURPOSE OF THE STUDY

NIDDY as a clinical syndrome does apparently occur in South Africa but as is clear from the literature reviewed (Section 1.4), it has been almost casually studied. The purpose of the present study is to select, in the first instance, patients from the Indian population who meet defined criteria to be classified as having NIDDY and, having collected the patients, the clinical features of the disorder will be detailed.

## 4.2 PATIENTS

Patients used in this study attended the diabetic clinics of the R.K. Khan and King Edward VIII Hospitals to which the author has been attached since 1978.

### 4.2.1 Criteria for the Diagnosis of Non-Insulin Dependent Diabetes in the Young

The patients were categorised as having NIDDY if they met the following criteria: onset of diabetes before the age of 35 years, symptomatic presentation, prevention of ketonuria and satisfactory [illegible] therapy for at least one year. [illegible] patients had to [illegible] the

ERRATUM

Page 73 : last line to read:

'glucose ≥ 12 m mol/l, 2 h after taking 100 g glucose'

(1978 - 1980) 85 Indian patients were found at the clinics who met all the above criteria and were entered into the study.

## 4.3 STUDY PROCEDURE

### 4.3.1 History and Clinical Examination

A careful history was taken from all patients. For the purpose of the present study, age at presentation and the age at which symptomatic diabetes was first positively diagnosed were noted; particular attention was paid to the duration of diabetes. A detailed family history of diabetes as far as possible was taken, particularly the occurrence of diabetes among forebears. All patients were questioned directly about symptoms which could suggest the presence of vascular complications of diabetes (angina pectoris, intermittent claudication, strokes, visual disturbances and urinary symptoms).

Full systematic examination was performed on all patients by the author and a physician. The aspects relevant to this study which received particular attention were: sex, height (mm), mass (kg), systolic and diastolic (fourth phase) 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 urine examination for glucose, ketones and protein performed.

### 4.3.2 Special Investigations

A 12 lead electrocardiogram (ECG) was recorded at rest in all 85 patients. In addition to the fundal examination performed in the clinic, fundal photographs were taken after full mydriasis in the

Department of Ophthalmology and, when indicated, flourescein angiography was performed. Renal function was assessed in all patients by a 24 hour urine protein excretion, serum creatinine and $\beta_2$-microglobulin levels. The majority of patients also had their glomerular filtration rate assessed by the single shot $^{51}$Cr-EDTA technique (Chantler et al, 1969).

## 4.4 Analytical Methods

The methods used to determine plasma glucose, creatinine, $\beta_2$-microglobulin concentration and urinary protein excretion are described in Appendix A: plasma glucose (A-1), creatinine (A-2), $\beta_2$-microglobulin (A-3) and urinary protein (A-4).

## 4.5 Results

### 4.5.1 Sex Ratio, Age of Onset, Duration of Diabetes and Body Mass of Patients with NIDDY

All patients presented with symptomatic diabetes (polyuria, polydypsia, lethargy, pruritis vulvae and skin infections). Of the 85 patients studied 68 (80%) were women. The age at which diabetes was positively diagnosed ranged from 14 years to 34 years, with a mean age of onset of 26,8 years; the majority of patients were diagnosed between the ages of 21 and 34 years (Figure 4.1). Whilst the mean duration of diabetes was 5,9 years, the range was from 1 to 35 years, but the majority of patients (83%) had diabetes for less than 10 years (Figure 4.2). Desirable mass was obtained from tables of desirable mass for men and women (Documenta Geigy, 1962); for a given height the mean of the range for "medium frame" was considered as 100% (Seltzer et al, 1967). The definition of obesity was an actual mass $\geq$ 120% of desirable mass (National Diabetes Data Group, 1979).

Obesity was present in 42 patients (50%) and the mean percentage desirable mass of the entire group was 122,2% (Figure 4.3). It can also be seen from Figure 4.3 that, although the majority of patients exceeded their desirable mass, 19 percent had a desirable mass equal to or less than 100 percent of the ideal.

### 4.5.2 Familial Occurrence of Diabetes in Patients with NIDDY

The prevalence of diabetes in the families of patients with NIDDY is summarised in Table 4.1. A positive family history of diabetes was present in 71 patients (84%); 64 patients (75%) had a diabetic parent, the more commonly affected parent was the mother (mother diabetic in 60% and father diabetic in 41%). Both parents were diabetic in 24% of the patients. Whilst 45% of patients had a diabetic sibling, 40% had both a diabetic sibling and parent. Three generation vertical transmission of diabetes was present in 7 patients (8%).

### 4.5.3 Vascular and Other Related Complications

To date, none of the patients in this series has presented with either diabetic ketoacidosis or hyperosmolar non-ketotic coma. The frequency of vascular and other related complications are shown in Table 4.2 and the relevant clinical data of the patients with vascular disease in Table 4.3. The mean age of the patients with vascular complications was 4,3 years greater than for the whole group; mean duration of diabetes was 5,2 years longer than the duration in the whole group; 19% of the patients with vascular disease were men, virtually the same percentage as in the whole group (20%) and the mean percentage of desirable mass was similar to that of the whole group.

#### 4.5.3.1 Macrovascular Complications

Ischaemic heart disease was diagnosed if there was a history of angina pectoris, or a previous myocardial infarction together with compatible electrocardiographic changes. Supporting ECG evidence included ischaemic changes, left bundle branch block and pathological left axis deviation. According to these criteria, ischaemic heart disease was present in 3 patients (4%). While none of the patients had evidence of peripheral vascular disease, one patient had evidence of cerebrovascular disease.

#### 4.5.3.2 Microvascular Complications

##### 4.5.3.2.1 Retinopathy

The commonest vascular complication present in NIDDY was retinopathy which was found in 14 patients (17%). Of the 14 patients with retinopathy, 12 had background changes only whilst the remaining 2 also had proliferative changes. Patients with hypertension and retinal haemorrhages and exudates as the only changes were not considered to have diabetic retinopathy.

##### 4.5.3.2.2 Nephropathy

Nephropathy was diagnosed if the 24 hour urine protein excretion was in excess of 0,5 grams. Six patients (7%) had nephropathy according to this criterion and in 5 it was associated with retinopathy whereas the sixth patient had bilateral cataracts and her retinae could not be visualised. In addition to having a 24 hour urine protein excretion in excess of 0,5 grams, the $^{51}$Cr-EDTA GFR was less than 80 ml/min in all 6 patients (Table 4.4). Serum creatinine concentrations were increased in 3 patients ( > 133 $\mu mol/\ell$) and serum $\beta_2$-microglobulin levels

were increased in all 6 patients (upper limit of reference range 2,3 mg/ℓ). Although the fundi could not be visualised in the one patient with nephropathy because of bilateral cataracts, an alternative cause of nephropathy could not be established by clinical examination or other special investigations.

#### 4.5.3.3 Hypertension in NIDDY

The W.H.O. definition of hypertension was adopted (Strasser, 1972): systolic pressure $\geq$ 160 mm Hg and/or a diastolic pressure $\geq$ 95 mm Hg in subjects over 30 years; in subjects under 30 years the values were taken as $\geq$ 150 mm Hg systolic and/or $\geq$ 90 mm Hg diastolic. A patient was only labelled hypertensive when increased blood pressure was recorded on two different occasions at least one month apart. Using these criteria, hypertension was present in 15 patients (18%); 5 of the 16 patients with vascular complications had hypertension (31%).

#### 4.5.3.4 Peripheral Neuropathy

Peripheral neuropathy as diagnosed by history and clinical examination was present in 20 patients (24%). This was the commonest chronic complication present.

#### 4.5.3.5 Infections

The patients were prone to infections and the common infections encountered were urinary tract infections, monilial vaginitis and infections of the skin. These infections were treated appropriately with anti-bacterial and anti-fungal agents and posed no major problems. In addition, none of the patients were precipitated into ketosis by infections.

FIGURE 4.1

THE AGE OF ONSET OF DIABETES IN THE PATIENTS WITH NIDDY

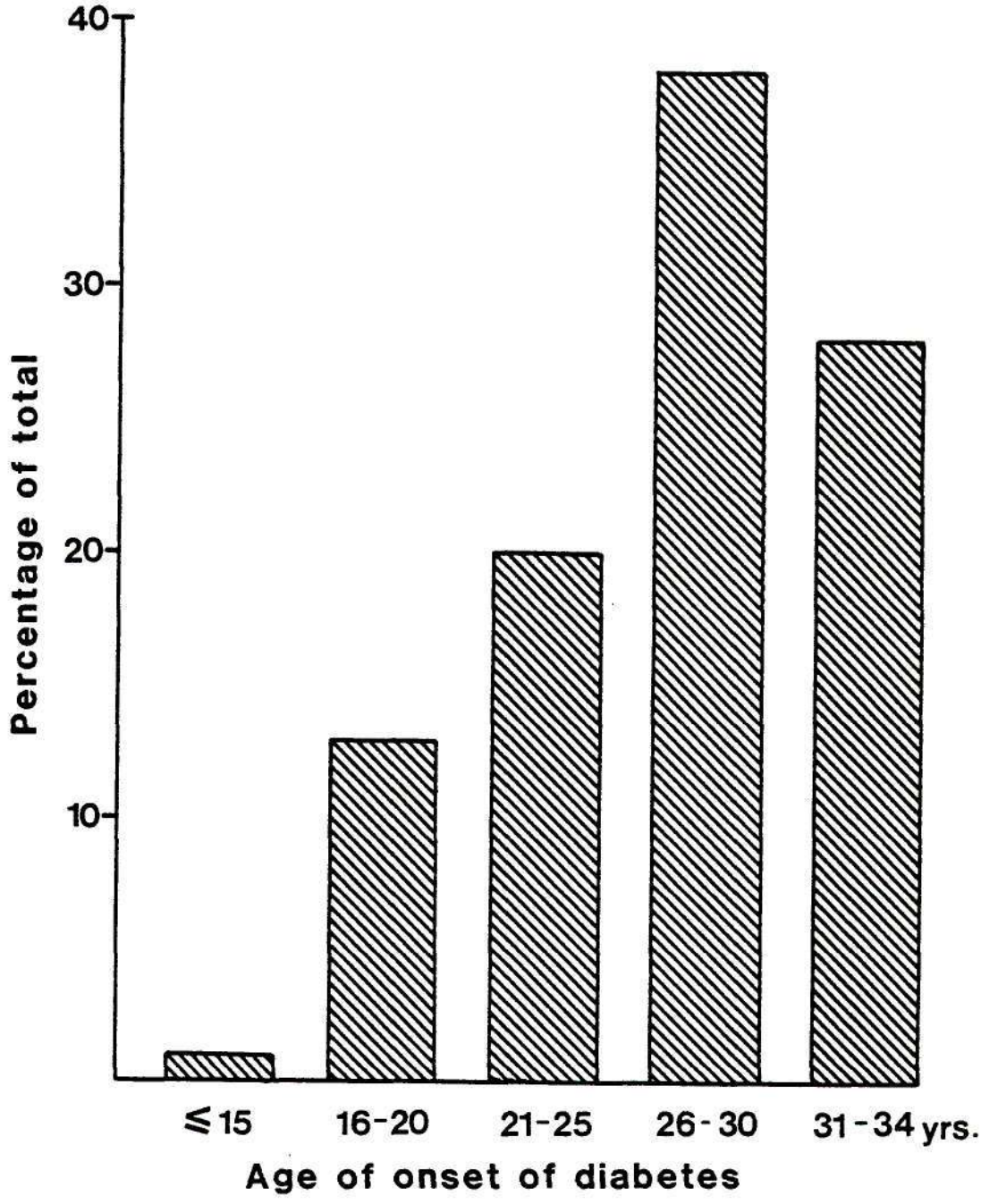


FIGURE 4.2

THE DURATION OF DIABETES IN THE PATIENTS WITH NIDDY

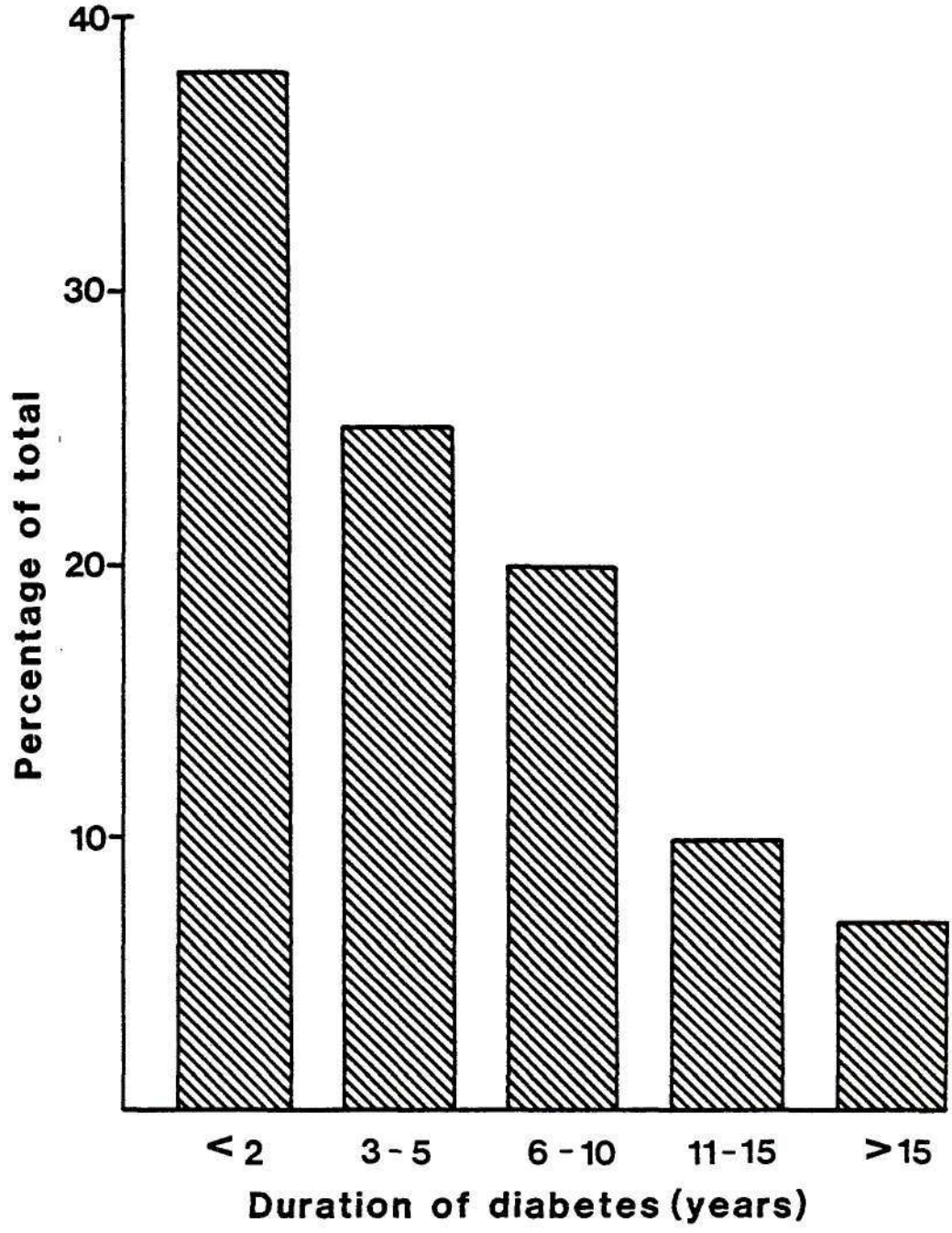


FIGURE 4.3

THE PERCENT DESIRABLE MASS OF THE PATIENTS WITH NIDDY

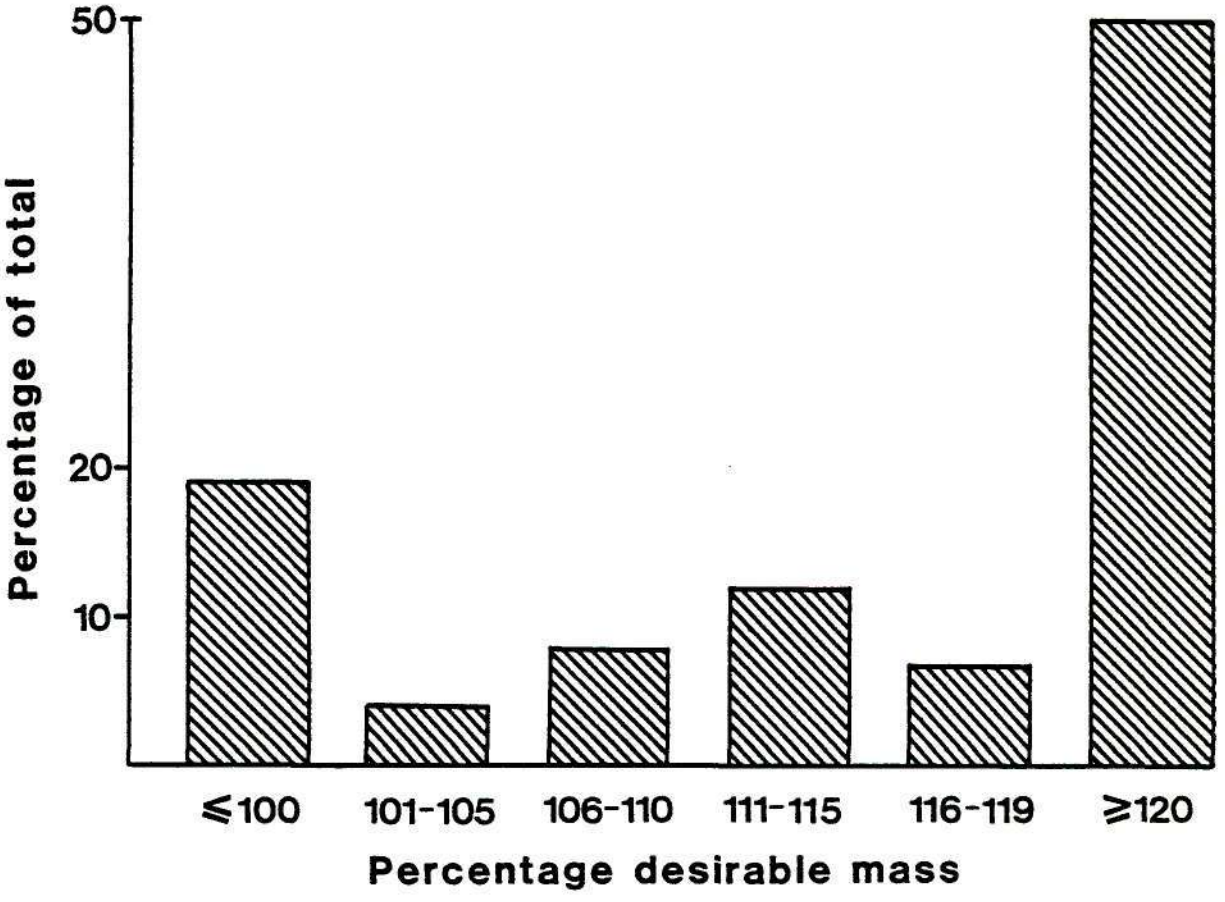


TABLE 4.1

PREVALENCE OF DIABETES IN THE FAMILIES OF PATIENTS WITH NIDDY

| | No. | (%) |
|---|---|---|
| Positive family history | 71 | (84) |
| Diabetic parent | 64 | (75) |
| Mother diabetic | 51 | (60) |
| Father diabetic | 35 | (41) |
| Both parents diabetic | 20 | (24) |
| Sibling diabetic | 38 | (45) |
| Sibling and parent diabetic | 34 | (40) |
| Grandparent and parent diabetic | 7 | ( 8) |

TABLE 4.2

THE FREQUENCY AND NATURE OF VASCULAR COMPLICATIONS, HYPERTENSION AND PERIPHERAL NEUROPATHY IN NON-INSULIN DEPENDENT DIABETES IN THE YOUNG

| | Number of Patients | (%) |
|---|---|---|
| MICROANGIOPATHY | 15 | (18) |
| RETINOPATHY | 14 | (17) |
| (i) background | 12 | |
| (ii) proliferative | 2 | |
| NEPHROPATHY | 6 | (7) |
| MACROANGIOPATHY | 4 | (5) |
| Ischaemic heart disease | 3 | |
| Cerebro-vascular disease | 1 | |
| Peripheral vascular disease | 0 | |
| PERIPHERAL NEUROPATHY | 20 | (24) |
| HYPERTENSION | 15 | (18) |

TABLE 4.3

RELEVANT CLINICAL INFORMATION ON PATIENTS WITH VASCULAR COMPLICATIONS

| Patient No. | Sex | Age (years) | Duration (years) | BP (mmHg) | % Ideal Body Mass |
|---|---|---|---|---|---|
| 1 | F | 36 | 6 | 140/90 | 134 |
| 2 | F | 34 | 14 | 150/100 | 115 |
| 3 | M | 54 | 35 | 150/90 | 123 |
| 4 | F | 28 | 8 | 130/90 | 157 |
| 5 | F | 38 | 15 | 110/70 | 145 |
| 6 | F | 41 | 15 | 120/90 | 137 |
| 7 | F | 27 | 6 | 130/90 | 90 |
| 8 | F | 45 | 15 | 180/100 | 95 |
| 9 | F | 38 | 10 | 150/90 | 105 |
| 10 | F | 38 | 14 | 120/85 | 124 |
| 11 | F | 24 | 1 | 140/90 | 95 |
| 12 | F | 27 | 9 | 120/80 | 113 |
| 13 | F | 30 | 1 | 120/80 | 144 |
| 14 | M | 39 | 7 | 120/90 | 118 |
| 15 | F | 36 | 5 | 110/80 | 111 |
| 16 | M | 49 | 16 | 140/80 | 105 |
| Mean | | 36,5 | 11,1 | 133/92 | 119,4 |
| Range | | (24-54) | (1-35) | (110/70-180/100) | (90-157) |
| NIDDY (all patients) | | | | | |
| Mean | | 32,2 | 5,9 | | 122,2 |
| Range | | (14-54) | (1-35) | | (86-171) |

TABLE 4.4

ASSESSMENT OF RENAL FUNCTION IN NIDDY PATIENTS WITH NEPHROPATHY

| Patient | 24 Urine Protein (G) | Serum Creatinine (μmol/L) | Glomerular Filtration Rate (ml/min) | Serum $\beta_2$-Micro-globulin (mg/L) |
|---|---|---|---|---|
| S S | 1,86 | 115 | 52 | 2,81 |
| P N | 6,05 | 370 | 18 | 22,5 |
| P P | 1,98 | 65 | 66 | 2,4 |
| N S | 0,91 | 150 | 70 | 2,91 |
| F S | 2,30 | 140 | 24 | 5,03 |
| N S | 0,60 | 125 | 70 | 2,71 |

## 4.6 DISCUSSION

The rigid diagnostic criteria adopted in the selection of patients leave no doubt whatsoever that NIDDY is an entity in the spectrum of diabetic types found in the local Indian community and confirms the earlier reports discussed in Section 1.4. The choice of 35 years as the upper limit of youth reflected the prerogatives exercised by earlier workers rather than any conviction, but is necessary for broad comparative purposes (Campbell, 1960; Fajans and Conn, 1960; Fajans et al, 1976).

Clinically, the patients were in many respects similar to patients with NIDDY described elsewhere but there were also differences. It is relevant to highlight some of these similarities and differences.

In common with most other reports on the condition, women were the dominant sex (Campbell, 1960; Tattersall, 1974; Pond, 1975; Barbosa et al, 1978). However, it would be quite wrong to conclude that there is sexual dimorphism in the prevalence of NIDDY because it is well known among Indians in Natal that women tend to support hospital clinics whereas men prefer private or company medical services. This personal bias may well account for the dominance of women in this group.

An aspect which should be stressed is that diabetes was first diagnosed in most patients between the ages of 21 and 34 years; the youngest patient being 14 years old. NIDDY in the Indian community is therefore essentially a disorder found in young adults and not in children and confirms the observation of Jackson (1978b) that NIDDY is not found in Indians during childhood. However, this finding is at variance with most previous reports, for the majority of patients in these series were under 25 years of age with the youngest patient being diagnosed

at the age of 3 years (Chiumello et al, 1969; Fajans et al, 1969; Tattersall, 1974; Pond, 1975; Tulloch and Adamson, 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978; Panzram and Adolph, 1981).

In the present series of 85 patients only 50% were obese by definition, but the mean mass of the group was 122,2% of the desirable which indicates that NIDDY as a group could be categorised as obese. This finding contrasts with most previous reports on NIDDY in which the percentage obese patients were substantially lower (0 - 23%) than reported here (Tattersall, 1974; Fajans et al, 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978). Jackson (1978b) pointed out that in the local Indian population, increasing body mass is associated with an increasing frequency of diabetes. However, such considerations do not explain why the present group differs so much from patients described by others. Whereas it is known that increasing body mass is not necessarily associated with an increasing frequency of diabetes in all population groups, the Indians are not unique in this association (Jackson, 1978b). Campbell (1960) also commented on the obesity of his patients, but did not define obesity and hence no direct comparisons are possible. On the evidence it should however be concluded that in Indians with NIDDY, the patients tend to obesity.

By far the most striking clinical finding in Indian patients with NIDDY is the prevalence of diabetes in the families of the patients (84%). In the present series 75% of patients had a diabetic parent and 45% a diabetic sibling as opposed to 85% with a diabetic parent and 53% of tested siblings having diabetes in the report by Tattersall and Fajans (1975). In these respects the groups therefore closely resemble each other. The present study differs from previous reports in that only 8% of the patients were found to have three generation transmission of diabetes as opposed to the 46% reported in previous series (Tatter-

sall, 1974; Tattersall and Fajans, 1975). However, it should be borne in mind that the socio-economic circumstances of Indians 40 - 60 years ago and the availability of medical services generally in South Africa were such that it would be surprising if Indians of the present generation knew what medical disorders their grandparents suffered. Hence, great reliance cannot be placed on this aspect of the history. As pointed out in Section 1.3.1, Tattersall (1976) argued that transmission of diabetes through three consecutive generations favours a dominant mode of inheritance and he did indeed interpret the fact that 46% of patients gave such a history as indicative of autosomal dominant transmission in NIDDY. But there is, of course, quite a different side to the coin. Even if reliable family histories could be obtained in the Indian patients with NIDDY, the problem of studying the inheritance of NIDDY in a population in which diabetes has a high prevalence is indeed formidable, if not impossible. This aspect has already been dealt with in Section 1.4, and in the population under consideration it is unlikely that any major advance in the inheritance of NIDDY will be forthcoming until such time as true genetic markers are identifiable. Yet another aspect is that the present study is not alone in failing to demonstrate unequivocal evidence for autosomal dominant inheritance. Recently, workers from Germany found that 75% of their 58 patients with NIDDY had a diabetic parent, 14% a diabetic sibling and 30% vertical transmission of diabetes through three generations (Panzram and Adolph, 1981). They interpreted their findings as pointing to heterogeneity in the mode of inheritance and hence heterogeneity of diabetic type. Manifestly, their data correspond more closely to the findings presented in Indian patients with NIDDY. Indeed it would be inconceivable in the Indian community if there was not genetic transfer of the various types of diabetes and it is conceivable that people

are born with a genome predisposing to more than one type of diabetes. Be that as it may, the familial aggregation of diabetes in the present study is unique enough to differentiate the condition from NIDDM in the older age group, since the presence of a positive family history in 84% is far in excess of the 29 to 48% reported in various series in older Indian patients with NIDDM (Cosnett, 1959; Campbell and McKechnie, 1961; Walker et al, 1963; Campbell, 1963).

Despite the usual proneness of the NIDDY patients to intercurrent infections of various kinds, not a single patient ever presented with ketonuria which is clearly a remarkable feature, having regard to the fact that the group as a whole were severe diabetics. However, patients with NIDDY do not stand alone in this regard for previous workers have highlighted the rarity of ketosis in Indian diabetics, both IDDM and NIDDM (Cosnett, 1959; Campbell, 1960; Jackson, 1978b).

The overall prevalence of vascular complications was 19% in the patients with NIDDY which is significant and supports the impression expressed by Jackson (1978a) that vascular complications are not uncommon in Indian patients with NIDDY. Mean age and duration of diabetes in the patients with vascular complications were greater than that of the whole group. However, many patients in whom the duration of diabetes exceeded the mean duration of those with complications had no evidence of vascular disease. Microvascular involvement was manifestly the commonest complication and 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.

Microangiopathy presented mainly as retinopathy and this reflects the experience of others reporting on NIDDY. However, the overall prevalence of retinopathy (17%) seems greater than previously reported

(Section 1.6.2). In the present series, 12 of the 14 patients with retinopathy had background changes which is in agreement with those reports in which retinopathy was found; where present, background changes were usually seen, whilst proliferative retinopathy was a rare finding (Tattersall, 1974; Tulloch and Adamson, 1976; Pyke, 1977; Johansen and Gregersen, 1977; Barbosa et al, 1978; Fajans et al, 1978; Kobberling et al, 1980). Thus, in keeping with previous reports, retinopathy in Indian NIDDY is a relatively benign degenerative process. Whereas retinopathy was present in 17% of the patients with NIDDY, Indian patients with NIDDM in South Africa appear to have a prevalence of 27 to 61% in different series (Cosnett, 1959; McKechnie, 1964; Seftel, 1967; Jackson et al, 1970). Although these broad comparisons suggest that retinopathy is a less frequent complication in NIDDY than NIDDM, it should be borne in mind that the duration and severity of diabetes have marked effects on the expression of vascular disease (Pirart, 1978; West, 1978; Skyler, 1979) and both duration and severity varied in these different studies. The patients with NIDDY were certainly severe diabetics, when untreated, but the reasonable conclusion cannot be pressed further than that on the whole retinopathy is mild in NIDDY.

On the basis of the criteria used, 7% of the patients had nephropathy and this finding contrasts with previous reports on NIDDY. 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; Kobberling et al, 1980). Indeed, a search of the literature has revealed a total of only 6 patients with nephropathy (Soler et al, 1969; Johansen et al, 1973; Steel et al, 1975; Fajans et al, 1978; Bigler and Adler, 1981). One reason for the higher prevalence of nephropathy in the current study is that a thorough and detailed search was made for renal

involvement. By contrast, as pointed out in Section 1.6.2.2, no previous studies really addressed this aspect in great depth. The interesting aspect is, however, that despite this thorough search for renal involvement, only 7% of the patients were affected, which contrasts with a prevalence of 11 to 21% in older Indian patients with NIDDM (Cosnett, 1959; Seftel, 1967; Jackson et al, 1970). However, inasmuch as the basis of the lesion in nephropathy is vascular, it is not possible to make direct comparisons between NIDDM and NIDDY for the reasons set out when retinopathy was considered. It can merely be recorded that, as in NIDDM in the older patients, renal involvement can be expected in NIDDY.

Macroangiopathy was found in only 5% of patients in the present series which is much lower than the 23% reported by Fajans et al (1978). However, it is similar to most other reports on NIDDY for these workers found that very few patients have macrovascular disease (Tattersall, 1974; Tulloch and Adamson, 1976; Johansen and Gregersen, 1977; Barbosa et al, 1978; Kobberling et al, 1980). In fact, none of the patients in the present series had peripheral vascular disease which contrasts with the experience of Fajans et al (1978) who found that peripheral vascular involvement was the commonest form of macrovascular disease in their patients. However, Indian diabetics in South Africa appear to have some resistance to peripheral vascular disease and Jackson et al (1970) commented on the fact that peripheral vascular disease is much less common in Indian diabetics than in their White counterparts. Consequently, the absence of peripheral vascular disease in the patients with NIDDY in this study may relate to this peculiarity. However, the overall prevalence of macrovascular disease in the patients with NIDDY is much lower than in Indian patients with NIDDM in whom ischaemic heart disease may affect as much as 30% of patients, with peripheral vascular

disease and cerebrovascular disease affecting approximately 10% and 6% of patients respectively (Cosnett, 1959; Jackson et al, 1970).

Overtly, the presence of hypertension in 18% of the patients appears to be no different from the 19% prevalence reported by Seedat et al (1978) in the Indian population of Durban. However, the NIDDY patients were far younger than the age range in the Indian population reported by Seedat et al (1978). No comparisons can be made with the previous reports on NIDDY since none commented on the prevalence of hypertension. While the presence of hypertension in 18% of the present series accords with the 21 - 23% reported in two studies in much older Indian patients with NIDDM (Seftel, 1967; Jackson et al, 1970) it is much lower than that reported by Cosnett (1959) who found that 30% of his patients with NIDDM were hypertensive. The more significant finding in the present study is the fact that 31% of the NIDDY patients with vascular disease were hypertensive suggesting that hypertension is a serious additional risk factor in the development of vascular complications in NIDDY as in other diabetics (Williamson and Kilo, 1980).

The commonest chronic complication in NIDDY was neuropathy (24%) and this is in agreement with studies in Indian patients with NIDDY reported by Thandroyen et al (1980) and Asmal et al (1981). They found that 36% and 31% of the patients in their respective groups had peripheral neuropathy. However, a review of the literature reveals only a further 3 NIDDY patients with neuropathy (Soler et al, 1969; Tattersall, 1974; Bigler and Adler, 1981). The high prevalence in Indians with NIDDY is in agreement with the 38% prevalence of neuropathy reported by Jackson et al (1970) in much older NIDDM. It would thus appear that the prevalence of neuropathy in Indian NIDDY is similar to that in NIDDM but far commoner than that reported in White

patients with NIDDY. This could probably be an expression of the more severe degree of hyperglycaemia in Indian patients (Jialal et al, 1982).

## 4.7 CONCLUSION

Using strict, defined criteria, the study unequivocally confirms the occurrence of NIDDY in the Indian population in South Africa but, with the difference that the disorder was not found in children. A most striking finding was the frequency of diabetic family histories, far greater than that found in the NIDDM population as a whole. The mode of inheritance could not be established, but it is significant that, despite the obvious limitation of not having accurate medical histories of antecedants, a number of patients gave histories of diabetes in three consecutive generations, which suggest a dominant type of inheritance, in these patients, though not necessarily for the group. The prevalence of obesity was much higher than that reported by previous workers. Both micro and macrovascular complications were found, the commonest vasculopathy being retinopathy with background changes. Not a single patient was found with peripheral vascular disease. Nephropathy was present in a significant proportion of patients and hypertension was more common in patients with vascular complications. On the whole, while the complications were rather mild, the prevalence of microvascular complications was much higher than that reported by previous workers. Despite the usual proneness to intercurrent infections, not a single patient presented with ketonuria, which is clearly a remarkable feature, having regard for the fact that the group as a whole were severe diabetics. The commonest chronic complication in NIDDY was peripheral neuropathy. It would seem that the natural history of the disorder appeared to run a more benign course than in NIDDM with an onset at a more mature stage of life. Despite

many common features between patients, it is also clear that the patients did not represent an entirely homogenous group. This fact should be kept in mind, but should not detract from the more compelling observation that the patients differed from NIDDM in the older age group and had sufficient features in common to be characterised as a group suffering from a similar disorder.

# CHAPTER 5

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

## 5.1 THE INSULIN AND GLUCOSE RESPONSE TO A 100g ORAL GLUCOSE LOAD

### 5.1.1 Purpose of the Study

The clinical features of NIDDY, as seen in the group of Indian patients selected, were considered in some detail in the preceding chapter. In a number of aspects these features appear to differ from the clinical findings in NIDDM when it occurs in more mature patients or, as defined for the purpose of the study, patients in whom the onset of diabetes is after the age of 35 years.

Manifestly, it would be necessary to know whether, in biochemical terms, NIDDY can be characterised and whether such findings can be correlated with the clinical features which have been described.

The purpose of the present study is thus to define, in the first instance, the glucose intolerance and insulin status of the patients.

### 5.1.2 Patients and Reference Subjects

The 85 patients with NIDDY, described in the previous chapter, are under consideration. A group of 85 reference subjects composed of healthy Indian volunteers with normal mass, was selected for comparison. Although both groups were within the same age range (14 - 54 years) and the mean age of the reference subjects (28,1 years) was not significantly different from that of the patients with NIDDY (32,2 years), the reference subjects were not matched individually by sex, age and mass with the patients.

### 5.1.3 Study Procedure

Since the patients were on oral hypoglycaemic agents and nominally on low carbohydrate diets, they were instructed what a mixed diet, containing at least 300g carbohydrate was and requested not to take their medication and to eat a mixed diet for at least 3 days prior to glucose tolerance testing. All 85 patients had a glucose tolerance test (GTT). The reference subjects were also requested to have a mixed diet for 3 to 5 days prior to the GTT. Although fasting blood was withdrawn from all 85 reference subjects, only 50 (25 males and 25 females) submitted to a GTT. All participants in this study were ambulant and were not on any medication known to affect carbohydrate metabolism. After an overnight fast of about 12 hours, a cannula was inserted into a deep ante-cubital vein of those patients and reference subjects who submitted to a GTT. 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 100g of glucose, dissolved in 250 ml water were administered by the oral route over a period of 5 minutes. Further blood samples were withdrawn at 15 minute intervals for the first hour and at 30 minute intervals for the next 2 hours via the indwelling cannula. At each sampling time, the first 5 ml of blood obtained, were 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.

### 5.1.4 Methods

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

From the glucose and insulin values, a number of indices were calculated:

a) Areas under the insulin and glucose curves (Spitz et al, 1970).

$$\text{Area under curve} = \frac{a + 2b + 3c + 3d + e}{4}$$

where a represents fasting values and b - e values at 30 minute intervals, up to 150 minutes after oral glucose was administered.

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

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

c) The modified Seltzer insulinogenic index (MSI) (Glueck et al, 1969).

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

### 5.1.5 Results

#### 5.1.5.1 Glucose and Insulin Responses

The mean plasma glucose responses to 100 g glucose administered orally in both the patients with NIDDY and the reference subjects, are presented in Figure 5.1. It can be seen that the NIDDY group had a mean fasting plasma glucose of 12,3 mmol/$\ell$ and was unequivocally intolerant to glucose. Insulin responses are graphically presented in Figure 5.2. In the fasting state, insulin levels were significantly higher in the patients with NIDDY. (NIDDY 25,3 $\pm$ 1,4; reference subjects 14,6 $\pm$ 0,7 $\mu$U/ml; $p < 0,001$). Following the glucose challenge, the response in the NIDDY group was attenuated and the insulin levels did not peak, but plateaued after 90 minutes. This

response contrasts with the response in the reference subjects, who demonstrated mean peak insulin levels of 111,6 µU/ml at 30 minutes. Both the response curve and the actual insulin levels are significantly different from the NIDDY group, except at 180 minutes where there was no significant difference in the mean insulin levels (NIDDY 45,4 ± 3,4 µU/ml; reference subjects 39,9 ± 2,4 µU/ml; $p > 0,05$).

### 5.1.5.2 Glucose and Insulin Areas

The glucose and insulin areas are shown in Figure 5.3. While the area under the glucose curve was significantly higher in the NIDDY group, when compared to the reference subjects (47,5 ± 1,3 and 13,4 ± 0,3 mmol/ℓ, respectively; $p < 0,001$), the area under the insulin curve in NIDDY (108,2 ± 6,1 µU/ml) was substantially lower than that obtained in the reference subjects (200,7 ± 10,5 µU/ml; $p < 0,001$). There was a significant correlation between the duration of diabetes and the glucose area (Figure 5.4; $r = 0,23$; $p < 0,05$), but not between the duration and the insulin area ($r = 0,18$; $p > 0,1$). Eighty patients (94%) in the NIDDY group had an insulin area which was lower than the mean area obtained in the reference subjects. Of the 5 patients in whom the insulin areas exceeded the reference subjects' mean, 3 were obese and 2 were non-obese; in 1 patient (obese) the insulin area exceeded the mean + 1 standard deviation of the mean area in the reference subjects.

### 5.1.5.3 Insulin-Glucose Ratios

At all time intervals during the glucose loading, NIDDY exhibited significantly lower insulin-glucose ratios (Table 5.1). The smallest disparity between the ratios was in the fasting state, whilst the most marked differences were apparent at 60 minutes. 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 with NIDDY (NIDDY 0,14 ± 0,01; reference subjects 0,87 ± 0,05; $p < 0,001$).

### 5.1.5.4 Effect of Obesity on Insulin and Glucose Responses

Because of the well-known association between obesity and hyperinsulinism (Glass et al, 1981), insulin and glucose responses were analysed after subgrouping the patients with NIDDY into obese (≥ 120% of desirable mass) and non-obese (< 120% of desirable mass) categories. There were no significant differences between the two subgroups when both their glucose and insulin responses were compared (Table 5.2). In addition, there was no significant correlation between the fasting insulin level and the percentage desirable mass ($r = 0,14$; $p > 0,1$).

### 5.1.5.5 Effect of Severity of Diabetes on the Insulin and Glucose Responses

To determine the effect of the severity of diabetes on the glucose and insulin responses, the patients with NIDDY were divided into severe and moderate diabetes according to their fasting plasma glucose concentration (Seltzer, 1980). Severe diabetics constituted those with a fasting plasma glucose > 12,8 mmol/ℓ and moderate diabetics those with a fasting plasma glucose ≤ 12,8 mmol/ℓ. The responses in these subgroups are shown in Table 5.3. While glucose responses were significantly greater in the severe diabetics at all time intervals, the insulin levels were significantly lower at all time intervals in those with severe diabetes, except in the fasting state.

FIGURE 5.1 THE PLASMA GLUCOSE RESPONSE TO 100 G ORAL GLUCOSE (Mean ± S.E.M.)

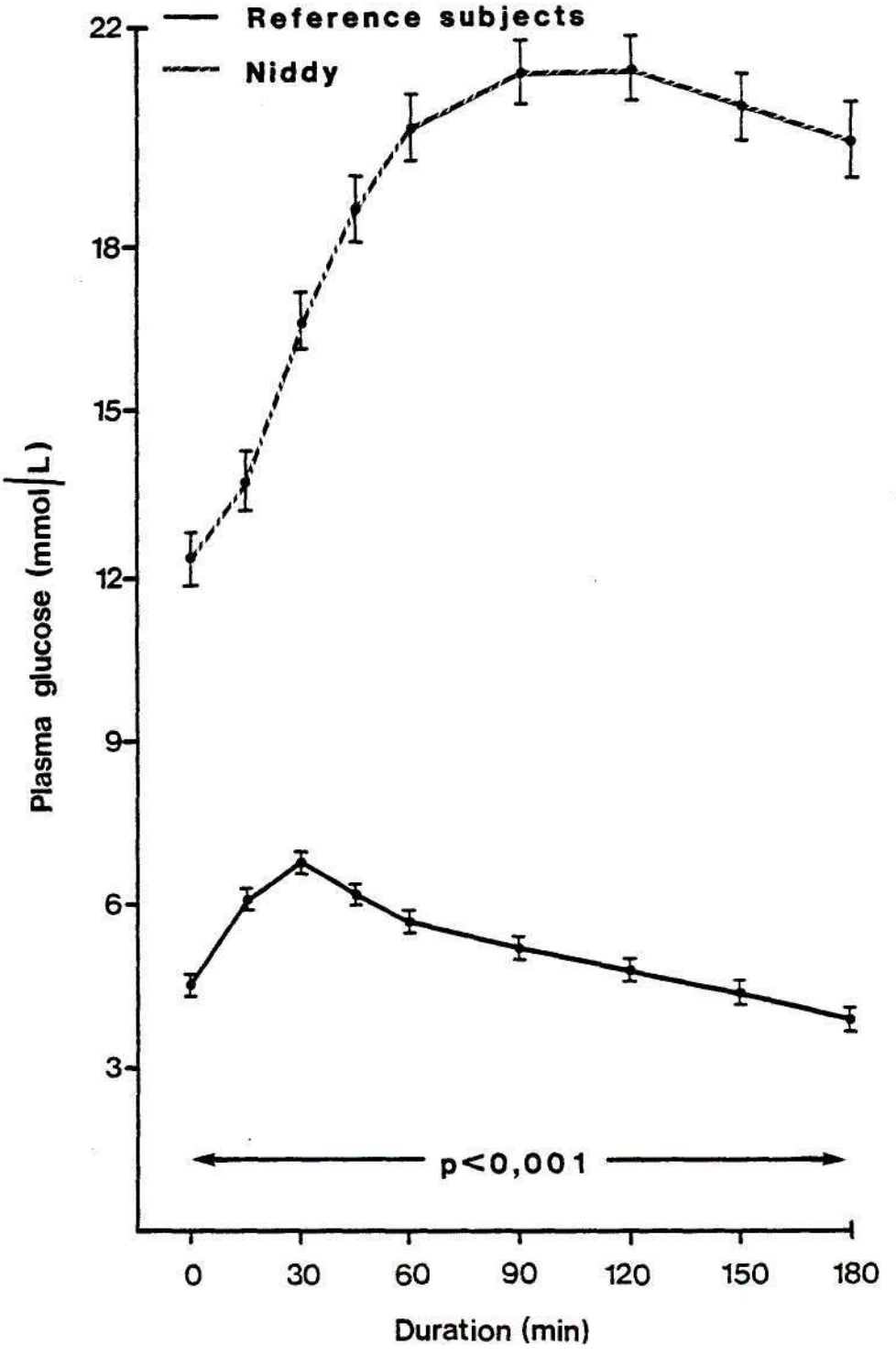


FIGURE 5.2

THE INSULIN RESPONSE TO A 100g ORAL GLUCOSE LOAD (Mean±S.E.)

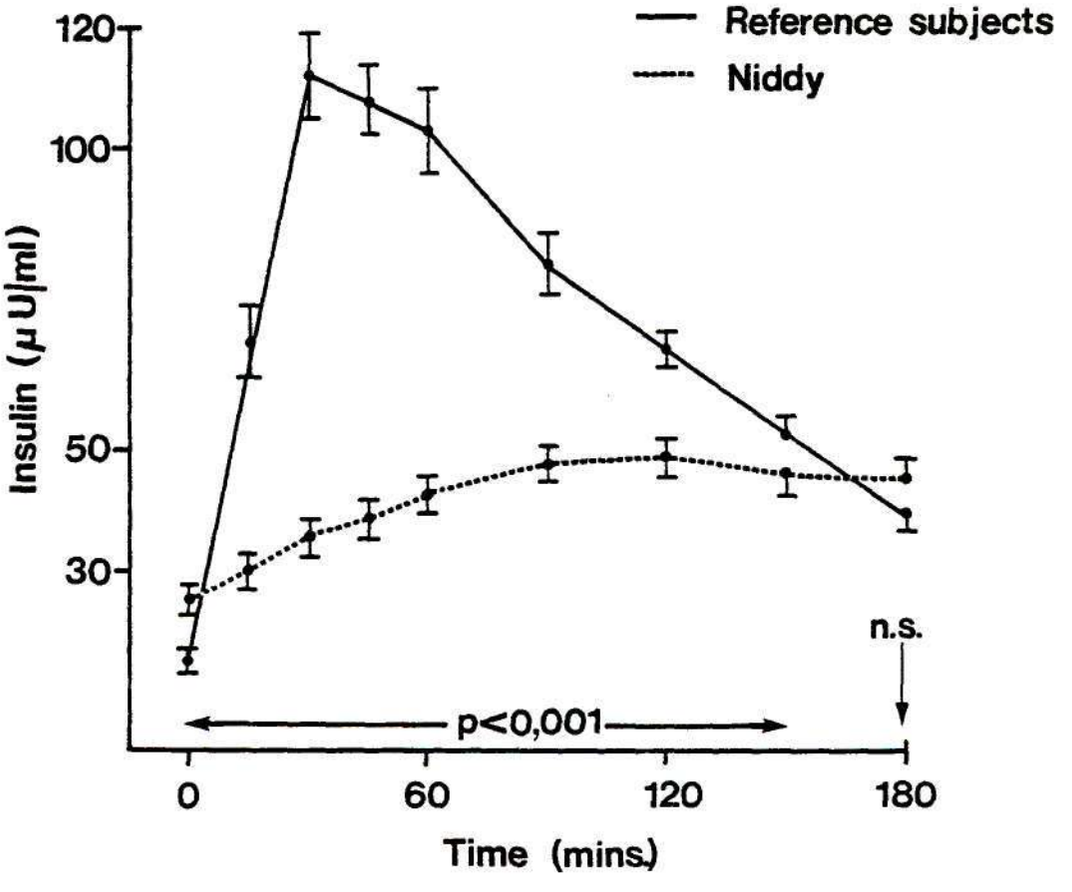


FIGURE 5.3

THE CALCULATED INSULIN AND GLUCOSE AREAS (Mean ± S.E.)

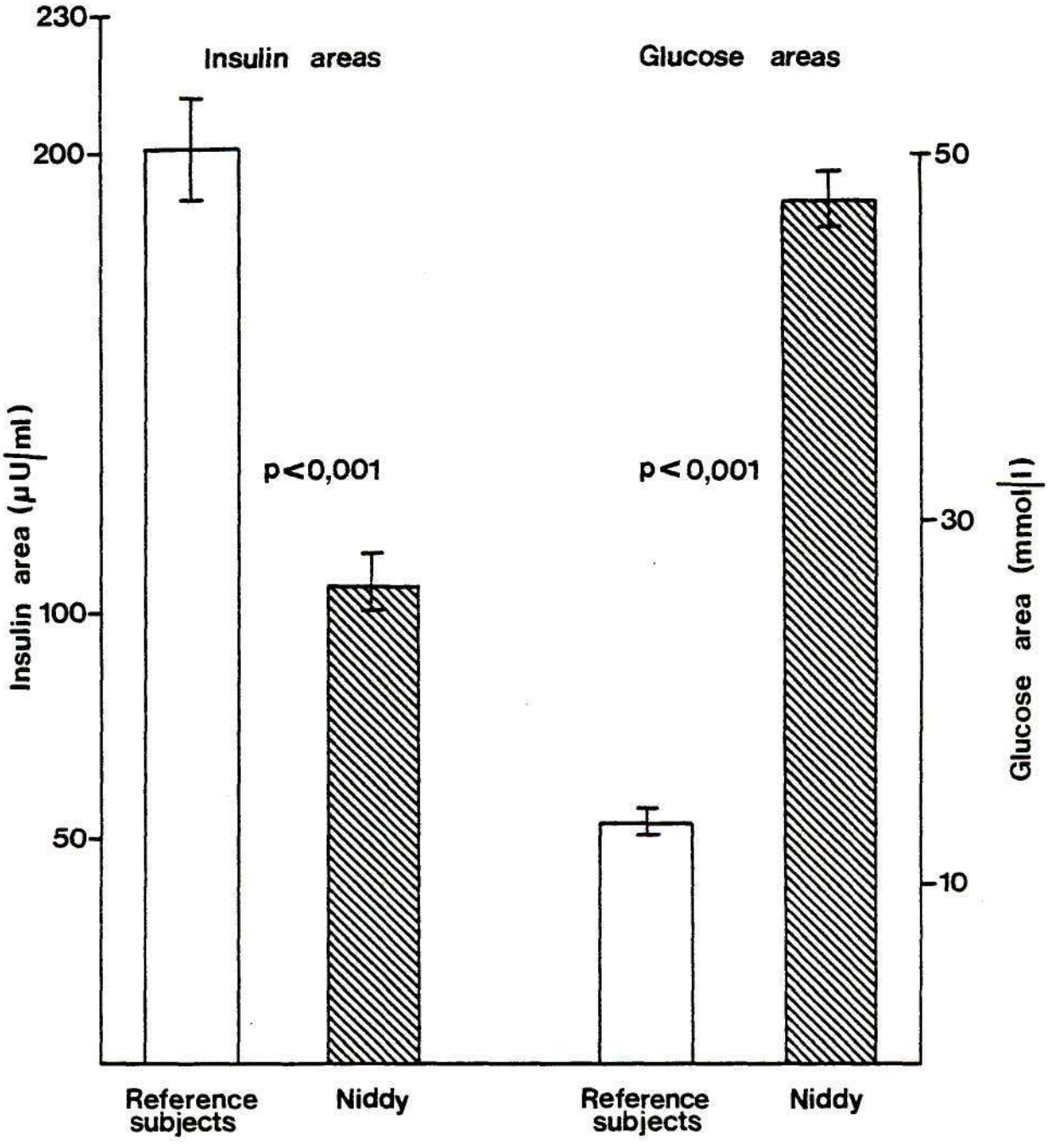


FIGURE 5.4

CORRELATION BETWEEN THE DURATION OF DIABETES AND THE GLUCOSE AREA IN NIDDY

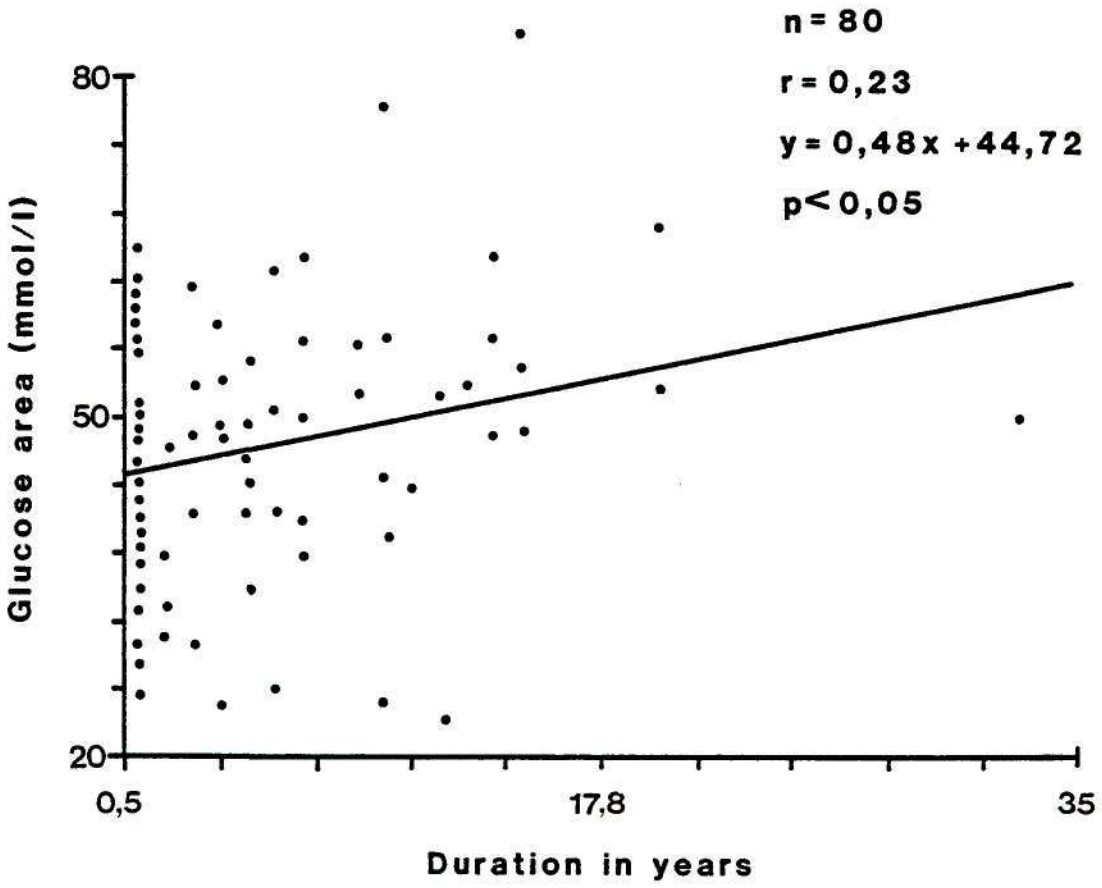


TABLE 5.1

INSULIN-GLUCOSE RATIOS AND MODIFIED INSULINOGENIC INDEX IN NON-DIABETIC CONTROLS AND NIDDY

| | Insulin-glucose ratios (mean ± SEM) | |
|---|---|---|
| | Controls (50) | NIDDY (81) |
| 0 min | 0,19 ± 0,01 | 0,13 ± 0,01* |
| 60 min | 1,05 ± 0,08 | 0,14 ± 0,01* |
| 120 min | 0,82 ± 0,05 | 0,15 ± 0,01* |
| 180 min | 0,59 ± 0,04 | 0,16 ± 0,02* |
| MSI | 0,87 ± 0,05 | 0,14 ± 0,01* |

* $p < 0,001$

MSI = modified Seltzer insulinogenic index

TABLE 5.2

PLASMA GLUCOSE AND SERUM INSULIN LEVELS
IN OBESE AND NON-OBESE NIDDY

| | Plasma Glucose (mmol/l) | |
|---|---|---|
| | Obese | Non-Obese |
| 0 min | 12,57 ± 0,69 | 11,88 ± 0,74* |
| 60 min | 20,24 ± 0,83 | 19,82 ± 0,82* |
| 120 min | 21,55 ± 0,92 | 20,67 ± 0,87* |
| 180 min | 19,72 ± 1,05 | 18,99 ± 1,07* |
| | Serum Insulin (μU/ml) | |
| | Obese | Non-Obese |
| 0 min | 26,23 ± 1,85 | 23,03 ± 1,91* |
| 60 min | 45,23 ± 4,40 | 40,40 ± 3,44* |
| 120 min | 50,81 ± 4,48 | 45,34 ± 5,30* |
| 180 min | 48,77 ± 4,60 | 41,13 ± 5,09* |

* $p > 0,05$

TABLE 5.3

PLASMA GLUCOSE AND SERUM INSULIN LEVELS (MEAN ± SEM)
IN MODERATE (PLASMA GLUCOSE ≤ 12,8 mmol/l)
AND SEVERE DIABETES (> 12,8 mmol/l)

| | Plasma Glucose (mmol/l) | |
|---|---|---|
| | Moderate (47) | Severe (34) |
| 0 min | 9,25 ± 0,33 | 16,39 ± 0,52* |
| 60 min | 17,23 ± 0,53 | 24,09 ± 0,68* |
| 120 min | 18,45 ± 0,68 | 25,13 ± 0,74* |
| 180 min | 16,46 ± 0,82 | 23,63 ± 0,87* |
| | Serum Insulin (µU/ml) | |
| | Moderate (47) | Severe (34) |
| 0 min | 25,32 ± 1,69 | 24,96 ± 2,31† |
| 60 min | 50,02 ± 4,12 | 33,2 ± 2,7 * |
| 120 min | 57,51 ± 4,78 | 35,63 ± 3,26* |
| 180 min | 54,44 ± 4,85 | 33,69 ± 3,48* |

* $p < 0,001$

† $p > 0,05$

NIDDY, because of the evidence for dominant inheritance in some patients, which implies an identifiable defect. Obesity does not seem to be a factor promoting insulin resistance or insulin secretion in patients with NIDDY.

## 5.2 THE INSULIN AND GLUCOSE RESPONSES DURING AN INTRAVENOUS INSULIN TOLERANCE TEST

### 5.2.1 Purpose of the Study

In the previous section, the importance of insulin resistance as a primary disorder or as a phenomenon secondary to severe hypoinsulinaemia, or both, were seen as central in the pathogenesis of non-insulin dependent diabetes. If, indeed, insulin resistance plays a significant role in the genesis of NIDDM, those patients with NIDDY in whom the disorder is apparently dominantly inherited could serve as models in which to identify the nature of what is inherited. Clearly, whatever the nature of the disorder inherited, it must have a biochemical basis and hence, should be potentially identifiable.

However, the basic requirement to study the suggested insulin resistance is clearly some method of identifying the resistance and measuring it in quantitative terms.

In this section insulin resistance in NIDDY will be measured by an accepted in-vivo technique and the results will be used to come to grips with what is really a very complex problem.

### 5.2.2 Patients and Reference Subjects

Because of the known effects of obesity on insulin resistance in non-diabetic individuals (Glass et al, 1981), only non-obese patients and reference subjects were chosen for this study. Accordingly, 16

non-obese patients with NIDDY were selected from the group of 85 patients and age and sex matched with 16 reference subjects.

### 5.2.3 Study Procedure

The precautions in respect of diet and oral hypoglycaemic agents in both patients and reference subjects were as detailed in Section 5.1.3. None of the patients or reference subjects had any evidence of hepatic or renal dysfunction. Insulin resistance was assessed by the technique of Lebovitz et al (1977). After an overnight fast of 12 hours, a cannula was inserted into a deep ante-cubital vein. The cannula was maintained as described in Section 5.1.3. Samples of blood were withdrawn as described in Section 5.1.3 at zero time for glucose and insulin estimation and at 5, 10, 15, 20, 25, 30, 45 and 60 minutes, after an intravenous injection of 0,1 U regular porcine insulin (Novo, Denmark) per kg body mass. Blood taken into fluoride-oxalate tubes were used for glucose estimation and in tubes containing no preservative for insulin assays. After withdrawal of the 60 minute specimen, 40 ml of a 50% dextrose solution was administered rapidly to terminate any relative hypoglycaemia. Insulin resistance was assessed by the glucose disposal constant ($K_1$), which was computed as described by Lebovitz et al (1977):

The $K_1$ expressed as %/min is a measure of the rate of fall in plasma glucose over time in response to intravenous insulin and is derived from the slope of the initial linear portion of the regression line of the natural logarithm of plasma glucose versus time.

### 5.2.4 Analytical Methods

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

5.2.5 Results

There was no significant difference in the mean percentage desirable mass of the two groups on which insulin tolerance tests were done (Table 5.4).

Fasting plasma glucose and insulin levels were significantly higher in the patients with NIDDY (Table 5.4). Following insulin administration, plasma glucose concentrations decreased significantly in both groups, as judged by the difference between fasting and nadir values ($p < 0,001$). However, the values obtained throughout the period of testing, were significantly higher in the groups with NIDDY (Fig. 5.5).

The index of insulin resistance ($K_1$), as calculated, is given in Figure 5.6. $K_1$ values obtained in NIDDY were significantly lower than that of the reference subjects ($2,57 \pm 0,40$ and $6,39 \pm 0,58$%/min respectively, $p < 0,001$). All the reference subjects had a $K_1$ value greater than 2,5, whilst the majority of patients with NIDDY (15/16) had values which were less than the mean value obtained in the reference subjects (6,39%/min); 7 of the 16 patients with NIDDY (44%) had a $K_1$ value below 2. There was no significant correlation between the $K_1$ and the fasting plasma insulin levels in the patients with NIDDY ($r = -0,20$; $p > 0,1$). However, as shown in Figure 5.7, there was a significant inverse correlation between the $K_1$ and the fasting plasma glucose ($r = -0,58$; $p < 0,02$).

The plasma insulin levels, following insulin administration, are shown in Figure 5.8. Insulin concentrations in the plasma were significantly higher throughout the period of testing in the NIDDY group.

TABLE 5.4

COMPARISON OF FASTING PLASMA GLUCOSE, INSULIN AND PERCENTAGE DESIRABLE MASS OF PATIENTS WITH NIDDY AND REFERENCE SUBJECTS (MEAN ± S.E.)

| | Reference Subjects | | NIDDY |
|---|---|---|---|
| No. | 16 | | 16 |
| Fasting Plasma Glucose (mmoℓ/L) | 4,64 ± 0,17 | * | 11,53 ± 0,90 |
| Fasting Serum Insulin (μU/mℓ) | 13,41 ± 1,42 | ** | 22,17 ± 3,08 |
| Percent Desirable Mass | 96,63 ± 2,72 | n.s. | 99,38 ± 3,13 |

* $p < 0,001$

** $p < 0,02$

n.s. not significant ($p > 0,05$)

FIGURE 5.5

THE GLUCOSE RESPONSE TO INTRAVENOUS INSULIN (0,1 U/kg)

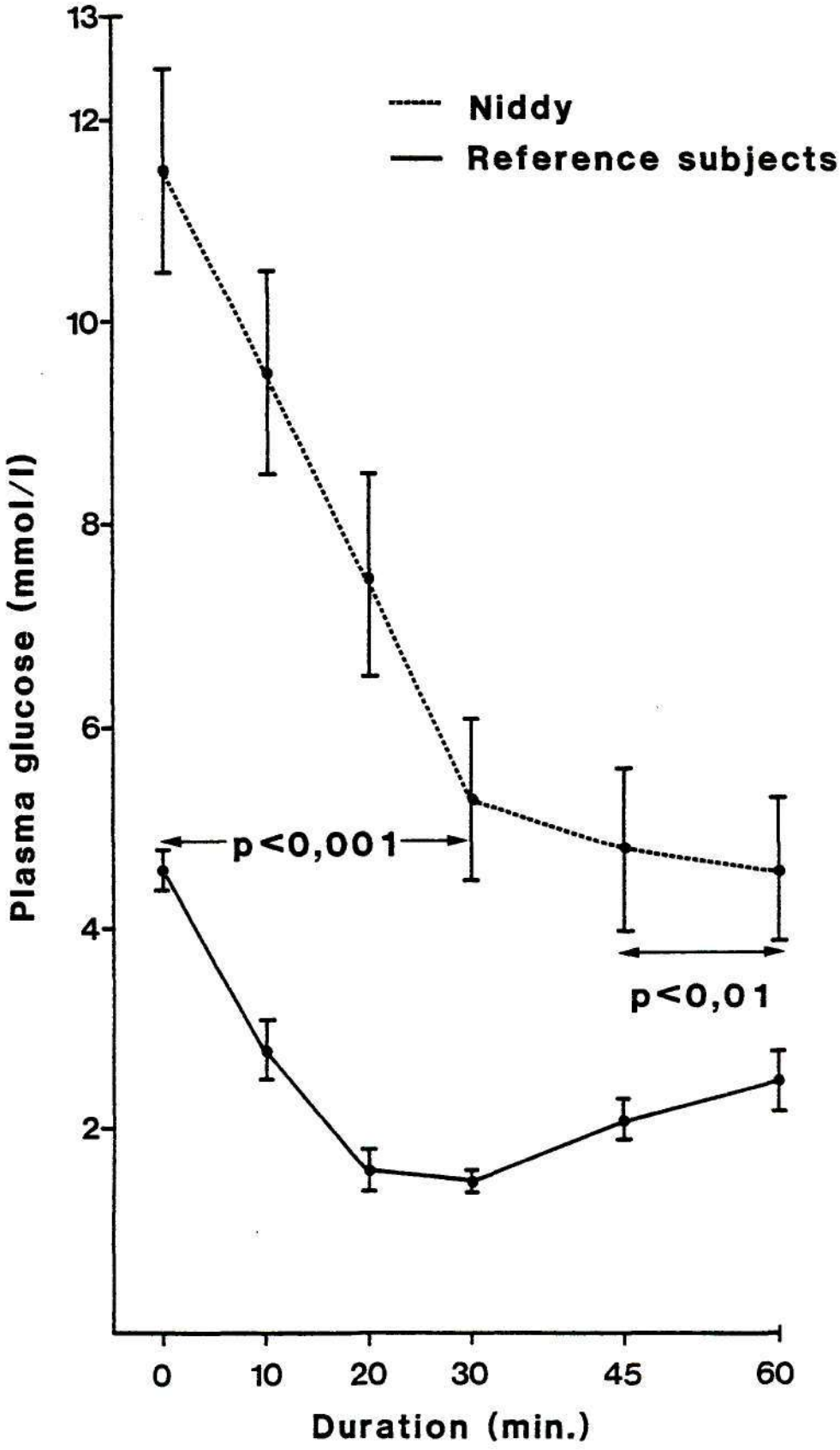


FIGURE 5.6

GLUCOSE DISPOSAL DURING AN INTRAVENOUS INSULIN TOLERANCE TEST IN NIDDY AND REFERENCE SUBJECTS.

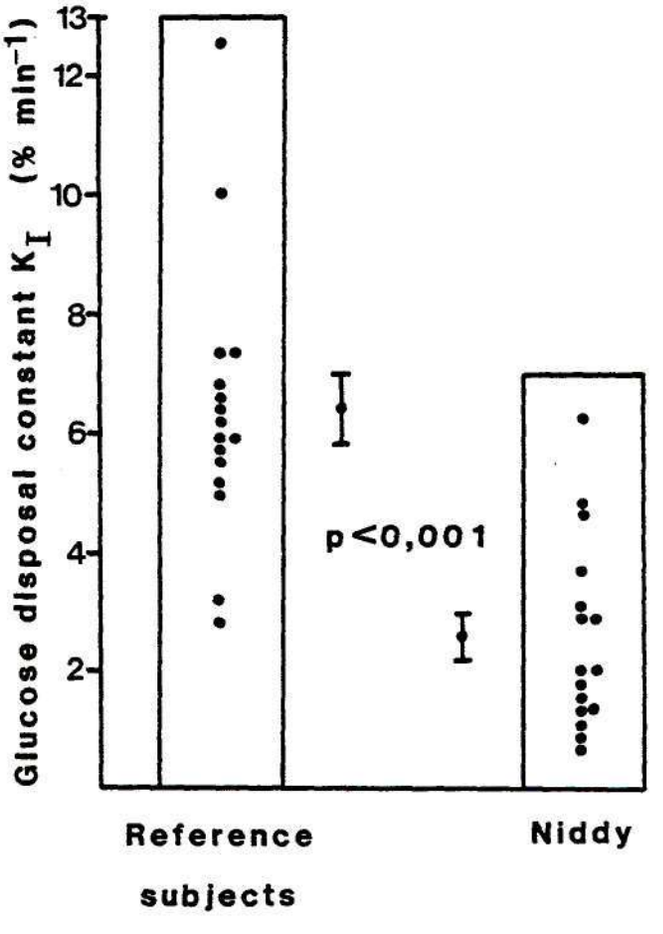


FIGURE 5.7

**RELATIONSHIP BETWEEN THE FASTING PLASMA GLUCOSE AND THE GLUCOSE DISPOSAL CONSTANT**

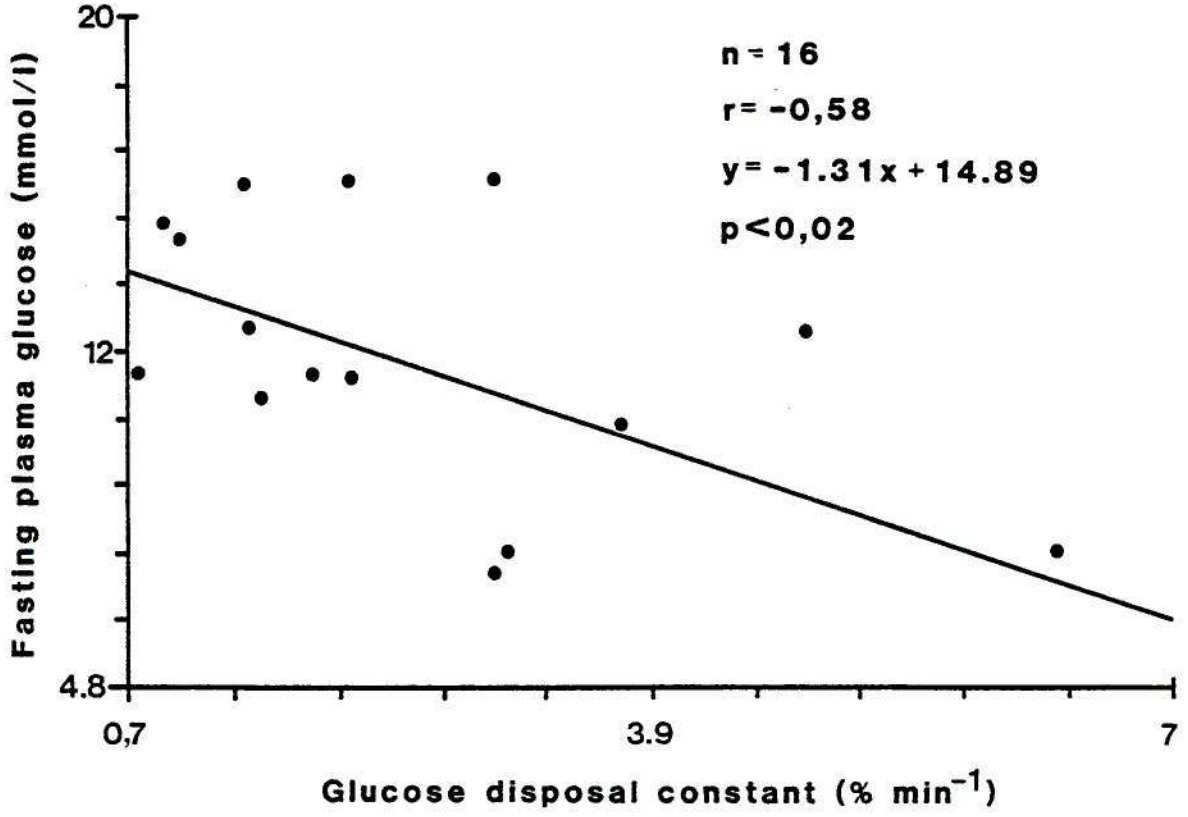
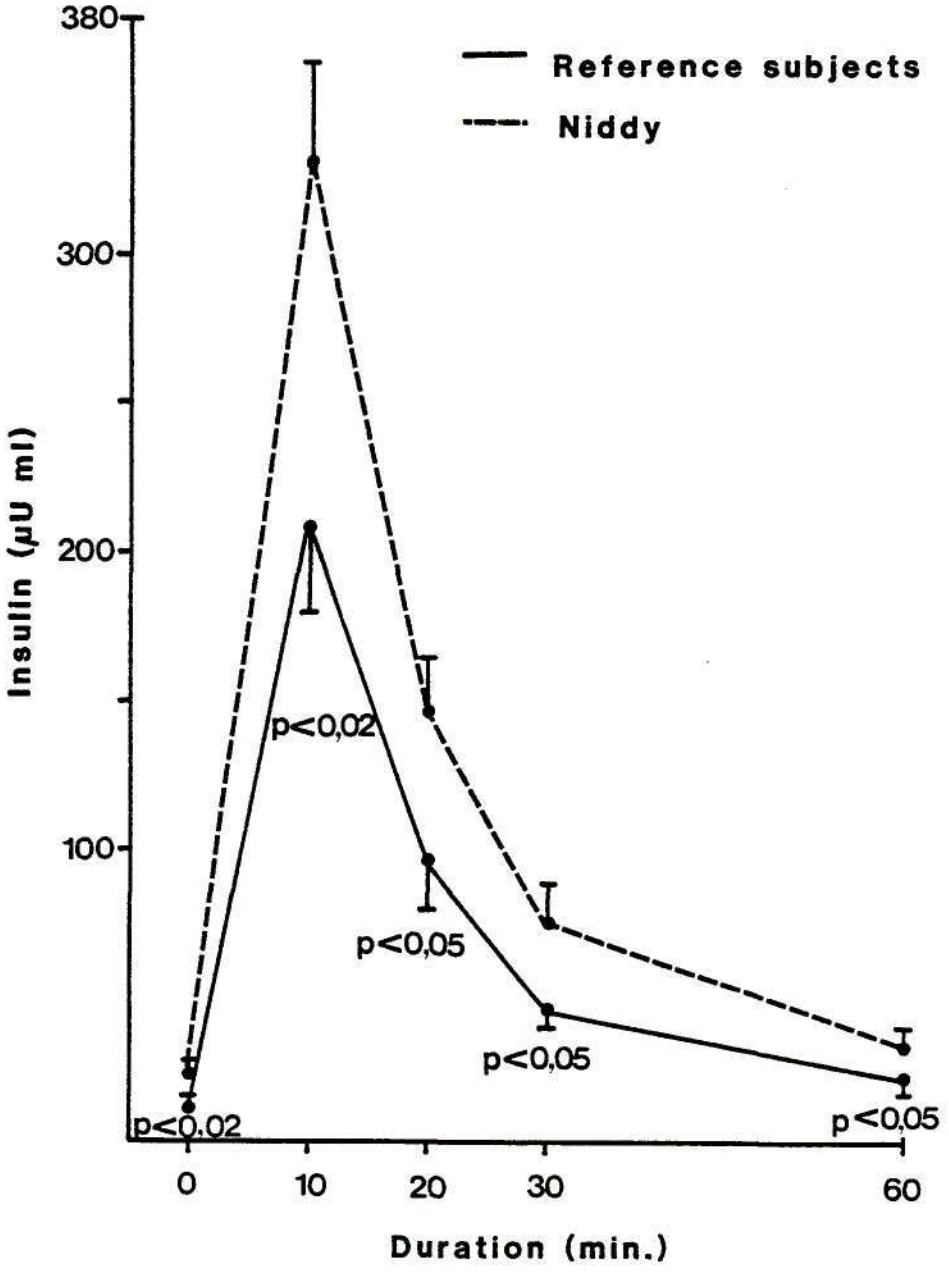


FIGURE 5.8

THE INSULIN RESPONSE DURING AN INTRAVENOUS INSULIN TOLERANCE TEST



### 5.2.6 Discussion

The notion that insulin resistance is aetiologically important in NIDDM, basically arises from the observation that some patients with mildly impaired glucose tolerance have greater than normal insulin responses during glucose tolerance testing and had already been put forward by Himsworth (1936). As discussed in Section 2.1.4.3, more recent work has shown that, although proinsulin secretion is increased in hyperinsulinaemic responses, the relative proportions of insulin and proinsulin are unaffected, and hence, the immunoreactive insulin measured represents an absolute increase in the insulin response as well.

Having regard to the index of insulin resistance ($K_1$) as measured in this study, the patients with NIDDY clearly had a much lower index than the reference subjects. Since there was a significant inverse correlation between the $K_1$ and the fasting plasma glucose, it would not be unreasonable to conclude that insulin resistance contributed to the fasting hyperglycaemia. There was close agreement when the $K_1$ values of the reference subjects in the present study were compared to the values obtained by Lebovitz and Feinglos (1980) in their controls, for in both groups the $K_1$ was greater than 2,5 in all individuals and the mean $K_1$ value in the reference subjects in the present study (6,39) was similar to that obtained in their controls (5,29). However, the fact that the majority of diabetic patients (96%) in their series had a $K_1$ value which was less than 2 (Lebovitz and Feinglos, 1980), contrasts with the findings in the present series in which only 7 of the 16 patients (44%) had values less than 2. This could be explained by the fact that, while all the patients with NIDDY in the present series were on sulphonylureas for a variable period prior to testing, none of the patients in their series had been on any

drug therapy. It has been shown that sulphonylureas improve glucose tolerance by decreasing insulin resistance (increasing receptor numbers) (Olefsky and Reaven, 1976; Lebovitz and Feinglos, 1978).

As pointed out in Section 5.1, in 2 reports on NIDDY, an exaggerated insulin response to oral glucose was obtained in 12 and 15% of the patients (Barbosa et al, 1978; Fajans et al, 1976). Although both groups of workers did not actually attempt to measure insulin resistance, Fajans (1981) interpreted the exaggerated insulin responses as evidence of insulin resistance. However, Thorell et al (1975), using a different technique, were unable to demonstrate any evidence of insulin resistance in 4 patients with NIDDY. But, as is obvious from Figure 5.6, there is an overlap between reference subjects and patients and thus 4 patients would not constitute an adequate number for a definitive conclusion.

If it is accepted that the technique used does identify insulin resistance (Stocks and Martin, 1969; Alford et al, 1971; Sensi and Capani, 1976; Lebovitz et al, 1977; Beck-Nielsen et al, 1979), it should be concluded that insulin resistance, whatever the nature, is a real factor in NIDDY.

But can the technique used really be considered acceptable to demonstrate insulin resistance? Firstly, the fasting glucose and insulin values in the two groups were significantly different and, as will be seen in subsequent sections, there were also differences in other hormonal parameters having a bearing on glucose homeostasis. Secondly, during the first 30 minutes after insulin administration, the patients with NIDDY lowered plasma glucose by 6,89 mmol/$\ell$, as opposed to the mean value of 3,13 mmol/$\ell$ achieved in the reference group. It is, of course, known that hyperglycaemia enhances glucose

uptake by mass action (Cherrington, 1978; Best et al, 1981; Reaven et al, 1982), but in quantitative terms, the exact effect of the initial hyperglycaemia cannot be computed, since there is no knowledge of glucose turnover during the present study. Also, the insulin values in the patients with NIDDY are significantly higher than that of the reference subjects, despite patients and reference subjects being matched for sex, age and mass.

It, therefore, seems somewhat unscientific to interpret the $K_1$ values as a measure of insulin resistance in the face of non-comparable steady state parameters and other variables.

If these criticisms are valid, the question is obviously whether better methods exist. Defronzo and Ferrannini (1982) reviewed the whole question of insulin resistance in NIDDM and listed the various in vivo methods used. All the methods used to measure insulin resistance have produced the same answer; there is apparently insulin resistance in NIDDM. However, every single method can be criticised on a variety of grounds and the simple truth of the matter is that at present insulin resistance cannot be measured in unequivocal quantitative terms. Yet, inferentially and intuitively one feels that it exists and studies of receptor and post receptor defects have confirmed the existence of insulin resistance in NIDDM (Olefsky and Kolterman, 1981). If, as has been argued, NIDDY is of particular interest because it holds the potential to identify the biochemical defect(s) underlying the disorder, a speculative look at the possible site of the lesion is instructive as to the problem involved.

Insulin receptors as the basis of the insulin resistance are obvious candidates for study in NIDDY, but immediately the problem of down regulation of insulin receptors by the fasting hyperinsulinism confounds the issue and could manifestly be mainly a secondary

phenomena (Lippe, 1982). If post-receptor inherited disorders are postulated, it must be admitted that such studies are in the early stages of development and give no guide at the present time (Olefsky and Kolterman, 1981). There is also the question of whether all tissues express this supposed insulin resistance equally and the reality that insulin action is never unopposed, but integral to a complex series of counter-regulatory responses.

### 5.2.7 Conclusion

Using an established in vivo method, insulin resistance has apparently been demonstrated in Indian patients with NIDDY. The sensible approach, therefore, seems to be to define whether tissues are equally sensitive or, for that matter, resistant to insulin action and to define the counter-regulatory responses to insulin action in NIDDY.

## 5.3 THE PLASMA FREE FATTY ACID RESPONSE TO A 100g ORAL GLUCOSE LOAD

### 5.3.1 Purpose of the Study

Differential tissue sensitivity, as discussed in the previous section, is particularly relevant in the South African Indian. More than a decade ago, it was found that, while non-diabetic Indian males had inferior glucose tolerances compared to Whites and Africans, they had a more pronounced fall in free fatty acid (FFA) levels in response to oral glucose (Rubenstein et al, 1969).

These findings suggested that in the Indian there is a co-existent relative resistance to insulin in muscle but retained sensitivity of adipose tissue to insulin. These circumstances in non-diabetic Indians are clearly relevant to diabetic patients as well and hence this aspect had to be investigated in the patients with NIDDY under study.

### 5.3.2 Patients and Reference Subjects

From the 85 patients and reference subjects described in Section 5.1.2 the FFA response to oral glucose was assessed in 35 non-obese patients with NIDDY and 35 reference subjects, who were matched for age, sex and mass with the patients.

### 5.3.3 Study Procedure

Therapeutic and dietary prescriptions before the glucose tolerance test, the detailed procedure with respect to maintenance of the intravenous cannula and the blood sampling technique, have been described in detail in Section 5.1.3. In the 35 patients with NIDDY and the 35 reference subjects blood samples were taken in the fasting state and then at half-hourly intervals for 3 hours after 100g of glucose was administered by the oral route. Glucose, insulin and FFA levels were assayed on these samples. Blood samples for FFA were collected in tri-potassium-EDTA tubes on ice, centrifuged at 4°C and the separated plasma stored at -20°C.

### 5.3.4 Analytical Methods

Methods used to measure glucose (A-1), insulin (A-5) and free fatty acids (A-6) are described in Appendix A.

### 5.3.5 Results

#### 5.3.5.1 The Glucose and Insulin Responses

The glucose responses of the 35 patients with NIDDY and the reference subjects were identical to the responses in the 85 patients and 50 reference subjects (Figure 5.1). While fasting insulin levels were

FIGURE 5.9

THE INSULIN RESPONSE TO A 100 G ORAL GLUCOSE LOAD

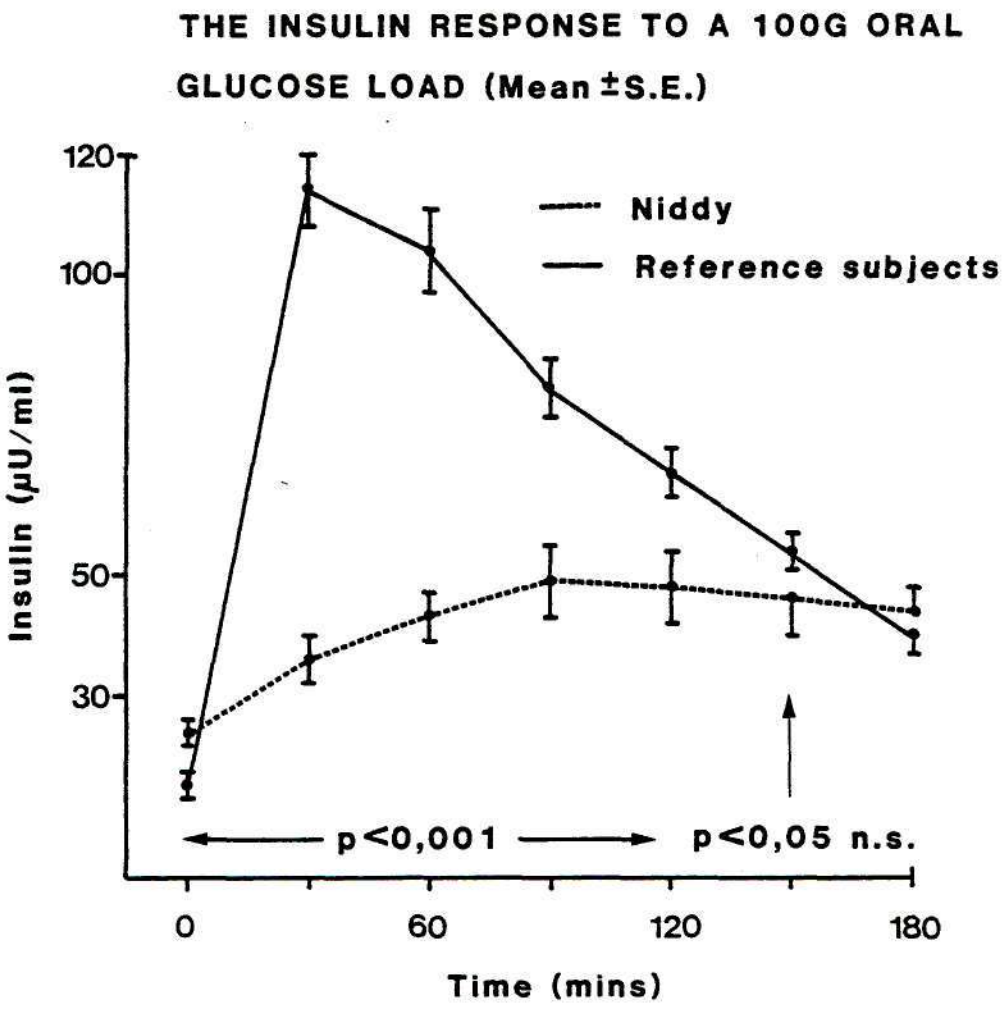
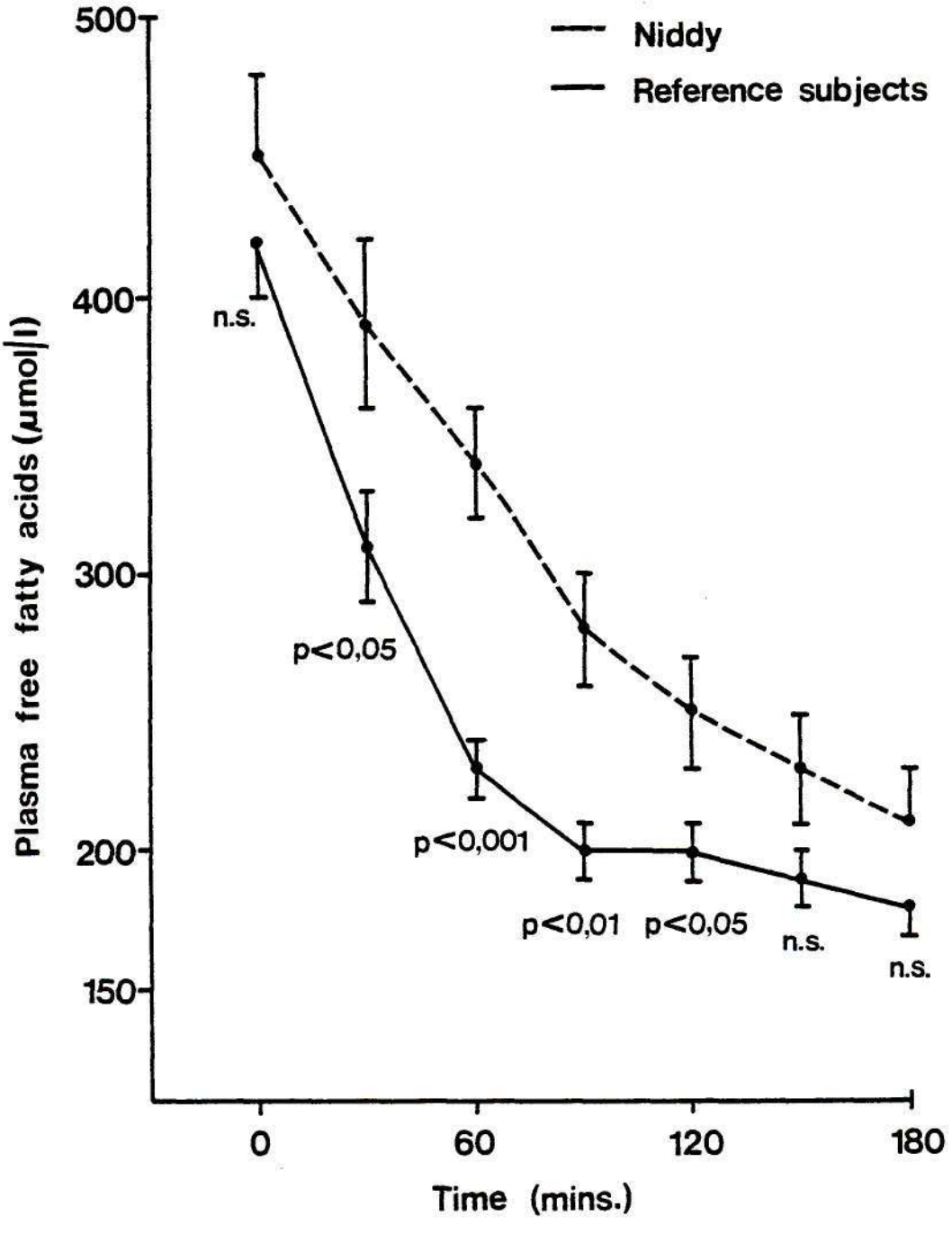


FIGURE 5.10

THE FFA RESPONSE TO A 100 G ORAL GLUCOSE LOAD



patients with NIDDY was no different from that obtained in the reference subjects and hence the hormonal basis for ketogenesis in the liver was lacking. It would therefore seem that the resistance to ketosis in Indian patients with NIDDY, already noted by Campbell (1960) is explicable on grounds of the unusual sensitivity of adipose tissue to the inhibitory action of insulin on lipolysis, thus limiting substrate for ketogenesis and the fortuitous limitation of the insulin-glucagon ratio in the fasting state to the ketogenic set of the liver.

The fasting hyperglucagonaemia also explains the anomalous finding of similar fasting FFA levels in patients and reference subjects in the face of the fasting hyperinsulinism in the patients.

In the present study no evidence was found that obesity influenced fasting glucagon levels and this finding concurs in the general consensus, although some workers have reported lower glucagon levels in obese individuals (Wise et al, 1973; Eaton and Schade, 1978; Sims, 1979).

The pathogenesis of the hyperglucagonaemia in patients with NIDDY is not clear from the present study. There are no leads as to whether there is a primary functional abnormality of the islet alpha cells or whether it is secondary to the inadequate insulin secretion.

## 6.6 THE CORTISOL RESPONSE TO 100 g GLUCOSE GIVEN ORALLY

### 6.6.1 Results

#### 6.6.1.1 The Cortisol Response of the Entire Group

Fasting serum cortisol concentrations were significantly higher in

FIGURE 6.2

THE CORTISOL RESPONSE TO A 100 G ORAL GLUCOSE LOAD

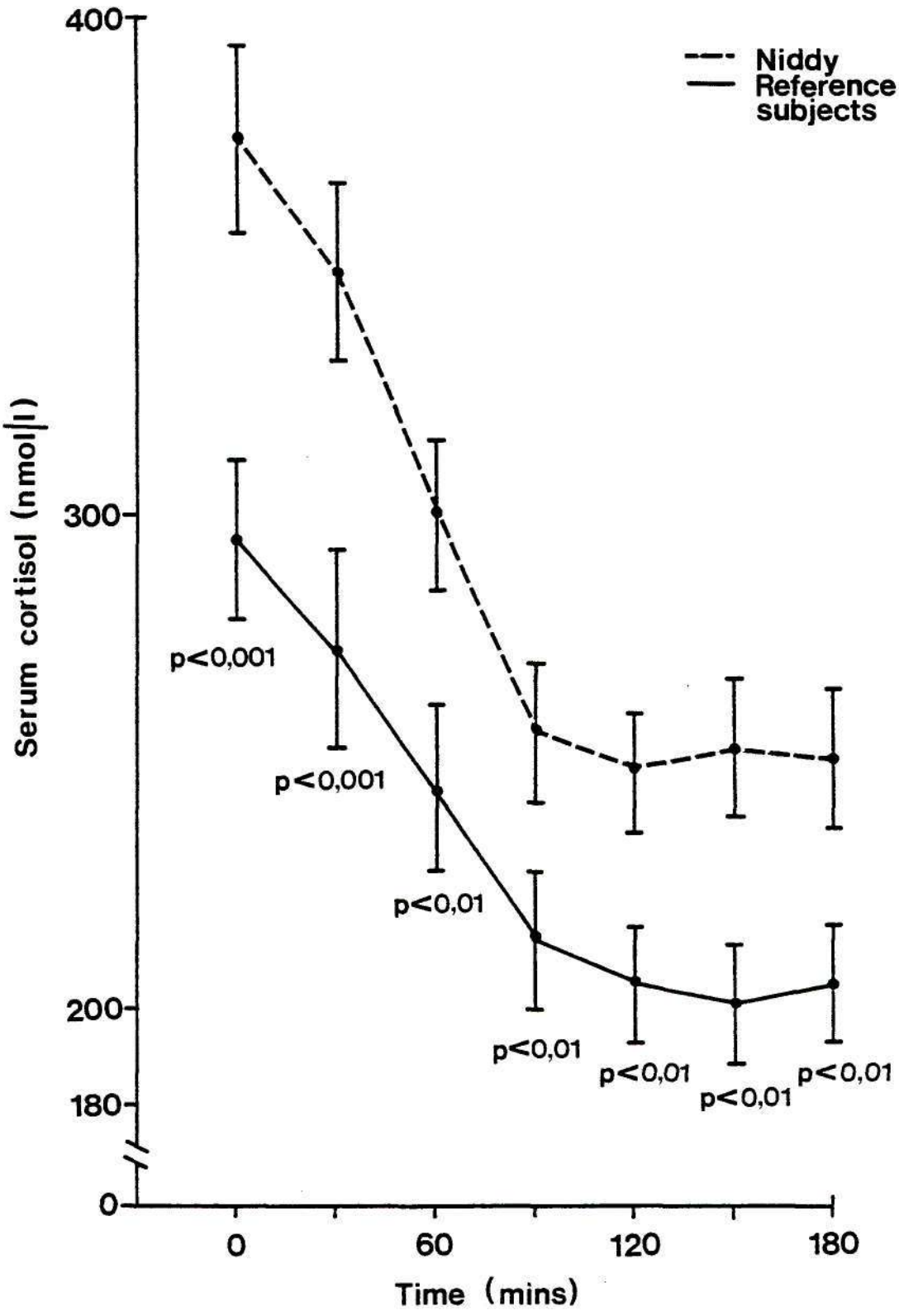


TABLE 6.2

COMPARISON OF THE CORTISOL RESPONSE OF OBESE AND NON-OBESE NIDDY WITH THE REFERENCE GROUP (MEAN ± S.E.)

CORTISOL (nmol/ℓ)

| TIME (MIN) | OBESE NIDDY | | REFERENCE SUBJECTS | | NON-OBESE NIDDY |
|---|---|---|---|---|---|
| 0 | 400 ± 28 | *** | 295 ± 17 | * | 348 ± 25 |
| 30 | 348 ± 28 | *** | 273 ± 19 | ** | 345 ± 25 |
| 60 | 304 ± 22 | ** | 246 ± 17 | * | 284 ± 22 |
| 90 | 251 ± 19 | * | 215 ± 14 | * | 257 ± 19 |
| 120 | 254 ± 19 | * | 204 ± 11 | * | 243 ± 19 |
| 150 | 246 ± 17 | ** | 201 ± 11 | * | 268 ± 25 |
| 180 | 257 ± 19 | ** | 204 ± 11 | * | 251 ± 22 |

*p < 0,05

**p < 0,01

***p < 0,001

TABLE 6.3

COMPARISON OF CORTISOL AND GROWTH HORMONE LEVELS IN NIDDY PATIENTS WITH AND WITHOUT MICROVASCULAR COMPLICATIONS ($\bar{X} \pm$ S.E.)

| | N I D D Y | | |
|---|---|---|---|
| | VASCULAR COMPLICATIONS | | |
| | PRESENT | | ABSENT |
| NO. | 15 | | 15 |
| M/F | 3/12 | | 3/12 |
| AGE (years) | 36,5 ± 2,2 | * | 35,5 ± 1,6 |
| PERCENT DESIRABLE MASS | 116,9 ± 4,5 | * | 117,5 ± 3,2 |
| DURATION (years) | 11,4 ± 2,1 | * | 7,6 ± 1,4 |
| CORTISOL (nmol/ℓ) | 328,2 ± 26,5 | * | 381,4 ± 48,6 |
| GH (ng/ml) | 0,9 ± 0,3 | * | 0,7 ± 0,2 |

* not significant ($p > 0,05$)

the patients with NIDDY when compared to the reference subjects (NIDDY, 370 ± 15 nmol/ℓ; reference subjects, 276 ± 12 nmol/ℓ; $p < 0,001$). The cortisol responses to 100 g glucose taken orally is shown in Figure 6.2. Whereas cortisol levels were suppressed in both patients and reference subjects by the hyperglycaemia, in fact the suppression curves were parallel, cortisol levels remained significantly higher at all time intervals in the patients with NIDDY.

### 6.6.1.2 The Effect of Obesity on the Cortisol Response

Fasting cortisol levels in the obese patients with NIDDY were significantly higher than in the non-obese patients (obese-NIDDY, 400 ± 28; non-obese NIDDY, 348 ± 25 nmol/ℓ; $p < 0,05$). The cortisol responses in the obese and non-obese subgroups were therefore compared independently with the response in the reference subjects (Table 6.2). Both obese and non-obese subgroups had significantly higher cortisol levels throughout the period of testing, but there was no significant difference in the cortisol response between the obese and non-obese subgroups.

### 6.6.1.3 Cortisol Levels in Patients with Microvascular Disease

The 15 patients with NIDDY who had microvascular complications were matched for sex, age, body mass and duration of diabetes with 15 patients with NIDDY who had no evidence of vascular disease (Table 6.3). There was no significant difference in the fasting serum cortisol levels between these two groups (Table 6.3).

## 6.6.2 Discussion

Serum cortisol behaved in much the same way as glucagon. Fasting cortisol levels were significantly higher than in the reference group

and in response to oral glucose, serum cortisol was not suppressed to normal levels.

Although the obese patients with NIDDY had increased fasting cortisol levels as compared with the non-obese patients, there was no significant differences between the groups in the cortisol responses after glucose challenge. When each subgroup was compared separately with the reference group, both the obese and non-obese groups had significantly higher cortisol levels during the GTT and these findings therefore suggest that the hypercortisolaemia in Indian patients with NIDDY and the inadequate suppression of cortisol levels during the GTT are largely independent of obesity.

Unfortunately, the current findings cannot be compared with previous studies in NIDDY because none of the previous workers examined the cortisol status in NIDDY. However, the finding of higher fasting serum cortisol levels in the obese patients with NIDDY when compared to the non-obese subgroup is at variance with the findings of Tai and Lee (1975) who found no significant differences in the cortisol levels of obese and non-obese diabetics (both NIDDM and IDDM). In non-diabetics, most studies on obese individuals have revealed normal fasting plasma cortisol levels and urinary free cortisol excretions (Cohen, 1977; Sims, 1979; Glass et al, 1981).

Diabetes is known to be associated with increased fasting cortisol levels and physiological increases in plasma cortisol aggravate the hyperglycaemia in diabetics as discussed in Section 2.4. The observed hypercortisolaemia can thus be interpreted as yet another factor which would tend to aggravate the hyperglycaemia in patients with NIDDY through the known effects of cortisol on gluconeogenesis and impaired peripheral glucose utilization as discussed in Section 2.4. Excess glucocorticoids decrease glucose utilization by decreasing insulin

binding to receptors and also by decreasing the post-receptor glucose transport system activity in peripheral tissues (Reaven and Olefsky, 1978).

There was no significant difference in the cortisol levels between patients with NIDDY who had microvascular complications and matched patients without vascular disease. This finding is in agreement with the reports of most previous workers although some investigators have recorded higher cortisol levels in diabetics with vascular complications as discussed in Section 2.4. Thus, an important role cannot be assigned to cortisol in the genesis of microvascular disease in Indian patients with NIDDY.

Although hypercortisolaemia has been demonstrated in the patients with NIDDY, its mechanism remains obscure. Since there is no decrease in the metabolic clearance rate of cortisol in diabetics (L'age et al, 1974), it is not unreasonable to propose that there is an increased activity of the hypothalamo-pituitary-adrenal axis resulting in the hypercortisolaemia. Supporting evidence for increased activity of the hypothalamo-pituitary-adrenal axis in diabetics with hypercortisolaemia is the increased cortisol production rates and exaggerated cortisol response during ACTH stimulation (Lentle and Thomas, 1964). Furthermore, in the hypercortisolaemic patients, there is only a partial response during the 1 mg dexamethasone suppression test (Asfeldt, 1972). Determination of plasma ACTH levels could possibly have given more insight into the hypercortisolaemia in the present study. However, the unavailability of a sensitive and specific ACTH assay and the fact that the patients with NIDDY were already heavily investigated, determined that such investigations should stand over presently. Although it has been suggested that the stress of metabolic decompensation is responsible for stimulating the hypothalamo-pituitary-adrenal

axis (Asfeldt, 1972), the normal response of this system to hyperglycaemia, is of course suppression of cortisol secretion. Hence, the hypercortisolaemia in the patients with NIDDY could not be explained on the basis of hyperglycaemia and probably some undefined mechanism mediates the hypercortisolaemia either by increasing cortisol secretion from the adrenal directly or by increasing the sensitivity of the adrenal cortex to ACTH.

Be that as it may, there can be no doubt that the hypercortisolaemia would aggravate the hyperglycaemia via gluconeogenesis and its interference with the delivery of glucose to peripheral cells.

## 6.7 THE GROWTH HORMONE RESPONSE TO 100 g GLUCOSE GIVEN ORALLY

### 6.7.1 Results

#### 6.7.1.1 The Growth Hormone Response of the Entire Group

Fasting GH levels were significantly lower in the patients with NIDDY than in the reference subjects (NIDDY, 1,12 $\pm$ 0,18; reference subjects, 1,91 $\pm$ 0,29 ng/m$\ell$; $p < 0,01$). The GH responses to oral glucose are set out in Figure 6.3. It can be seen that the two curves cross over. An analysis of variance revealed that the responses were significantly different in the patients with NIDDY and the reference group ($F = 7,57$; $p < 0,01$), the difference being due to inadequate suppression of GH by glucose loading in the patients with NIDDY. Although the nadir of the GH response occurred earlier in the patients with NIDDY (30 minutes compared to 120 minutes in the reference group), the mean value at this point (0,73 ng/m$\ell$) was substantially higher than the nadir value of the reference group (0,30 ng/m$\ell$).

FIGURE 6.3

THE GROWTH HORMONE RESPONSE TO A 100 G ORAL GLUCOSE LOAD

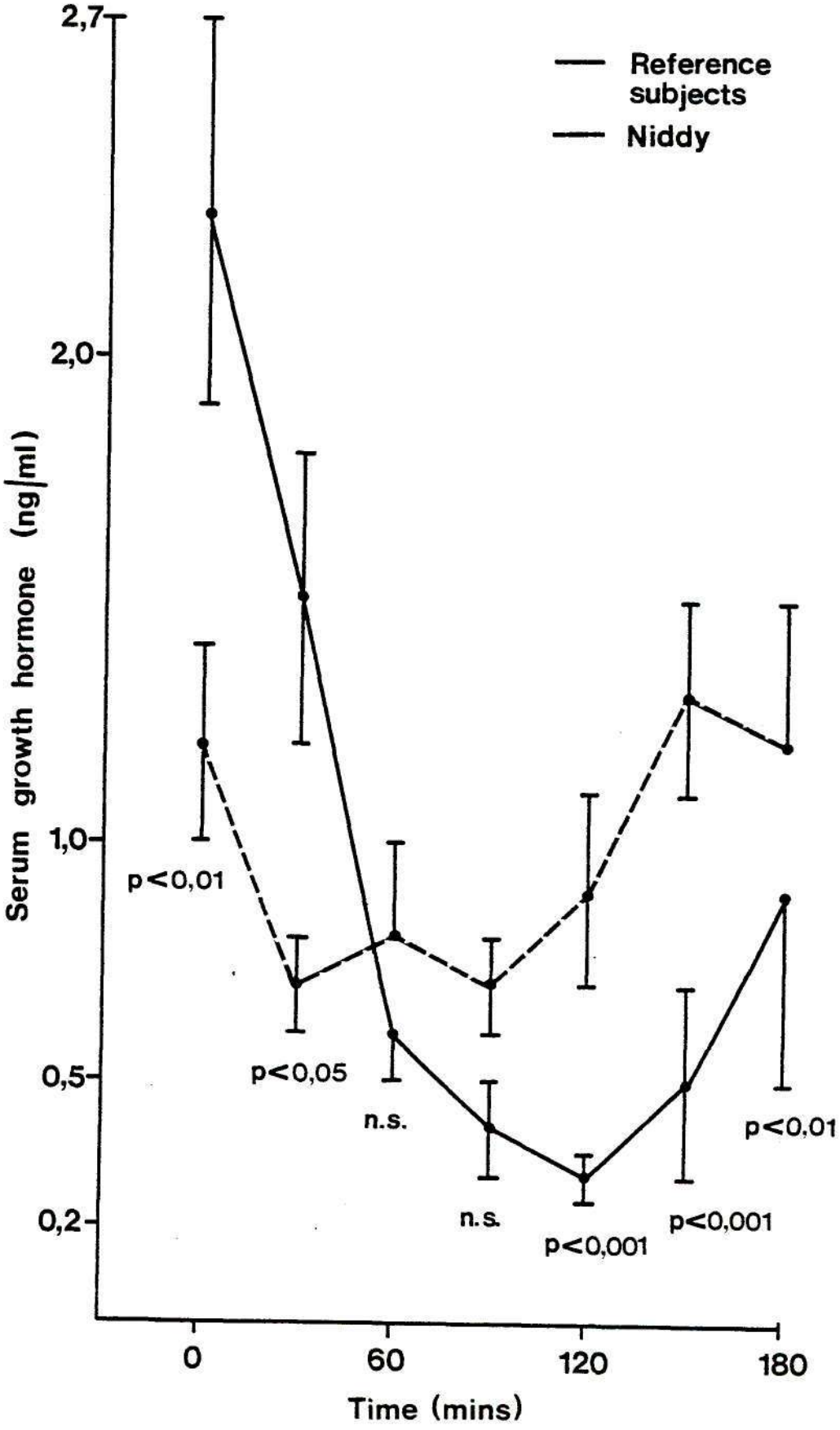


TABLE 6.4

COMPARISON OF THE GROWTH HORMONE RESPONSE OF THE OBESE AND NON-OBESE NIDDY WITH THE REFERENCE GROUP ($\bar{X} \pm$ S.E.)

| TIME (MIN) | OBESE NIDDY | | REFERENCE SUBJECTS | | NON-OBESE NIDDY |
|---|---|---|---|---|---|
| 0 | 1,3 ± 0,3 | N.S. | 2,2 ± 0,4 | * | 1,0 ± 0,3 |
| 30 | 0,8 ± 0,1 | N.S. | 1,4 ± 0,3 | N.S. | 0,7 ± 0,2 |
| 60 | 0,8 ± 0,2 | N.S. | 0,6 ± 0,1 | N.S. | 0,9 ± 0,3 |
| 90 | 0,7 ± 0,1 | N.S. | 0,4 ± 0,1 | N.S. | 0,8 ± 0,2 |
| 120 | 0,8 ± 0,1 | ** | 0,3 ± 0,1 | ** | 1,0 ± 0,3 |
| 150 | 1,0 ± 0,2 | ** | 0,5 ± 0,2 | *** | 1,4 ± 0,3 |
| 180 | 1,4 ± 0,4 | ** | 0,9 ± 0,3 | * | 1,1 ± 0,4 |

* $p < 0,05$

** $p < 0,01$

*** $p < 0,001$

N.S. not significant ($p > 0,05$)

6.7.1.2 The Effect of Obesity on the Growth Hormone Response

The patients with NIDDY were subdivided into obese and non-obese groups and their GH responses compared. The obese subgroup had significantly higher fasting GH levels (obese-NIDDY, 1,33 ± 0,28 ng/mℓ; non-obese NIDDY, 1,0 ± 0,3 ng/mℓ; $p < 0,05$). However, when these two subgroups were compared to the reference group, the mean fasting GH levels were significantly lower in the non-obese group while the GH levels in the obese subgroup was similar to that obtained in the reference group (Table 6.4). The obese and non-obese subgroups did not differ from each other in their GH responses and when these subgroups were compared independently with the response of the reference group, the differences of the responses were similar to the response seen when the entire group of patients with NIDDY were compared to the reference group (Table 6.4; Figure 6.3).

6.7.1.3 Growth Hormone Levels in Patients with Microvascular Disease

There was no significant difference between the GH levels of the patients with NIDDY who had microvascular complications as compared to patients matched for age, sex, mass and duration of diabetes (Table 6.3).

6.7.2 Discussion

The finding of decreased fasting GH levels in the patients with NIDDY is not surprising in view of the fasting hyperglycaemia, which normally suppresses pituitary secretion of GH. Differences in the GH response to oral glucose in the patients with NIDDY should perhaps be seen in this light. The challenge of increased blood glucose levels could well have a different meaning when a glucose level already exists in the fasting state (12,3mmol/ℓ) which is not achieved in the

reference group. Inadequate suppression of GH in the present study contrasts with the report of Drash (1973) who showed no difference in the GH responses between 12 patients with NIDDY who had mildly impaired glucose tolerances and controls. In yet another study, no significant difference in the fasting GH levels between patients with NIDDY and controls were reported (Barbosa et al, 1978) but, an oral glucose challenge did not suppress GH levels in the patients nor the controls which, as far as the controls are concerned, is a most unusual finding. The standard response to oral glucose is an initial suppression of GH levels followed by a rebound rise (Peake et al, 1979). A possible explanation for the results of Barbosa et al (1978) could be that the GH assay used had a sensitivity level of 2,5 ng/ml and would thus not detect lower values. As pointed out in previous sections, the patients with NIDDY in the present study were selected to be severe diabetics and constitute a more extreme example of NIDDY. Obviously endocrine responses in the more severely affected patient reflects the extreme and was not meant to be comparable to the milder diabetics studied by others.

However, the data presented here point to an increased rebound GH response in the patients with NIDDY during the period of observation and in this relative sense it could be argued that the GH behaviour could add to the hyperglycaemia in the patients with NIDDY on the basis of the diabetogenic potential of GH demonstrated since the classical studies of Houssay as described in Section 2.3. But whether GH levels lower than 2 ng/mℓ indeed have this potential is another matter and, on the existing evidence, potentiation of the diabetic state as a result of GH behaviour would at most be minimal and probably non-existent. In a study of a Tamilian Indian family with a high prevalence of diabetes (37% of individuals over the age of 25 years), Jackson et al (1974) remarked on a trend of inadequate suppression of

the GH levels during an oral GTT in this family when the GH response was compared to controls. However, the differences were not statistically significant. The present study in Indian patients with NIDDY demonstrates that there is a significant and real failure to suppress GH levels following oral glucose.

Although the obese subgroup of patients with NIDDY had significantly higher fasting GH levels, the response to the glucose load was similar to that of the non-obese group of patients with NIDDY. In fact, since the patients with NIDDY as a whole had lower fasting GH levels, the finding of higher GH levels in the obese subgroup partly masked the decreased GH levels present in the fasting state in the patients as a whole.

Lundbaek et al (1970) proposed that GH hypersecretion was important in the genesis of diabetic angiopathy. Although much support accrued over the years for the thesis, others have failed to demonstrate any abnormality in GH secretion in diabetics with vascular disease as discussed in Section 2.3.3. In the present study there was no significant difference in the GH levels between the patients with microvascular disease and matched patients without any evidence of vascular disease. Thus, an important role cannot be assigned to GH in the genesis of microvascular disease in the patients with NIDDY.

On the basis of the findings discussed here, GH behaviour is obviously not an important factor which would contribute to the hyperglycaemia or the insulin resistance in patients with NIDDY.

## 6.8 CONCLUSION

It has manifestly been demonstrated that the behaviour of the circulating hormonal insulin antagonists is abnormal in patients with

NIDDY. With respect to at least two of these hormones, glucagon and cortisol, the increased levels and inadequate suppression in response to oral glucose would tend to aggravate the hyperglycaemia quite apart from the primary contribution of insulin insufficiency. Both these hormones, in addition to increasing glucose production, are well-known circulating insulin antagonists and would therefore contribute further to the hyperglycaemia via insulin resistance. As far as GH is concerned, it would appear that its contribution is minimal for fasting levels were significantly lower and, quite apart from inadequate suppression in response to oral glucose, the values were below 2 ng/mℓ. Obesity did not seem to affect the behaviour of these insulin antagonists except in the fasting state, when differences were observed; fasting cortisol levels were higher in the obese patients with NIDDY and in this way obesity could have aggravated the hyperglycaemia.

# CHAPTER 7

# LIPID AND LIPOPROTEIN ABERRATIONS IN NON-INSULIN DEPENDENT DIABETES IN THE YOUNG

## 7.1 INTRODUCTION

In the previous chapters the clinical features (prevalence of vascular complications) and the hormonal aspects of Indian patients with NIDDY were described. Since these patients appeared to have a much higher prevalence of microvascular complications than that reported by other investigators in patients with NIDDY and abnormalities of lipid metabolism have been incriminated in the genesis of microvascular disease (Section 3.4.5), a study of some aspects of lipoprotein metabolism in these patients are relevant to the overall picture. The present study was undertaken to examine the lipid and lipoprotein status of patients with NIDDY, which has hitherto not been explored and, in addition, to determine what relationship existed between microvascular complications and lipoprotein aberrations in patients with NIDDY.

## 7.2 PATIENTS AND REFERENCE SUBJECTS

The 85 patients with NIDDY described in detail in Chapter 4 are under consideration. These patients were individually matched for sex and age with 85 non-obese apparently healthy volunteers; 35 of these volunteers were the reference subjects described in Section 5.1.2.

## 7.3 STUDY PROCEDURE

A full clinical examination was performed on all patients to exclude other causes of secondary hyperlipidaemia and the patients were

specifically asked about their smoking habits and alcohol consumption. All participants were encouraged to adhere to the diet they normally consumed for at least 2 weeks prior to sampling and to omit the morning dose of the oral hypoglycaemic agent they were taking on the day on which blood samples were withdrawn. None of the patients or reference subjects were on any other medication known to affect lipid metabolism and all were ambulant at the time of sampling.

Blood samples were obtained, after an overnight fast of 12 to 14 hours, from the 85 patients and reference subjects with minimal venostasis. Samples for lipid and lipoprotein estimations were collected in tubes containing no preservative while the samples for glycosylated haemoglobin ($HbA_1$) were taken in potassium-EDTA tubes. The samples for $HbA_1$ estimation were immediately stored at 4°C. After allowing the samples for lipid and lipoprotein quantitation to clot at room temperature, they were centrifuged within 2 hours. The sera obtained were stored at 4°C for total cholesterol, triglycerides, high density lipoprotein (HDL)-cholesterol and low density lipoprotein (LDL)-cholesterol determinations; these assays were performed within 3 days from the time of sampling. However, the sera for Apoprotein A-1 (Apo A-1) and Apoprotein B (Apo B) assays were stored at -20°C and assays carried out when convenient.

The refrigerator test (storage of sera at 4°C for 18 hours) was undertaken as described by Lewis (1976) on the sera of the 9 patients with total triglyceride concentrations greater than 5 mmol/ℓ.

Although blood samples were taken in all patients and reference subjects, results were not obtained in all patients for a variety of reasons, but on a non-selective and random basis. The effective numbers of patients and reference subjects in the various studies are indicated in the relevant tables.

Because of the known effects of obesity on lipid and lipoprotein levels (Lewis, 1976; Sims, 1979), the patients were subdivided into obese and non-obese groups and their lipid and lipoprotein levels compared.

To examine the effect of sulphonylureas on cholesterol, triglycerides and HDL-cholesterol levels, 20 patients had their cholesterol, triglycerides, HDL-cholesterol and plasma glucose levels measured before the commencement of therapy and after one year of treatment.

Since only 9 of the 85 patients (11%) admitted to smoking or taking alcohol, the effect of cigarette smoking and alcohol consumption on lipid profiles could not be analysed statistically because of the small sample size.

## 7.4 ANALYTICAL METHODS

The methods used to measure glucose (A-1), cholesterol (A-10), triglycerides (A-11), HDL-cholesterol (A-12), LDL-cholesterol (A-13), Apo A-1 (A-14), Apo B (A-15) and $HbA_1$ (A-16) are described in Appendix A.

## 7.5 RESULTS

### 7.5.1 Lipid and Lipoprotein Levels

Total serum cholesterol and LDL-cholesterol levels were not significantly different in patients and reference subjects irrespective of the sex of the patients (Tables 7.1 and 7.2). However, serum total triglycerides were significantly higher in the patients and HDL-cholesterol levels lower than in the reference subjects. This was true for both sexes (Tables 7.1 and 7.2).

There were no significant differences in the Apo A-1 concentrations in both the male and female patients when compared to their respective reference group. While the Apo B levels in the female patients with NIDDY were significantly higher than that obtained in the female reference subjects (Table 7.1), there was no significant difference between the Apo B levels of the male patients with NIDDY and the male reference subjects, although the male patients had higher levels (Table 7.2).

Both the hypertriglyceridaemia and decreased HDL-cholesterol levels were more pronounced in the male patients with NIDDY than in the female patients when compared to their respective reference groups (Tables 7.1 and 7.2).

### 7.5.2 Refrigerator Test Results

The refrigerator test was carried out in the 9 patients (3 males) in whom total triglyceride concentrations were in excess of 5 mmol/ℓ. In 8 of the 9 patients, inspection of the sera revealed diffuse lactescence while the remaining patients' serum showed diffuse lactescence with a creamy layer on top of the sample.

### 7.5.3 The Effect of Obesity on Lipid and Lipoprotein Levels in NIDDY

There were no significant differences in any of the lipid or lipoprotein concentrations between the obese and non-obese subgroups (Table 7.3).

### 7.5.4 The Effect of Oral Hypoglycaemic Agents on Lipid and Lipoprotein Levels in NIDDY

The findings with respect to the fasting plasma glucose, total serum cholesterol, total serum triglycerides and HDL-cholesterol levels in

20 patients with NIDDY prior to therapy with oral sulphonylureas and after 1 year of treatment are present in Figure 7.4. Although there was a significant fall in the plasma glucose levels, the drugs did not effect any significant change in the lipid and lipoprotein levels.

### 7.5.5 Inter-Relationship Between Lipids, Lipoproteins and Glycosylated Haemoglobin Levels

There was a significant positive correlation between glycosylated haemoglobin ($HbA_1$) levels and serum triglyceride levels (Figure 7.1; $r = 0,23$; $p < 0,05$). However, while $HbA_1$ levels correlated marginally with the Apo B levels ($r = 0,23$; $p = 0,05$), there was no correlation between $HbA_1$ levels and serum HDL-cholesterol ($r = -0,02$; $p > 0,05$). Serum triglycerides correlated significantly with the HDL-cholesterol levels (Figure 7.2; $r = -0,37$; $p < 0,001$) but not with Apo A-1 levels ($r = 0,13$; $p > 0,05$). The serum Apo B levels correlated significantly with the serum LDL-cholesterol (Figure 7.3; $r = 0,52$; $p < 0,001$) and serum triglycerides (Figure 7.4; $r = 0,42$; $p < 0,001$).

### 7.5.6 Lipid and Lipoprotein Levels in Patients With and Without Vascular Complications

The 15 patients with NIDDY who had microvascular disease were matched with 15 patients without any evidence of vascular disease for age, sex, body mass and duration of diabetes (Table 6.3) and their lipid and lipoprotein levels were compared. There were no significant differences in any of the lipid and lipoprotein concentrations between these 2 groups (Table 7.5). However, both the fasting plasma glucose and glycosylated haemoglobin ($HbA_1$) levels were significantly higher in the patients with microvascular disease.

TABLE 7.1

LIPID AND LIPOPROTEIN LEVELS IN FEMALE PATIENTS WITH NIDDY AND REFERENCE SUBJECTS

| | NIDDY | REFERENCE SUBJECTS |
|---|---|---|
| TOTAL SERUM CHOLESTEROL (mmol/l) | | |
| N | 66 | 66 |
| $\bar{X} \pm S E$ | 5,25 ± 0,17 | 4,85 ± 0,11 |
| | N S | |
| TOTAL SERUM TRIGLYCERIDES (mmol/l) | | |
| N | 68 | 68 |
| $\bar{X} \pm S E$ | 1,84 ± 0,14 | 1,18 ± 0,10 |
| | p < 0,001 | |
| HDL-CHOLESTEROL (mmol/l) | | |
| N | 68 | 68 |
| $\bar{X} \pm S E$ | 0,89 ± 0,02 | 1,14 ± 0,03 |
| | p < 0,001 | |
| LDL-CHOLESTEROL (mmol/l) | | |
| N | 65 | 67 |
| $\bar{X} \pm S E$ | 3,50 ± 0,15 | 3,17 ± 0,10 |
| | N S | |
| APOPROTEIN A-1 (mg %) | | |
| N | 55 | 55 |
| $\bar{X} \pm S E$ | 91,14 ± 2,52 | 94,05 ± 2,45 |
| | N S | |
| APOPROTEIN B (mg %) | | |
| N | 62 | 62 |
| $\bar{X} \pm S E$ | 117,61 ± 3,22 | 92,45 ± 3,51 |
| | p < 0,001 | |

TABLE 7.2

LIPID AND LIPOPROTEIN LEVELS IN MALE PATIENTS WITH NIDDY AND REFERENCE SUBJECTS

| | NIDDY | REFERENCE SUBJECTS |
|---|---|---|
| TOTAL SERUM CHOLESTEROL (mmol/l) | | |
| N | 17 | 17 |
| $\bar{X} \pm S E$ | 6,29 ± 0,89 | 5,19 ± 0,24 |
| | N S | |
| TOTAL SERUM TRIGLYCERIDES (mmol/l) | | |
| N | 17 | 17 |
| $\bar{X} \pm S E$ | 4,16 ± 1,65 | 1,07 ± 0.09 |
| | p < 0,002 | |
| HDL-CHOLESTEROL (mmol/l) | | |
| N | 17 | 17 |
| $\bar{X} \pm S E$ | 0,81 ± 0,05 | 1,09 ± 0,05 |
| | p < 0,002 | |
| LDL-CHOLESTEROL (mmol/l) | | |
| N | 17 | 17 |
| $\bar{X} \pm S E$ | 3,69 ± 0,30 | 3,63 ± 0,23 |
| | N S | |
| APOPROTEIN A-1 (mg %) | | |
| N | 14 | 14 |
| $\bar{X} \pm S E$ | 85,86 ± 3,86 | 88,93 ± 5,92 |
| | N S | |
| APOPROTEIN B (mg %) | | |
| N | 14 | 14 |
| $\bar{X} \pm S E$ | 122,45 ± 8.03 | 105,11 ± 5,97 |
| | N S | |

TABLE 7.3

COMPARISON OF LIPID AND LIPOPROTEIN CONCENTRATIONS BETWEEN NON-OBESE AND OBESE NIDDY PATIENTS

| | NON-OBESE | OBESE |
|---|---|---|
| TOTAL SERUM CHOLESTEROL (mmol/l) | | |
| N | 41 | 42 |
| $\bar{X} \pm S E$ | 5,71 ± 0,40 | 5,07 ± 0,20 |
| | * | |
| TOTAL SERUM TRIGLYCERIDES (mmol/l) | | |
| N | 41 | 44 |
| $\bar{X} \pm S E$ | 2,71 ± 0,71 | 1,95 ± 0,18 |
| | * | |
| HDL-CHOLESTEROL (mmol/l) | | |
| N | 41 | 44 |
| $\bar{X} \pm S E$ | 0,88 ± 0,03 | 0,87 ± 0,03 |
| | * | |
| LDL-CHOLESTEROL (mmol/l) | | |
| N | 41 | 41 |
| $\bar{X} \pm S E$ | 3,69 ± 0,20 | 3,42 ± 0,18 |
| | * | |
| APOPROTEIN A-1 (mg %) | | |
| N | 34 | 35 |
| $\bar{X} \pm S E$ | 90,66 ± 3,19 | 89,49 ± 2,96 |
| | * | |
| APOPROTEIN B (mg %) | | |
| N | 36 | 39 |
| $\bar{X} \pm S E$ | 119,10 ± 4,48 | 117,88 ± 4,18 |
| | * | |
| $HbA_1$ (%) | | |
| N | 40 | 37 |
| $\bar{X} \pm S E$ | 12,85 ± 0,45 | 13,31 ± 0,38 |
| | * | |

* N S ($p > 0,05$)

TABLE 7.4

EFFECT OF ORAL HYPOGLYCAEMIC AGENTS ON PLASMA GLUCOSE, CHOLESTEROL, TRIGLYCERIDE AND HDL CHOLESTEROL LEVELS

| | Pretreatment (mean ± S E) | Post-treatment (mean ± S E) |
|---|---|---|
| PLASMA GLUCOSE (mmol/l) | 11,87 ± 0,66 | 9,37 ± 0,65** |
| TOTAL CHOLESTEROL (mmol/l) | 4,79 ± 0,26 | 4,93 ± 0,22* |
| TOTAL TRIGLYCERIDES (mmol/l) | 1,83 ± 0,28 | 2,07 ± 0,42* |
| HDL-CHOLESTEROL (mmol/l) | 0,84 ± 0,04 | 0,87 ± 0,05* |

* $p > 0,05$

** $p < 0,05$

TABLE 7.5

COMPARISON OF LIPID AND LIPOPROTEIN LEVELS BETWEEN NIDDY PATIENTS WITH AND WITHOUT VASCULAR COMPLICATIONS

| | NIDDY | | |
|---|---|---|---|
| | VASCULAR COMPLICATIONS | | |
| | PRESENT | | ABSENT |
| TOTAL SERUM CHOLESTEROL (mmol/l) | N | 15 | 15 |
| | $\bar{X} \pm S E$ | 6,11 ± 0,41 | * 5,60 ± 0,37 |
| TOTAL SERUM TRIGLYCERIDES (mmol/l) | N | 15 | 15 |
| | $\bar{X} \pm S E$ | 2,68 ± 0,31 | * 2,09 ± 0,34 |
| HDL-CHOLESTEROL (mmol/l) | N | 15 | 15 |
| | $\bar{X} \pm S E$ | 0,85 ± 0,06 | * 0,92 ± 0,06 |
| LDL-CHOLESTEROL (mmol/l) | N | 15 | 15 |
| | $\bar{X} \pm S E$ | 4,33 ± 0,37 | * 3,86 ± 0,39 |
| APOPROTEIN A-1 (mg %) | N | 13 | 15 |
| | $\bar{X} \pm S E$ | 94,38 ± 5,71 | * 95,27 ± 3,76 |
| APOPROTEIN B (mg %) | N | 14 | 15 |
| | $\bar{X} \pm S E$ | 128,89 ± 5,29 | *111,32 ± 7.14 |
| $HbA_1$ (%) | N | 15 | 15 |
| | $\bar{X} \pm S E$ | 12,1 ± 0,8 | *** 10,3 ± 0,3 |
| FASTING PLASMA GLUCOSE (mmol/l) | N | 15 | 15 |
| | $\bar{X} \pm S E$ | 14,72 ± 1,23 | ** 11,65 ± 1,24 |

* N S ($p > 0,05$)

** $p < 0,05$

*** $p < 0,01$

FIGURE 7.2

RELATIONSHIP BETWEEN FASTING SERUM TRIGLYCERIDES AND HDL-CHOLESTEROL LEVELS

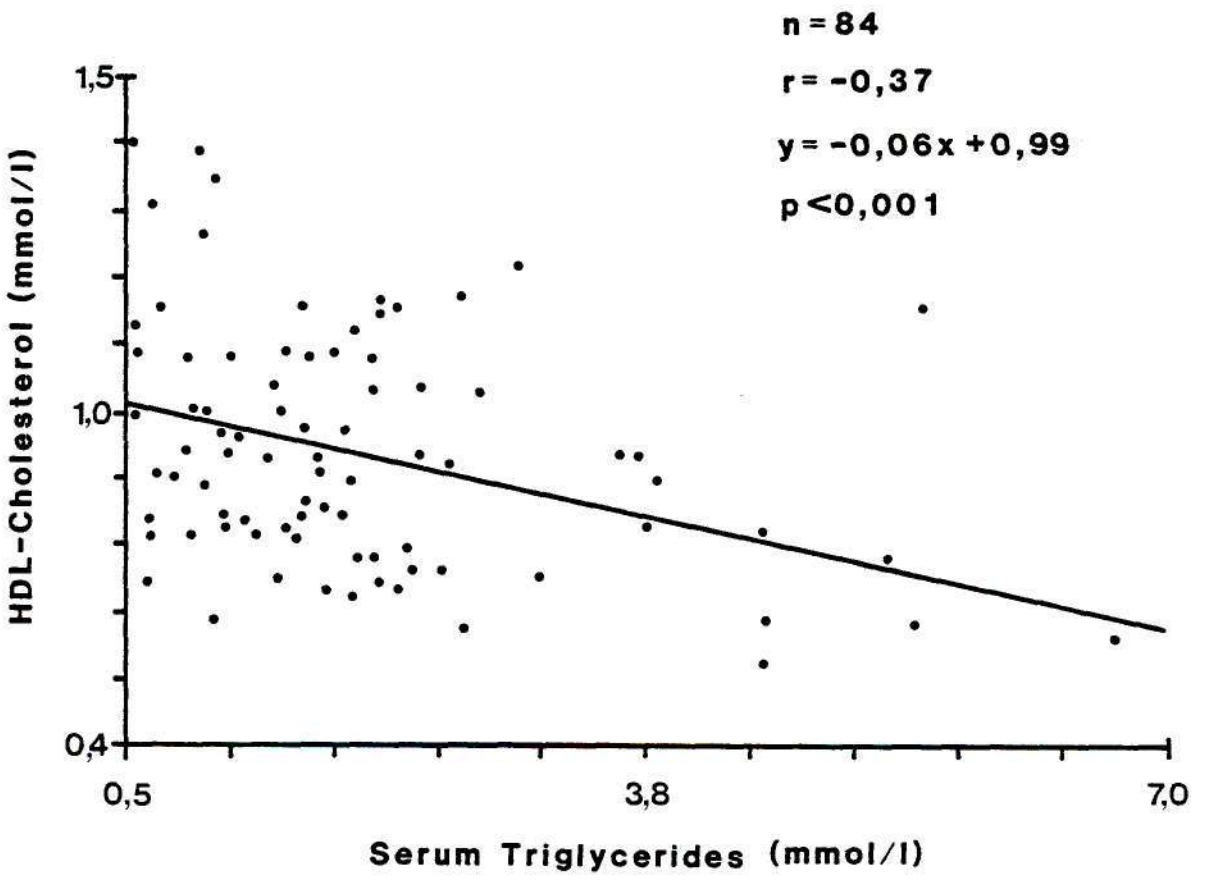


FIGURE 7.3

RELATIONSHIP BETWEEN SERUM APOPROTEIN B LEVELS AND LDL-CHOLESTEROL

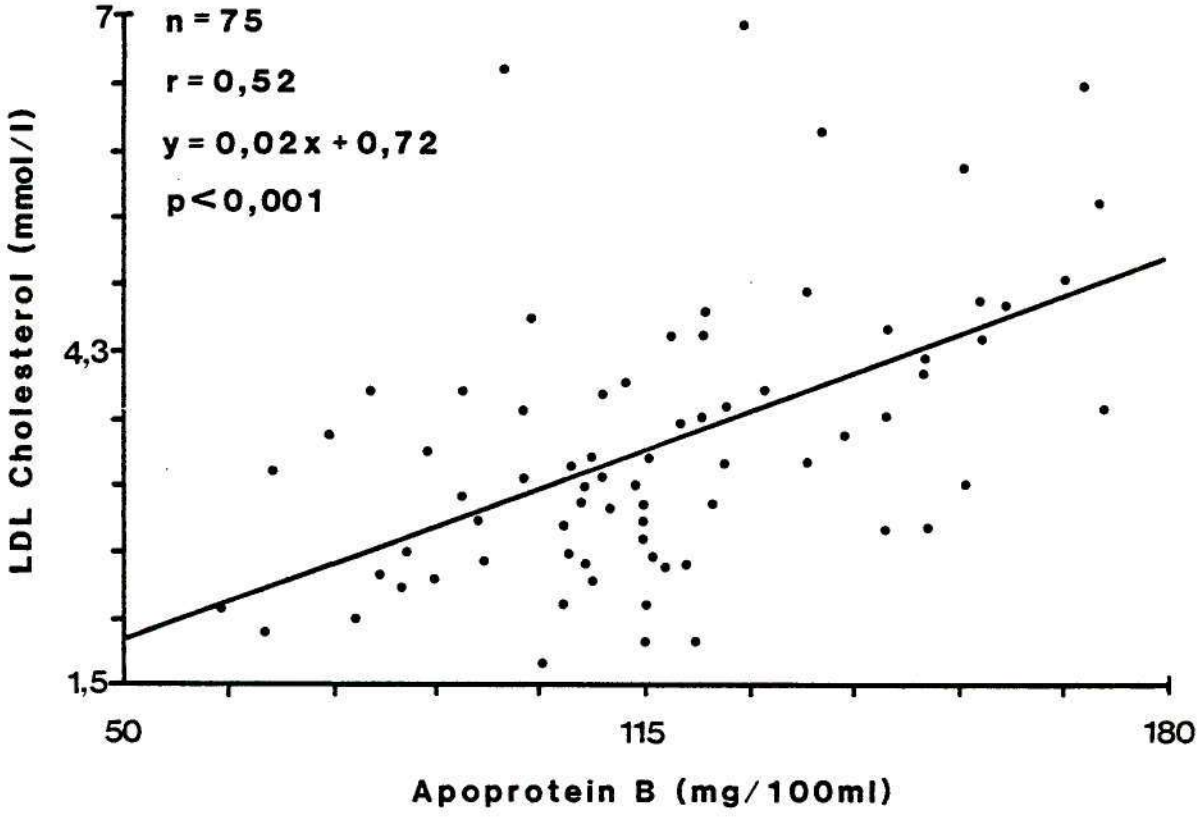
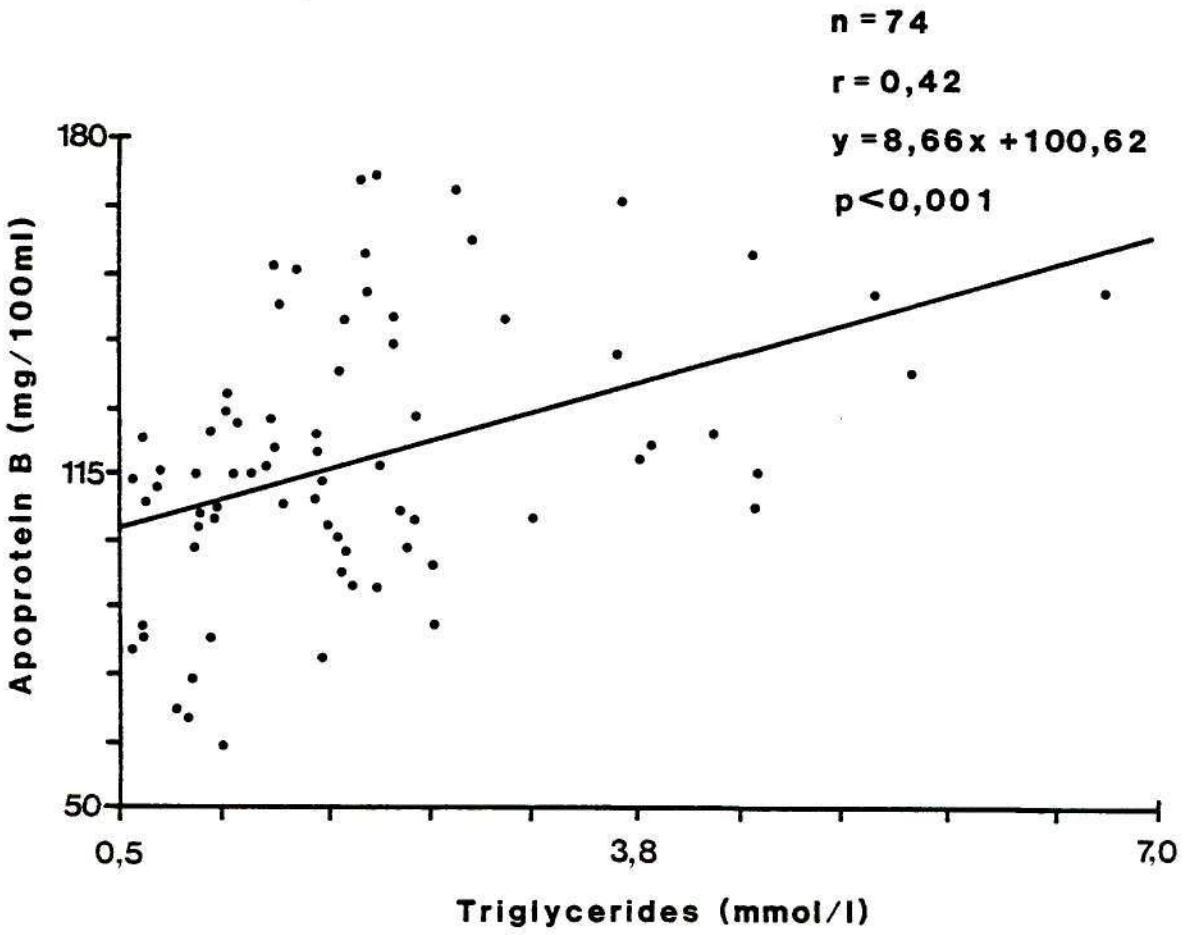


FIGURE 7.4

RELATIONSHIP BETWEEN SERUM TRIGLYCERIDES AND APOPROTEIN B



positive family history of diabetes, it is usual to assign a diagnosis of hypertriglyceridaemia secondary to diabetes than that of primary hyperlipoproteinaemia. If these proposals are adopted in the present study, it can be inferred that the hypertriglyceridaemia is secondary to the diabetic state since a positive family history of diabetes was present in 84% of the patients and all 85 patients had a fasting plasma glucose greater than 6,6 mmol/ℓ. In addition, the finding of a significant positive correlation between the serum triglyceride levels and the $HbA_1$ levels suggests that the increased triglyceride levels is related to the diabetic state. A positive correlation has been recorded by previous workers between triglycerides and $HbA_1$ (Peterson et al, 1977; Calvert et al, 1978; Aleyaissaine et al, 1980). Inasmuch as hypertriglyceridaemia is considered important in the development of vascular disease (Santen et al, 1972; Reckless et al, 1978; Brownlee and Cerami, 1981), this finding emphasises the importance of adequate control in this group of young patients.

The genesis of the hypertriglyceridaemia is undetermined. Increased triglyceride levels have been reported in obesity (Lewis, 1976; Sims, 1979) and the patients in the present study as a group were certainly obese. However, on dividing the patients into obese and non-obese and comparing their triglyceride levels no significant differences were found between these 2 subgroups. Obesity as a cause of the hypertriglyceridaemia is thus decidedly an unlikely explanation in the present study.

Insulin is known to promote lipogenesis (Section 2.1.3). The patients in the present study had significantly greater fasting insulin levels than the reference group and, on this basis, it could be argued that the increased insulin levels promoted hepatic VLDL secretion. The

raised triglyceride levels and Apo B concentrations in the present study would be consistent with an increased hepatic secretion of VLDL. But the same results will manifestly be obtained if the clearance of VLDL is impaired. There have been many studies on patients with NIDDM which attempted to answer whether the hypertriglyceridaemia is due to triglyceride overproduction or underutilization, but none have produced decisive evidence in support of either proposal (Lewis, 1976; Ganda, 1980; Goldberg, 1981).

In both male and female patients, the serum HDL-cholesterol levels were significantly lower than the values obtained in the reference subjects. This finding accords with the experience of most investigators who reports on HDL-cholesterol levels in NIDDM (Lopes-Virella et al, 1977a; Calvert et al, 1978; Paisey et al, 1978; Howard et al, 1978; Goldberg and Rubenstein, 1980; Taylor et al, 1981; Lisch and Sailer, 1981; Biesbroeck et al, 1982). Although lower levels of HDL-cholesterol have been recorded in obese individuals, as compared to non-obese controls (Carlson and Ericsson, 1975; Williams et al, 1979; Krauss, 1982), the lower HDL-cholesterol levels in patients with NIDDY cannot be explained on this basis for there was no significant difference between the HDL-cholesterol levels of the obese and non-obese NIDDY subgroups. This is in agreement with the results of many previous workers who failed to demonstrate any relationship between HDL-cholesterol and obesity in patients with NIDDM (Howard et al, 1978; Mattock et al, 1979; Goldberg and Rubenstein, 1980; Taylor et al, 1981).

In accordance with the findings of most previous workers reporting on NIDDM, the present study failed to demonstrate any significant correlation between HDL-cholesterol levels and $HbA_1$ (Kennedy et al, 1978; Yudkin et al, 1978; Aleyassine et al, 1980; Taylor et

levels of these parameters between the obese and non-obese subgroups. Thus, although the patients are obese as a group, obesity cannot be incriminated as a factor accounting for the aberrations recorded.

In accordance with the experience of most previous workers as described in Section 3.4.5, the present study failed to demonstrate any differences in the lipid and lipoprotein levels between the patients with NIDDY in whom microvascular disease was present and those in whom it was absent.

However, the finding of significantly higher fasting plasma glucose and glycosylated haemoglobin levels in the patients with microvascular disease is in agreement with the reports of some workers (Flock et al, 1979; Koenig and Cerami, 1980; Day, 1981) but not others (Trivelli et al, 1971; Coller et al, 1978). The higher values of $HbA_1$ in the patients with microvascular disease would suggest that non-enzymatic glycosylation of intracellular proteins may be important in the genesis of microvascular complications (Koenig and Cerami, 1980; Bunn, 1981).

Lipid aberrations are a major factor in the genesis of macrovascular disease in diabetics (Steiner, 1978; Bierman and Brunzell, 1978; Ganda, 1980; Alberti and Press, 1982). Unfortunately, from the point of view of this study, there were too few patients with macrovascular complications to permit an examination of the relationship between the lipid aberrations and such complications.

## 7.7 CONCLUSION

In the present study, aberrations in lipid metabolism were demonstrated in patients with NIDDY. The lipid and lipoprotein aberrations manifested as hypertriglyceridaemia, increased Apo B levels

# SUMMARY AND GENERAL CONCLUSIONS

Using strict defined criteria, the study unequivocally confirms the occurrence of non-insulin dependent diabetes in the young (NIDDY) in the Indian population of South Africa.

The clinical features manifest in the patients studied reflect in part the selection criteria applied. All were symptomatic diabetics and some were indeed very severe diabetics; the mean fasting glucose level of the group (12,3 mmol/ℓ) certainly categorised the patients as severe diabetics. Obviously, severe diabetes does occur in Indian patients with NIDDY. Unlike studies on patients with mild diabetes, no patient with a childhood onset was found in the present study. The lowest age at which symptomatic diabetes occurred was 14 years and the majority developed symptomatic diabetes in early adulthood.

As a group, the patients were obese and 50% of the group had a body mass which was 20% greater than the ideal. However, obesity did not correlate with the severity of diabetes.

There were striking aggregations of diabetes in the families of the patients and 84% had a positive family history. But, only 8% of patients had histories of diabetes transmitted vertically through three consecutive generations, a finding which may relate to inadequate medical histories about the ancestors.

The mean duration of symptomatic diabetes was only 5,9 years with a range of 1 to 35 years.

Despite the fact that the patients as a group were not well controlled and the mean age of the patients was 32,2 years, macrovascular complications were extremely rare: a mere 4% had evidence of ischaemic heart disease and not a single patient had peripheral vascular disease.

By contrast, unlike findings in NIDDY with mild diabetes, microvascular complications were relatively common: 18% overall had microvascular complications (17% had retinopathy and 7% nephropathy). Of the 14 patients with retinopathy, 12 had background changes only, whilst the remaining 2 also had proliferative changes. By far the commonest chronic complication on simple physical examination was, however, peripheral neuropathy which affected 24% of the group.

Despite the usual proneness of the patients to intercurrent infections, not a single patient presented with ketonuria during the entire period of the study which exceeded 4 years. This is clearly a remarkable feature, bearing in mind that the group as a whole were severe diabetics.

In a general sense the clinical picture does not differ very much from NIDDM except for the early age of onset of diabetes, the very strong family histories, lesser propensity to vascular complications and remarkable resistance to ketosis.

Having defined the clinical presentation of NIDDY in the group selected, biochemical features of the disorder were studied.

Glucose and insulin responses during a 100 g oral GTT were assessed in the 85 patients with NIDDY. The patients as a group displayed a fasting hyperglycaemia of 12,3 mmol/ℓ and a severe degree of glucose intolerance as evidenced by the marked hyperglycaemia during the GTT. Although the patients had fasting hyperinsulinism, the insulin response during the GTT was delayed and attenuated. This insulin deficiency was confirmed by the calculated insulin areas and insulin-glucose ratios. There was no significant difference between the obese and non-obese subgroups of NIDDY with respect to their insulin and glucose responses. However, when the patients were subdivided in terms of the severity of the disorder, the more severe diabetics had a more attenuated insulin

response than the moderate diabetics. Classification of the insulin responses into 'high' and 'low' responders revealed that 94% were 'low' insulin responders, and only 1 patient was a true 'high' insulin responder. Considered in conjunction with the other groups with much milder degrees of glucose intolerance described by others, it would appear as though NIDDY spans a wide spectrum of severity.

Non-obese patients with NIDDY proved to be resistant to insulin as assessed by the glucose disposal during an intravenous insulin tolerance test.

The role which the circulating hormonal antagonists of insulin play in the demonstrated insulin resistance was investigated. Patients with NIDDY manifested fasting hyperglucagonaemia and hypercortisolaemia. Fasting levels of glucagon and cortisol were also inadequately suppressed during glucose tolerance testing. Thus, these two hormones contributed to the hyperglycaemia by antagonising the action of the already deficient insulin secretion. However, fasting insulin-glucagon molar ratios were normal. The behaviour of growth hormone by contrast was such that it could not contribute to the hyperglycaemia.

Despite the decreased insulin response to glucose challenge, the FFA response was equivalent to that of the reference subjects during oral glucose tolerance testing. This apparent sensitivity of the patient's adipose tissue to the anti-lipolytic action of insulin, together with the normal insulin-glucagon molar ratios may account for the resistance of the patients to ketosis.

An investigation into the lipid and lipoprotein status of these patients revealed aberrations which manifested as hypertriglyceridaemia, low levels of HDL-cholesterol and increased Apo-B concentrations. Serum total cholesterol, LDL-cholesterol and Apoprotein A-1 levels were

similar to that obtained in the reference group. Obesity did not exert any influence on the lipid and related protein aberrations, nor did sulphonylurea therapy seem to affect lipid profiles. The hypertriglyceridaemia and increased Apoprotein-B levels appear to emanate from the VLDL class. Since HDL-cholesterol levels were decreased but Apoprotein A-1 levels normal, these findings could be interpreted as a reflection of an abnormal composition of the HDL class of lipoproteins.

There were no significant differences in the cortisol, growth hormone, lipid and lipoprotein levels in the patients with microvascular disease and matched patients without vascular disease. However, the fasting plasma glucose and glycosylated haemoglobin concentrations were significantly higher in the patients with microvascular disease, suggesting that non-enzymatic glycosylation might be important in the genesis of microvascular disease.

While 50% of the patients were obese, obesity did not relate to any of the biochemical aberrations found in the patients with NIDDY.

The biochemical findings in this study differ in no way from the expected findings in symptomatic non-insulin dependent diabetes with a mature onset. NIDDY is therefore not characterised by any unique biochemical aberration.

A major contribution of the present study is the demonstration that NIDDY can occur in severely diabetic forms and the disorder thus spans the entire spectrum of clinical and biochemical variation found in non-insulin dependent diabetes. Since the inheritance of the disorder can be accurately traced in some instances and appears to be autosomal dominant, the reported similarity to NIDDM gives hope that the genetic locus, and thus the biochemical identity of the disorder in non-

insulin dependent diabetes may probably be elucidated in the not too distant future.

# A P P E N D I X

## APPENDIX A : METHODS

### APPENDIX A-1 : DETERMINATION OF PLASMA GLUCOSE

Auto Analyser Methodology

#### A-1.1 References

Auto Analyser Methodology (1962)

Hoffman (1937)

#### A-1.2 Principle

Glucose was determined by a procedure utilising the potassium ferricyanide-potassium ferrocyanide oxidation-reduction reaction. The yellow solution of potassium ferricyanide was reduced to the colourless ferrocyanide. The change in absorbance was measured at 420 nm using a flow cuvette with a 15 mm light path.

#### A-1.3 Reagents

(1) Alkaline Potassium Ferricyanide

Sodium Chloride 9,0 g

Potassium Ferricyanide 0,25 g

Sodium carbonate 20,0 g

Distilled water q.s. 1000 ml

(2) Physiological saline

9 g NaCL per 1000 ml distilled $H_2O$

(3) Stock Glucose Solution (60 mmol/ℓ)

Dextrose (anhydrous) 21,62 g

Saturated benzoic acid q.s. 2000 ml

(4) Working Glucose Standards

The stock glucose solution was diluted with benzoic acid as follows:

| ml of stock solution | Diluted to | mmol/ℓ glucose |
|---|---|---|
| 10 | 200 ml | 3,0 |
| 10 | 100 ml | 6,0 |
| 15 | 100 ml | 9,0 |
| 20 | 100 ml | 12,0 |
| 25 | 100 ml | 15,0 |

A-1.4 Procedure

The flow diagram of the methodology employed is given in Figure A.1. For optimum bubble pattern 0,5 ml Brij-35 was added per litre of saline diluent and potassium ferricyanide.

A-1.5 Comment

Blood was collected into plastic 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.

Dialysis of the plasma sample removed much of the non-glucose reducing substances so that values near to the true glucose are obtained (Wootton, 1974).

Glucose standards are made up in saturated benzoic acid to prevent the growth of moulds which have a glycolytic effect.

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 1,74% and the inter-assay coefficient of variation was 2,33%.

## 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.

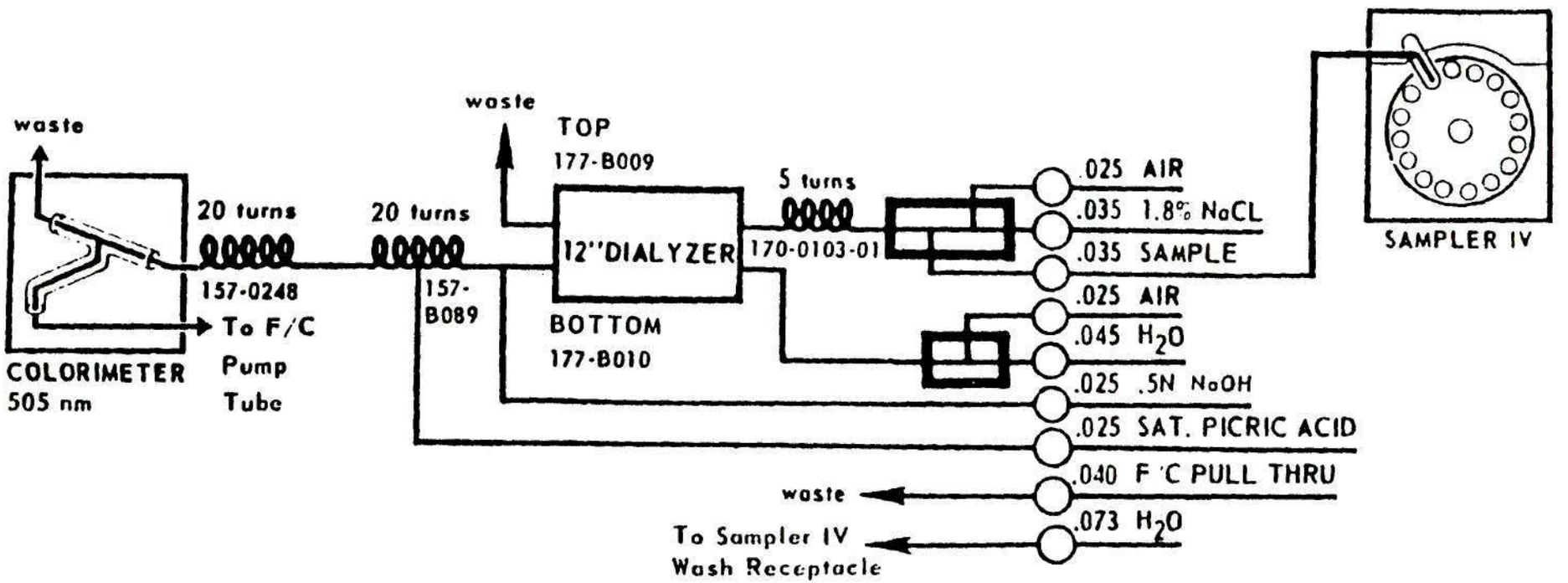
### A-2.3 Reagents

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

18 g NaCl

FIGURE A.2

FLOW DIAGRAM OF CREATININE METHODOLOGY



Distilled $H_2O$ 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 | µmoℓ/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 extinction 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 : DETERMINATION OF SERUM BETA$_2$-MICROGLOBULIN CONCENTRATIONS

Beta$_2$-microglobulin levels were determined using the Phadebas $\beta_2$-micro test from Pharmacia Diagnostics, Uppsala, Sweden.

### A-3.1 Principle

The Phadebas $\beta_2$-microtest is a competitive radioimmunoassay, $\beta_2$-microglobulin in the sample competes with a fixed amount of $^{125}$I-labelled $\beta_2$-microglobulin for the binding sites of anti-$\beta_2$ microglobulin antibodies covalently bound to sephadex particles. The competitive capacity is then compared with that of $\beta_2$-microglobulin standards of known concentration. The bound and free $\beta_2$-microglobulin are separated by centrifugation. The radioactivity of the labelled $\beta_2$-microglobulin bound to the sedimented sephadex particles is then measured and this radioactivity is inversely proportional to the quantity of unlabelled $\beta_2$-microglobulin in the sample.

### A-3.2 Reagents

(1) $^{125}$I-$\beta_2$-microglobulin

33 ng (114,7 kBq) of lyophilized $^{125}$I-$\beta_2$-microglobulin was reconstituted by adding 5,5 ml redistilled water.

(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 W.H.O. insulin standard yielded superimposable standard curves.

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

| Final Std. Concentration | | Buffer Solution | | Std. Solution |
|---|---|---|---|---|
| A | 320 µU/ml | - | | 4 ml |
| B | 160 µU/ml | 500 µℓ | + | 500 µℓ of A |
| C | 80 µU/ml | 500 µℓ | + | 500 µℓ of B |
| D | 40 µU/ml | 500 µℓ | + | 500 µℓ of C |
| E | 20 µU/ml | 500 µℓ | + | 500 µℓ of D |
| F | 10 µU/ml | 500 µℓ | + | 500 µℓ of E |
| G | 5 µU/ml | 500 µℓ | + | 500 µℓ 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 | - | 1,10 ml | 100 µℓ | - | - |
| Std. (µℓ) | - | - | - | 100 | - |
| Control/Samples (µℓ) | - | - | - | - | 100 |
| $^{125}$I-Insulin (µℓ) | 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 xg 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 xg 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).

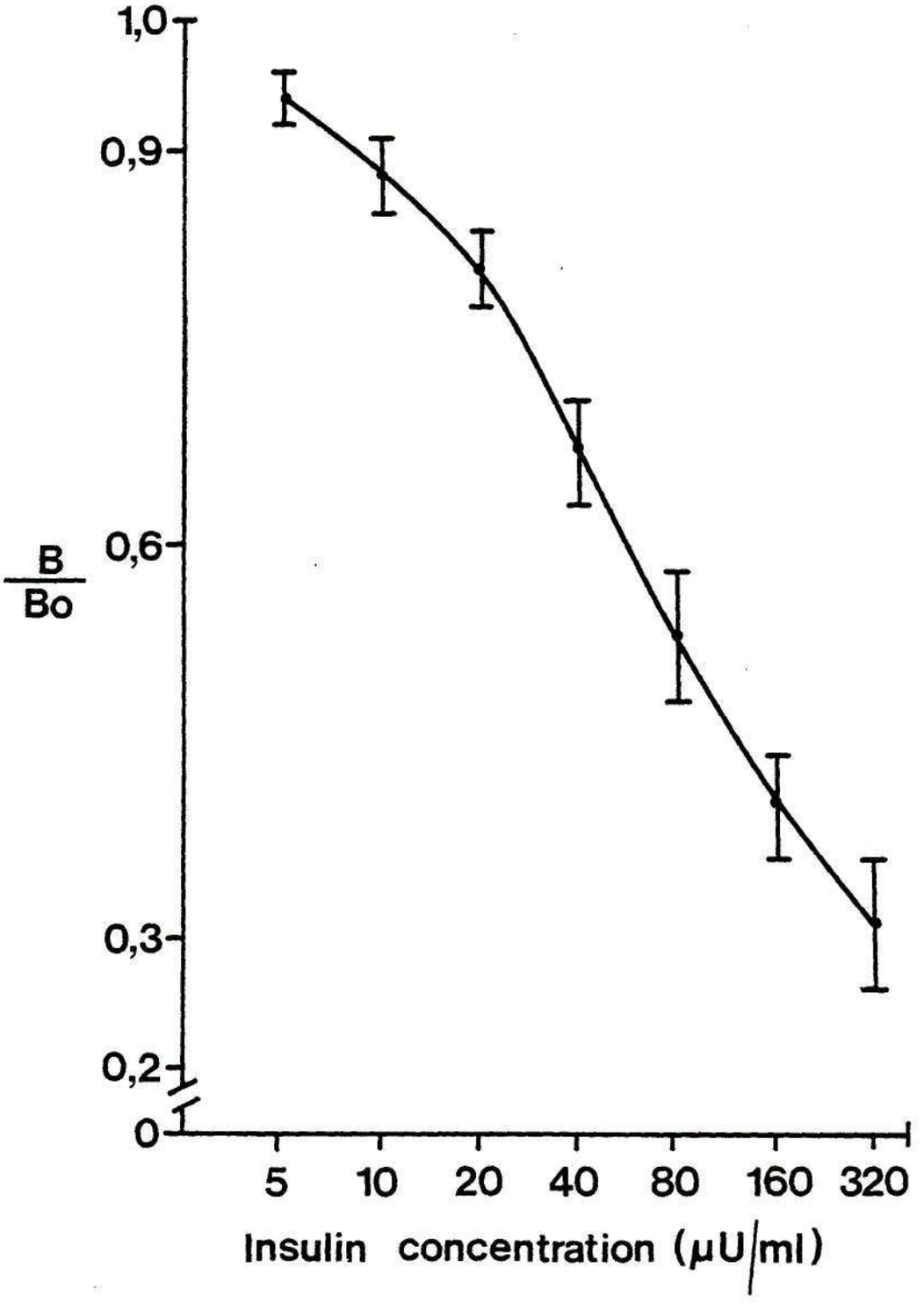
## A-5.4 Calculation

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.

FIGURE A.5

**COMPOSITE STANDARD CURVE GENERATED FROM 10 CONSECUTIVE ASSAYS (mean ± 1 SD)**



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.

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

a) Maximum binding (Bo/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 PLASMA FREE FATTY ACIDS

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

### A-6.1 References

Duncombe (1964)

### A-6.2 Principle

Free fatty acids are converted to chloroform-soluble copper salts. The

copper in the organic layer is subsequently measured colorimetrically 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-6.3 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 diethydithiocarbamate in redistilled butanol

(3) Chloroform

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

(4) Stock Standard Solution (500 µmoℓ/ℓ)

12,82 mg of palmitic acid in 100 ml chloroform. This stock solution was stored in the dark at room temperature.

## A-6.4 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 gree 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 absorbances (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-6.5 Calculation

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

$$\text{concentration of FFA in sample } (\mu mol/\ell) = \frac{A \text{ sample}}{A \text{ standard}} \times \frac{500}{1}$$

### A-6.6 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-7 : DETERMINATION OF PLASMA GLUCAGON CONCENTRATIONS

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

## A-7.1 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-7.2 Reagents

(1) Trasylol (Bayer)

10,000 KIU/ml (10 ml)

(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) $^{125}$I-glucagon

The lyophilized $^{125}$I-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 concentration 2,000, 1,000, 500, 250, 125, 62,5 pg/mℓ were prepared from the stock solution (2,000 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 |

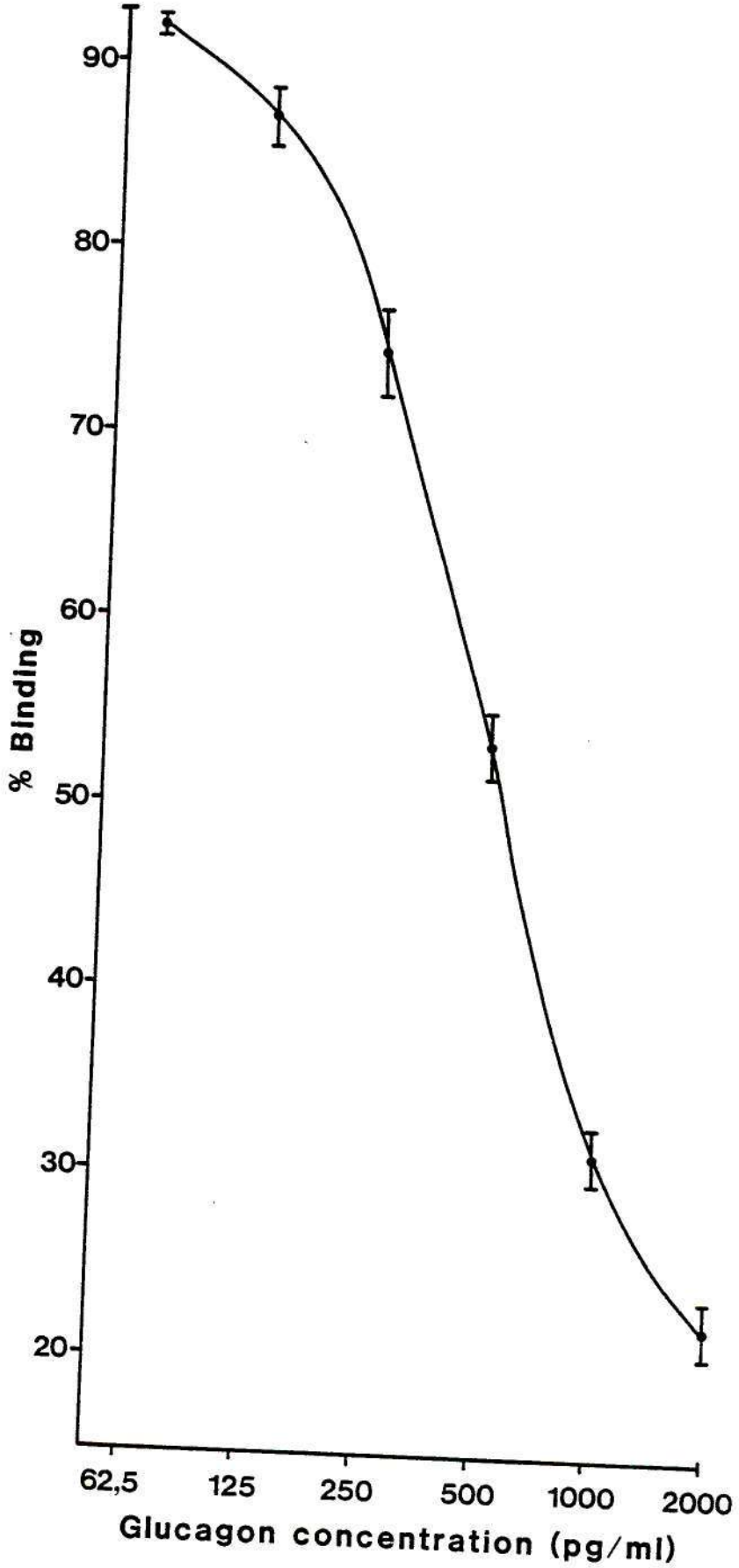
A-7.3 Procedure

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 |

FIGURE A.7

**COMPOSITE GLUCAGON STANDARD CURVE GENERATED FROM 10 CONSECUTIVE ASSAYS (Mean ± 1 SD)**



| | | | | | |
|---|---|---|---|---|---|
| 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-7.4 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 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.7.

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-7.5 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.

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

a) Maximum binding (Bo/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 6,9% and 9,2% respectively.

## APPENDIX A-8 : DETERMINATION OF SERUM CORTISOL CONCENTRATIONS

Serum cortisol levels were determined by a radioimmunoassay technique using the Gammacoat $^{125}$I-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 $^{125}$I-cortisol tracer in antibody coated tubes where the antibody is immobilised onto the lower inner wall of the Gamma-coat 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.

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 µℓ 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-9.4 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.9.

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

### A-9.5 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-10 : DETERMINATION OF TOTAL SERUM CHOLESTEROL LEVELS

Cholesterol was determined in serum by the CHOD-PAP Method Kit (Boehringer Mannheim GmbH Diagnostica).

### A-10.1 Reference

Trinder P. (1969)

### A-10.2 Principle

The method used is an enzymatic colorimetric method and the test principle is as follows:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \Delta 4 \text{ cholestenone} + H_2O_2$$

$$2\ H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} 4\ H_2O + \text{4 aminophenazone-p - benzoquinoneimine} *$$

* The colour intensity of this red dye is directly proportional to the concentration of cholesterol in the sample.

A-10.3 Reagents

(1) Solution 1

Potassium phosphate buffer (0,4M pH 7,7) containing phenol (20 mmol/ℓ) and methanol (1,85 mol/ℓ). Total volume 100 ml.

(2) Solution 2

Potassium phosphate buffer (0,4M pH 7,7) containing 4 amino-phenazone (2 mmol/ℓ), methanol (1,85 mol/ℓ) and hydroxypoly-ethoxydodecane (0,4%). Total volume 100 ml.

(3) Solution 3

Enzyme solution

Solution containing cholesterol esterase (≥ 40 U/ml), cholesterol oxidase (≥ 12 U/ml) and peroxidase (≥ 8 U/ml). Total volume 1,5 ml.

(4) Solution 4

Cholesterol reagent solution

The cholesterol reagent solution was prepared by mixing 1 part of Solution 1, 1 part of Solution 2 and 0,01 part of Solution 3 according to the volume of reagent required.

A-10.4 Procedure

A Precilip control (Boehringer Mannheim Gmb Diagnostica) was incorporated in each assay.

20 µℓ of each sample and control was pipetted into plastic test tubes. Thereafter, 2,0 ml of Solution 4 was added to each test tube and the mixture vortexed. A reagent blank containing only Solution 4 was included in each assay.

All tubes were incubated at 37°C for 15 minutes. Thereafter the absorbances of the samples and control were read against the reagent blank at 500 nm using a glass cuvette with a 1 cm light path on a Beckman Acta V spectrophotometer.

### A-10.5 Calculation

The concentration of cholesterol in each sample was calculated according to the following formula:

$$\text{Cholesterol concentration } (\text{mmol}/\ell) = \text{Absorbance sample} \times 15{,}1$$

### A-10.6 Comment

Serum was separated from blood as soon as possible after collection and stored at 4°C. Assays were done within 3 days.

All determinations were carried out in duplicate.

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

Glucose concentrations up to 55 mmol/ℓ caused no interference with the assay.

The intra- and inter-assay coefficients of variation of the cholesterol assay were 2,01% and 2,3% respectively.

## APPENDIX A-11 : DETERMINATION OF SERUM TRIGLYCERIDE LEVELS

Serum triglycerides were determined with a fully enzymatic triglyceride kit (Boehringer Mannheim GmbH Diagnostica).

A-11.1 Reference

Wahlefeld (1974)

A-11.2 Principle

Neutral fats are assayed enzymatically by way of their glycerol content. The test principle is as follows:

$$\text{Triglycerides} \xrightarrow[\text{esterase}]{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} \text{glycerol-3-PO4} + \text{ADP}$$

$$\text{ADP} + \text{Phosphoenolpyruvate} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{L-Lactate} + \text{NAD}^+$$

The amount of NADH consumed is equivalent to the amount of glycerol present in the specimen. The oxidation of NADH is determined by means of its absorption at 340 nm.

A-11.3 Reagents

(1) Solution 1

20 mmol/ℓ phosphate buffer pH 7 containing $MgSO_4$ (4 mmol/ℓ) and sodium dodecyl sulphate 0,35 mmol/ℓ.

(2) Solution 2

Solution 2 contained NADH (10 mmol/ℓ), ATP (22 mmol/ℓ), phosphoenol-pyruvate (18 mmol/ℓ).

(3) Solution 3

Solution 3 contained Lactate Dehydrogenase ($\geq$ 300 U/ml), pyruvate

kinase ($\geq$ 50 U/ml), lipase ($\geq$ 4,000 U/ml) and esterase ($\geq$ 30 U/ml).

(4) Solution 4

Solution 4 contained glycerokinase ($\geq$ 150 U/ml).

(5) Stock solution

After bringing the solutions to room temperature, volumes of Solutions 1, 2 and 3 were mixed in the proportions 100 : 2 : 2.

A-11.4 Procedure

A Precilip control (Boehringer Mannheim Gmb Diagnostica) was incorporated in each assay.

2,5 ml of stock solution and 50 µℓ of samples and control were pipetted into plastic test tubes. The contents of the tubes were mixed and this constituted the sample blank. 1,0 ml of this mixture was then pipetted into another test tube for each sample assayed and 5 µℓ of Solution 4 (glycerokinase) added to this solution. The contents of the tubes were mixed and incubated at room temperature for 10 minutes. This constituted the sample.

A reagent blank was determined with each assay by using redistilled water instead of sample. The absorbances of the sample blank and sample were read against air at 340 nm using a glass cuvette with a 1 cm light path on a Beckman Acta V spectrophotometer.

A-11.5 Calculation

The concentration of triglycerides in each sample was calculated according to the following formula:

Triglyceride concentration (mmol/ℓ) = Δ Absorbance x 8,13

Δ Absorbance = Absorbance sample blank - Absorbance sample - $Absorbance_{RB}$

where $absorbance_{RB}$ is the absorbance of the reagent blank.

A-11.6 Comment

Serum was separated from blood as soon as possible after collection and stored at 4°C. Assays were done within 3 days.

All determinations were carried out in duplicate.

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

The intra- and inter-assay coefficients of variation of the triglyceride assay were 2,37% and 4,8% respectively.

## APPENDIX A-12 : DETERMINATION OF SERUM HIGH DENSITY LIPOPROTEIN CHOLESTEROL LEVELS

A-12.1 References

Burnstein et al (1970)

Lopes-Virella et al (1977c)

A-12.2 Principle

The principle of the HDL-cholesterol determination is based on the quantitative separation of HDL from other lipoproteins with the aid of a suitable precipitant. In the method used phosphotungstate in the presence of magnesum ions precipitates LDL, VLDL and chylomicrons from sera. The cholesterol measured in the supernatant is equivalent to the HDL-cholesterol concentration in the sample.

A-12.3 Reagents

(1) Sodium phosphotungstate solution

40 g of phosphotungstate per litre of a mixture of 1 M NaOH and redistilled water (160 : 840) by volume.

(2) Magnesium chloride solution

2 molar $MgCl_2$,6 $H_2O$ (406,62 g in 2,0 litres of redistilled water)

Since $MgCL_2$ is hygroscopic the magnesium concentration was checked by atomic absorption spectroscopy.

A-12.4 Procedure

A pooled serum control was incorporated in each assay.

1,0 ml of sample was pipetted into plastic test tubes. To each tube 100 µℓ of phosphotungstate solution and 25 µℓ of $MgCL_2$ solution was added and the tubes were mixed on a vortex. After a 15 min incubation at room temperature, the tubes were centrifuged at 4°C for 30 mins at 1500 xg in a refrigerated centrifuge. The cholesterol content of the supernatant was determined as described in Appendix A.10 using 100 µℓ of sample instead of 20 µℓ and 1,92 ml reagent.

A-12.5 Calculation

To obtain the HDL-cholesterol, the cholesterol value obtained was multiplied by the dilution factor (1,125).

$$\text{HDL cholesterol} = \frac{15,1 \times 1,125}{5}$$

A-12-6 Comment

Serum was separated from blood as soon as possible after collection

and stored at 4°C. Assays were done within 3 days.

All determinations were carried out in duplicate.

If the supernatant was not clear the serum sample was diluted 1 : 1 with 0,9% saline and the assay repeated.

The intra- and inter-assay coefficients of variation for the HDL-cholesterol assay were 3,5% and 7,9% respectively.

## APPENDIX A-13 : DETERMINATION OF SERUM LOW DENSITY LIPOPROTEIN CHOLESTEROL LEVELS

### A-13.1 References

Wilson and Spiger (1973)

Ononogbu and Lewis (1976)

### A-13.2 Principle

In this method a 10% sodium dodecyl sulphate (SDS) solution results in the differential aggregation of VLDL from LDL and HDL in serum samples. The VLDL aggregates as a pellicle at the top of the tube while the LDL and HDL remain in the subnatant. The cholesterol content of the subnatant is equivalent to the LDL + HDL cholesterol. In another aliquot of serum the HDL-cholesterol concentration is determined as described in Appendix A.12. The LDL cholesterol concentration is obtained by subtracting the HDL-cholesterol from the LDL + HDL cholesterol.

### A-13.3 Reagents

10% SDS Solution

10 g of SDS in 100 ml of 0,15 M saline pH 9.

A-13.4 Procedure

A pooled serum control was incorporated in each assay.

To 1 ml of serum in polycarbonate centrifuge tubes was added 75 µℓ of a 10% SDS solution and the mixture vortexed.

The tubes were then incubated at 35°C for 2 hours in a waterbath. After this incubation, the tubes were centrifuged at 10,000 xg for 10 minutes in a JA 21 rotor of a Beckman Model J 2-21 centrifuge.

The VLDL particles aggregated as a pellicle at the top of the sample.

An aliquot of the subnatant was removed by gentle aspiration with a pasteur pipette.

The cholesterol content of the subnatant was determined as described in Appendix A.10.

In another aliquot of the same serum sample, the HDL-cholesterol concentration was determined as described in Appendix A.12.

A-13.5 Calculation

Cholesterol concentration
of LDL + HDL (mmol/ℓ) = Absorbance sample x 16,233*

* 15,1 x 1,075 (dilution factor)

To obtain the LDL-cholesterol the HDL-cholesterol obtained as described in Appendix 12 was subtracted from the LDL + HDL cholesterol value.

A-13.6 Comment

Serum was separated from blood as soon as possible after collection and stored at 4°C. Assays were done within 3 days.

All determinations were carried out in duplicate.

The intra- and inter-assay coefficients of variation for the (LDL + HDL-cholesterol) assays were 2,45% and 2,74% respectively.

## APPENDIX A-14 : DETERMINATION OF SERUM APOPROTEIN A-1 LEVELS

### A-14.1 References

Schonfeld and Pfleger (1974)

Karlin et al (1976)

Karlin et al (1978)

### A-14.2 Principle

The assay is based on the competitive binding principles of radio-immunoassay. Standards and unknown samples are incubated with $^{125}$I-Apo A-1 tracer and fixed amounts of anti-Apo A-1 antibody. At the end of the incubation period the bound Apo A-1 is separated from free Apo A-1 using the second antibody technique (normal rabbit serum and donkey anti-rabbit gamma globulin). The quantity of Apo A-1 in the sample is inversely related to the bound radioactivity.

### A-14.3 Reagents

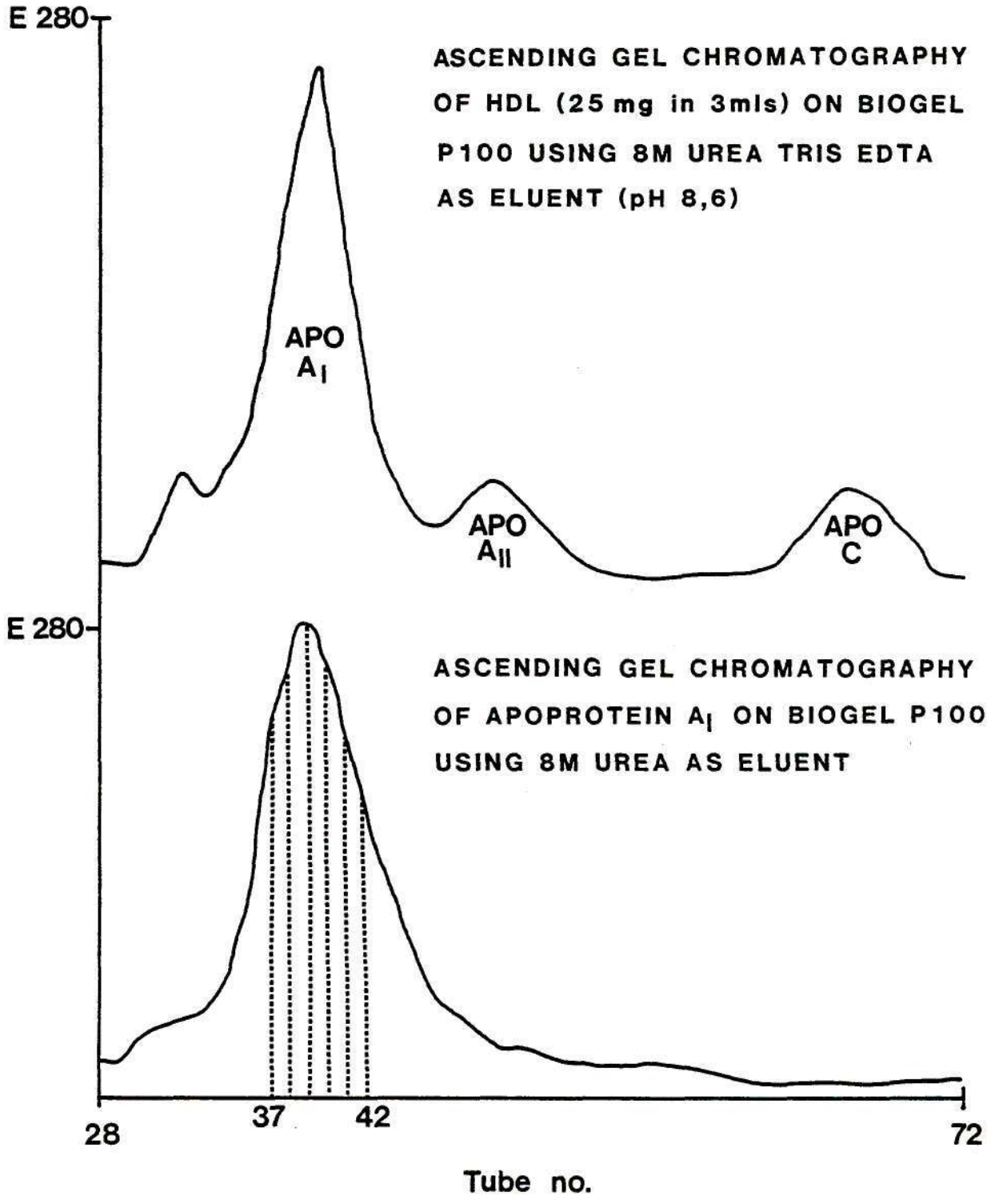
(1) Preparation of Apoprotein A-1 Standards

a) Preparation and Delipidation of HDL:

High density lipoproteins were isolated from pooled human serum by preparative ultracentrifugation at selected densities according to Havel et al (1955). Centrifugation was carried out at 20°C on a Beckman L2 65 B ultracentrifuge with a SW 27 rotor. The serum was centrifuged twice at densities of

FIGURE A.14.1

GEL CHROMATOGRAPHY OF APO-HDL



1,08 g/ml and at 1,18 g/ml (135,000 xg for 48 hours). This narrow density range was chosen to minimize contamination by other lipoproteins and albumen. The HDL fraction thus obtained was dialysed for 48 hours against repeated exchanges of 0,1 M Tris HCL buffer pH 8,6 containing 1,0 mmolar EDTA (TRIS-EDTA Buffer). The HDL fraction was tested for albumen contamination by immunodiffusion against goat anti-human albumen serum (Hyland, Los Angeles, USA) and was found to be albumen free. After being lyophilized, the HDL fraction was delipidated with ether ethanol according to the procedure of Scanu and Edelstein (1971). The delipidated HDL fraction was redissolved in a small volume of TRIS-EDTA buffer and the protein concentration determined by a modified Lowry procedure (Markwell et al 1978).

b) Separation of Apo A-1 from other apoproteins:
Separation of the apoproteins was achieved by ascending gel chromatography on a Biogel P-100 column using 8 M Urea in TRIS-EDTA Buffer as eluant (Scanu et al, 1969; Schonfeld and Pfleger, 1974); eluates were monitored at 280 nm. The elution profile is shown in Figure A-14.1. The fractions corresponding to Apoprotein A-1 were pooled, dialysed, lyophilized and redissolved in TRIS-EDTA Buffer, when it was rechromatographed on the same system under the same conditions (Figure A.14.1). Fractions obtained from the centre of the single elution peak were pooled, dialysed and lyophilized. The protein concentration was determined on the redissolved Apoprotein A-1 solution as described previously. The Apoprotein A-1 thus isolated migrated as a single band on polyacrylamide gel electrophoresis (9%) which was done according to Schonfeld and Pfleger (1974). Immunoelectrophoresis against goat anti-human serum (Hyland, Los Angeles, USA) yielded a single precipitin arc. The amino

TABLE A.14.1

AMINO ACID ANALYSIS OF APOPROTEIN A1 (RESIDUES PER MOL.)

| Amino Acids | Jialal et al (1979) | Brewer et al (1978) | Schonfeld et al (1976) |
|---|---|---|---|
| Aspartate & Asparagine | 20 | 21 | 20 |
| Threonine | 10 | 10 | 10 |
| Serine | 15 | 15 | 15 |
| Glutumate & Glutamine | 46 | 46 | 49 |
| Proline | 9 | 10 | 10 |
| Glycine | 11 | 10 | 10 |
| Alanine | 20 | 19 | 18 |
| Valine | 13 | 13 | 13 |
| Methionine | 3 | 3 | 3 |
| Leucine | 36 | 37 | 38 |
| Tyrosine | 7 | 7 | 7 |
| Phenylalanine | 6 | 6 | 6 |
| Lysine | 20 | 21 | 19 |
| Histidine | 5 | 5 | 5 |
| Arginine | 15 | 16 | 15 |
| Isoleucine | - | - | - |
| Tryptophan | - | 4 | - |
| No. of amino acids | 236 | 243 | 238 |

acid analysis of the isolated Apoprotein A-1 which was undertaken by the National Chemical Research Laboratory was in close agreement with that obtained by previous workers (Table A.14.1).

(2) Preparation of antiserum to Apoprotein A-1.

Antibody to Apoprotein A-1 (Apo A-1) was raised in rabbits. Apo A-1 (1 mg/ml) emulsified in an equal volume of complete Freund's adjuvant was injected subcutaneously on 3 occasions, 2 weeks apart; 10 days after the final injection the rabbits were bled and the serum obtained was stored at -20°C in 100 µℓ aliquots. Monospecificity of the antisera was evidenced by the fact that a single precipitin arc was obtained on immuno-electrophoresis against normal human serum. An antibody dilution curve revealed that a 1 : 5000 dilution of the anti-Apo A-1 antibody bound 50% of the $^{125}$I-Apo A-1 tracer.

(3) Iodination of Apoprotein A-1.

Apo A-1 (15 µg in 10 µℓ of 0,1 M Borate Buffer pH 8,5) was iodinated by the indirect conjugation labelling method of Bolton and Hunter (1973). Bolton Hunter reagent was obtained from the Radiochemical Centre, Amersham, England. The labelled product which was purified on a 0,9 x 30 cm Sephadex G-75 column (Pharmacia) had a specific activity of approximately 1,11 MBq/µg. The $^{125}$I-Apo A-1 was stored at 4°C and was rechromatographed on the day of the assay on a 0,9 x 30 cm Sepharose 6B column (Pharmacia).

(4) Assay Buffers

Samples and standards were diluted in 0,05 M Barbital Buffer pH 8 containing 40 g Bovine serum albumen/ℓ. First and second antibody, $^{125}$I-Apo A-1, and normal rabbit serum were diluted in 0,05 M Barbital Buffer containing 5 g bovine serum albumen/ℓ.

(5) Normal Rabbit Serum (NRS)

Blood was obtained from normal rabbits, centrifuged and the sera stored in 500 µℓ aliquots at -20°C. On the day of the assay a 1 : 100 dilution was made.

(6) Second Antibody

The second antibody produced against rabbit serum in donkeys was obtained from Wellcome Laboratories, Beckenham, England. The donkey anti-rabbit serum was titrated against normal rabbit serum and used at an optimum dilution of 1 : 10.

A-14.4 Procedure

The $^{125}$I-Apo A-1 was rechromatographed on a Sepharose 6B column on the day of the assay and the radioactivity diluted to give approximately 15,000 counts per minute per 100 µℓ. The Apo A-1 stock standard (40 µg/ml) which was stored at -20°C was allowed to thaw and then diluted 1 : 200 to give a standard with a final concentration of 200 ng/ml. Standard solutions, final concentration 200, 100, 50, 25, 12,5 and 6,25 ng/ml were prepared by diluting the stock standard (200 ng/ml) in buffer according to the following schedule:

| Final Standard | Concentration | Buffer Solution | | Standard Solution |
|---|---|---|---|---|
| A | 200 ng/ml | - | | 2 ml |
| B | 100 ng/ml | 1 ml | + | 1 ml of A |
| C | 50 ng/ml | 1 ml | + | 1 ml of B |
| D | 25 ng/ml | 1 ml | + | 1 ml of C |
| E | 12,5 ng/ml | 1 ml | + | 1 ml of D |
| F | 6,25 ng/ml | 1 ml | + | 1 ml of E |

A pooled serum control was incorporated in each assay.

The unknown samples and control were extracted with ether ethanol

(2 : 3 vol/vol) according to Schonfeld and Pfleger (1974); 100 µℓ of sample were extracted in 10 ml of ether-ethanol at 4°C overnight. The protein precipitates were washed twice with ether, dried under nitrogen and solubilised in 8 M Urea. The solubilised samples were then further diluted in 40 G/L BSA Barbital Buffer to give a final dilution of 1 : 20,000.

Assays were carried out in polystyrene tubes (LKB, Stockholm, Sweden).

The test schedule was as follows:

| Reagent | Total | NSB | Bo | Std. | Control/Samples |
|---|---|---|---|---|---|
| 5 G/L BSA Buffer (µℓ) | - | 100 | - | - | - |
| 40 G/L BSA Buffer (µℓ) | - | 100 | 100 | - | - |
| $^{125}$I-Apo A-1 (µℓ) | 100 | 100 | 100 | 100 | 100 |
| Apo A-1 Stds (µℓ) | - | - | - | 100 | - |
| Control/Samples (µℓ) | - | - | - | - | 100 |
| Anti-Apo A-1 (µℓ) | - | - | 100 | 100 | 100 |
| NRS (µℓ) | - | 100 | 100 | 100 | 100 |
| Tubes were mixed and incubated at 4°C for 24 hours. | | | | | |
| Second Ab (µℓ) | - | 100 | 100 | 100 | 100 |
| Tubes were mixed and incubated at 4°C for ± 18 hours. | | | | | |

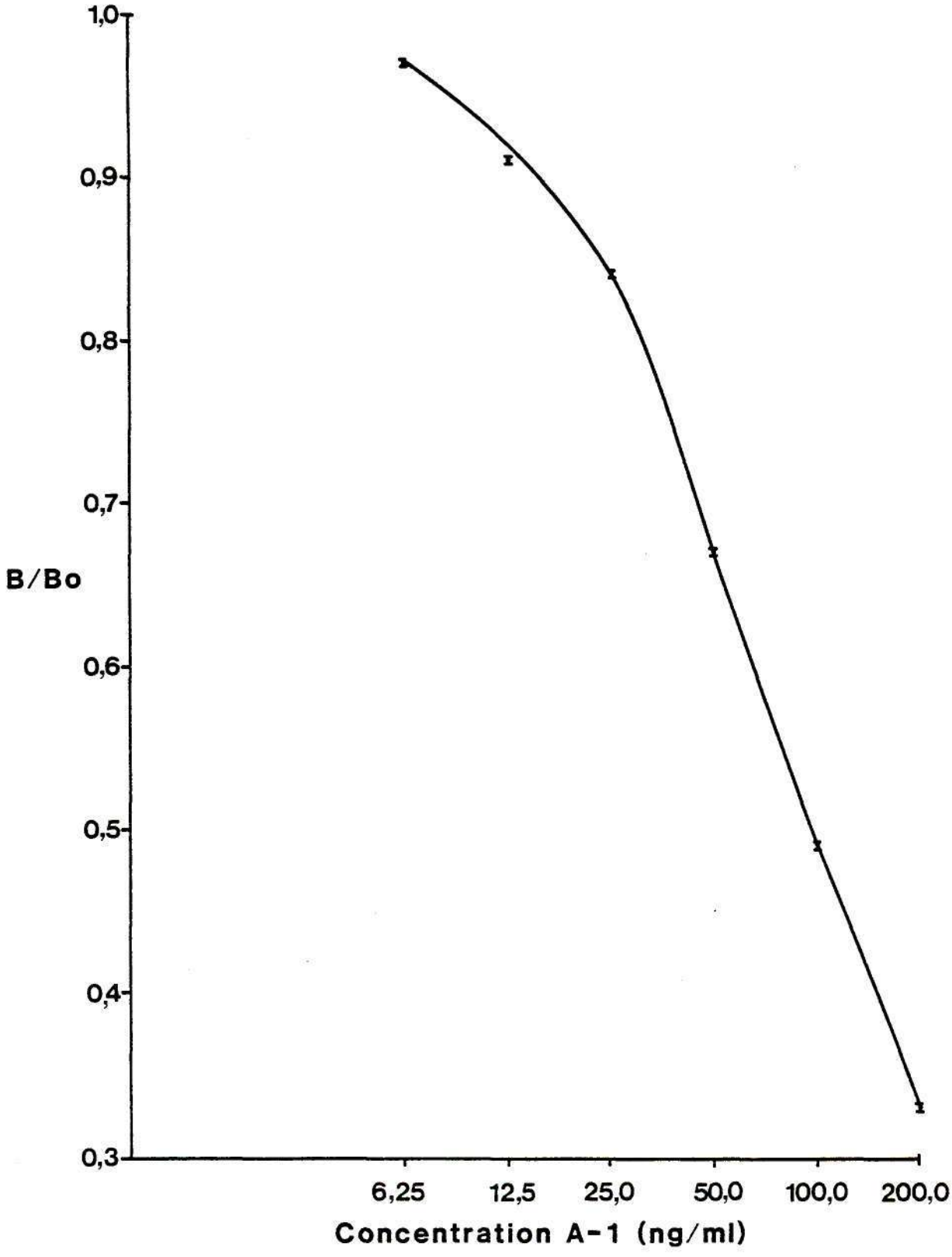
After this incubation, 2 mls of Tween 20-saline (0,1% Tween in 0,9% saline) was added to all tubes except the totals and the tubes were centrifuged at 4°C at 2400 xg for 60 minutes. After the supernatant was aspirated from all tubes except the totals, the radioactivity in each tube including the totals was determined using a gamma counter (5 min counts).

A-14-5 Calculation

The count rates for each of the standards was expressed as a percentage

FIGURE A.14.2

**COMPOSITE A-1 STANDARD CURVE GENERATED FROM FIVE CONSECUTIVE ASSAYS (Mean ± 1 S.D.)**



of the mean count rate of the zeros (B/Bo). The B/Bo for each Apo A-1 standard was plotted against its respective Apo A-1 concentration on lin-log paper and a standard curve constructed. A typical standard curve generated from 5 consecutive assays is shown in Figure A.14.2.

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 Apo A-1 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-14.6 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 (Bo/Total) had to be between 45 - 55%.

b) Non specific binding (NSB) had to be less than 5%.

The intra- and inter-assay coefficients of variation for the Apo A-1 assay were 3,8% and 9,8% respectively.

## APPENDIX A-15 : DETERMINATION OF SERUM APOPROTEIN B LEVELS

### A-15.1 References

Schonfeld et al (1974b)

Bautovich et al (1975)

Karlin et al (1978)

## A-15.2 Principle

The assay is based on the competitive binding principles of radio-immunoassay. Standards and unknown samples are incubated with $^{125}$I-Apo B tracer and fixed amounts of anti-Apo B antibody. At the end of the incubation period the bound Apo B is separated from the free Apo B using the second antibody technique (normal rabbit serum and donkey anti-rabbit gamma globulin). The quantity of Apo B in the sample is inversely related to the bound radioactivity.

## A-15.3 Reagents

(1) Preparation of Apo B standard

Low density lipoproteins were isolated from pooled human serum by preparative ultracentrifugation at selected densities according to Havel et al (1955). Centrifugation was carried out at 20°C in a Beckman L2 65B ultracentrifuge with a SW 27 rotor. The serum was centrifuged twice at densities of 1,030 g/ml and at 1,050 g/ml (135,000 xg for 48 hours). This narrow density range was chosen to minimise contamination by other lipoproteins and albumen. The LDL fraction isolated was dialysed overnight at 4°C against 0,165 M NaCL containing 1 mM EDTA and the protein concentration determined by the modified Lowry procedure (Markwell et al, 1978). On immunoelectrophoresis the LDL fraction produced a single precipitin arc against goat anti-human serum and goat anti-human β-lipoprotein (Hyland, Los Angeles, USA).

Since it has previously been shown that Apo B is the sole antigenic determinant of lipoprotein of density range 1,030 g/ml - 1,050 g/ml (Alaupovic et al 1972), the isolated LDL fraction was used as the immunising antigen, the Apo B standard in the assay and the source of $^{125}$I-Apo B.

(2) Preparation of Anti-Serum to Apo B.

Antibody to Apo B was raised in rabbits. Apo B (1 mg/ml) emulsified in an equal volume of complete Freunds adjuvant was injected subcutaneously on 3 occasions, 2 weeks apart; 10 days after the final injection the rabbits were bled and the serum obtained was stored at -20°C in 100 µℓ aliquots. The antibody appeared to be monospecific as evidenced by a single precipitin arc on immuno-electrophoresis against normal human serum. An antibody dilution curve revealed that a 1 : 100,000 dilution of the antibody bound 50% of the $^{125}$I-Apo B tracer.

(3) Iodination of Apo B.

Apo B (15 µg in 10 µℓ 0,1 m Borate Buffer pH 8,5) was iodinated by the indirect conjugation labelling method of Bolton and Hunter (1973). Bolton Hunter reagent was obtained from the Radiochemical Centre, Amersham, England. The labelled product which was purified on a 0,9 x 30 cm Sephadex G-75 column (Pharmacia) had a specific activity of approximately 1,11 MBq/µg. The $^{125}$I-Apo B was stored at 4°C and was rechromatographed on the day of the assay on a 0,9 x 30 cm Sephadex G-75 column.

(4) Assay Buffers

Samples and standards were diluted in 0,05 M Barbital Buffer pH 8 containing 40 g bovine serum albumen/ℓ. First and second antibody, $^{125}$I-Apo B and normal rabbit serum were diluted in 0,05 m Barbital Buffer containing 5 g bovine serum albumen/ℓ.

(5) Normal Rabbit Serum (NRS)

Blood was obtained from normal rabbits, centrifuged and the serum stored in 500 µℓ aliquots at -20°C. On the day of the assay a 1 : 100 dilution was made.

(6) Second Antibody

The second antibody produced against rabbit serum in donkeys was obtained from Wellcome Laboratories, Beckenham, England. The donkey anti-rabbit serum was titrated against normal rabbit serum and used at an optimum dilution of 1 : 10.

A-15.4 Procedure

The $^{125}$I-Apo B was rechromatographed on a Sephadex G-75 column on the day of the assay and the radioactivity diluted to give approximately 15,000 counts per minute per 100 µℓ.

The Apo B stock standard (9 µg/ml) which was stored at -20°C was allowed to thaw and then diluted 1 : 20 and 1 : 25 with 40 G/L BSA Barbital to give standards with final concentration 450 ng/ml and 360 ng/ml respectively. Further dilutions of these standards were made in 40 G/L BSA Barbital according to the following schedule:

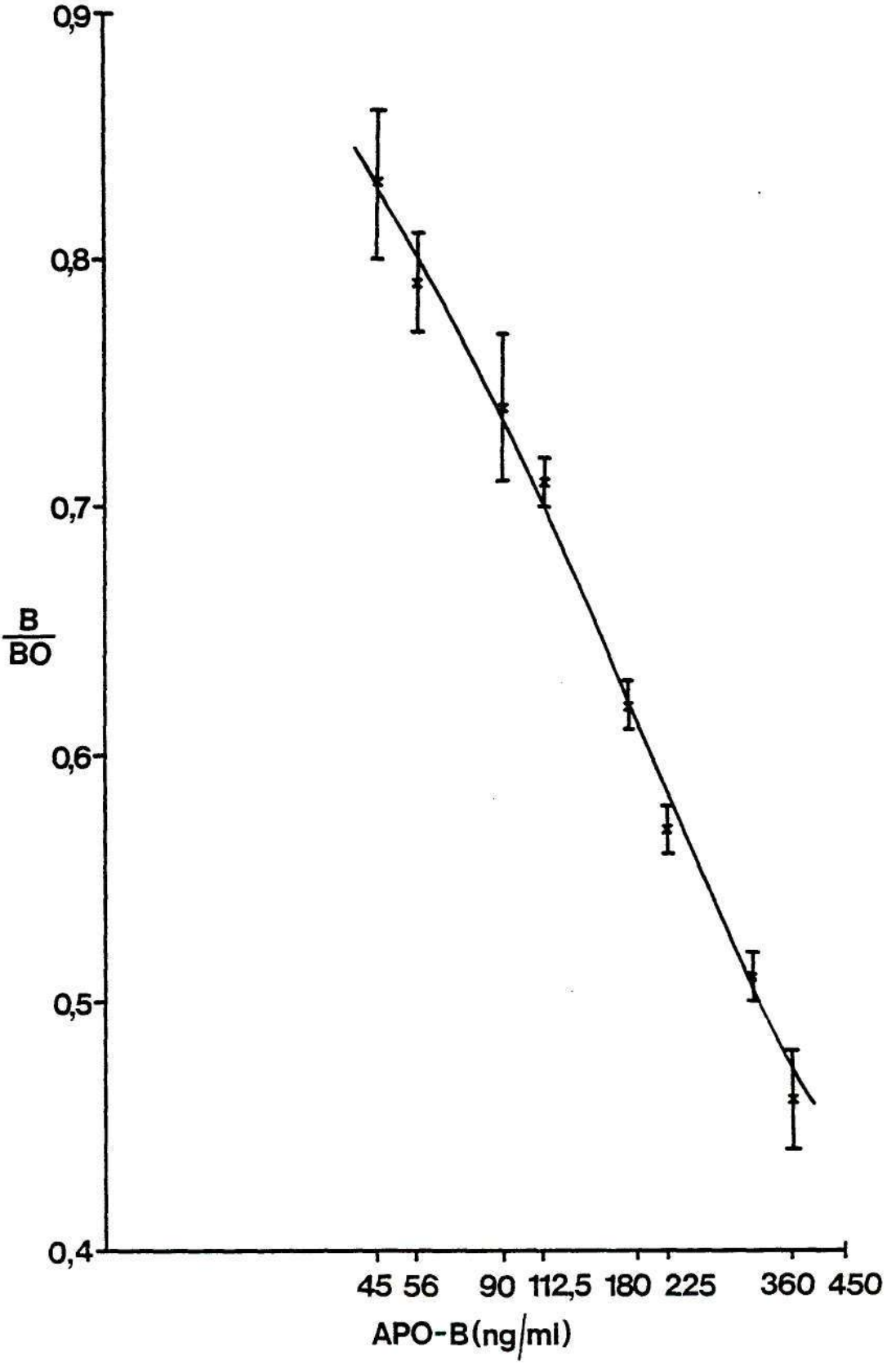
| Final Std. Concentration | | Buffer Solution | | Standard Solution |
|---|---|---|---|---|
| A | 450 ng/ml | - | | 2 ml |
| B | 225 ng/ml | 1,0 ml | + | 1,0 ml of A |
| C | 112,5 ng/ml | 1,0 ml | + | 1,0 ml of B |
| D | 56,3 ng/ml | 1,0 ml | + | 1,0 ml of C |
| E | 360 ng/ml | - | | 2 ml |
| F | 180 ng/ml | 1,0 ml | + | 1,0 ml of E |
| G | 90 ng/ml | 1,0 ml | + | 1,0 ml of F |
| H | 45 ng/ml | 1,0 ml | + | 1,0 ml of G |

A pooled serum control was incorporated in each assay.

The unknown samples and control were diluted 1 : 5,000 using 40 G/L BSA Barbital.

FIGURE A.15.1

**COMPOSITE STANDARD CURVES (±1SD) GENERATED IN FIVE CONSECUTIVE ASSAYS.**



Assays were carried out in polystyrene tubes (LKB, Stockholm, Sweden).

The test schedule was as follows:

| | Total | NSB | Bo | Std. | Control/Samples |
|---|---|---|---|---|---|
| BSA Buffer 5 G/L (µℓ) | - | 100 | - | - | - |
| BSA Buffer 40 G/L(µℓ) | - | 100 | 100 | - | - |
| $^{125}$I-Apo B (µℓ) | 100 | 100 | 100 | 100 | 100 |
| Apo B Stds (µℓ) | - | - | - | 100 | - |
| Control/Samples (µℓ) | - | - | - | - | 100 |
| Anti-Apo B (µℓ) | - | - | 100 | 100 | 100 |
| NRS (µℓ) | - | 100 | 100 | 100 | 100 |
| Tubes were mixed and incubated at 4°C for 24 hours | | | | | |
| Second Ab (µℓ) | - | 100 | 100 | 100 | 100 |
| Tubes were mixed and incubated at 4°C for ± 18 hours. | | | | | |

After this incubation, 2 mls of Tween 20-saline (0,1% Tween in 0.9% saline) was added to all tubes except the totals and the tubes were centrifuged at 4°C at 2,400 xg for 60 minutes.

The supernatant was aspirated from all tubes except totals and the radioactivity in each tube including totals was determined using a gamma counter (5 min counts).

A-15.5 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 Apo B standard was plotted against its respective Apo B concentration on lin-log paper and a standard curve constructed. A typical standard curve generated from 5 consecutive assays is shown in Figure A.15.1.

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 Apo B 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-15.6 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 (Bo/Total) had to be between 45 - 55%.

b) Non specific binding (NSB) had to be less than 5%.

The intra- and inter-assay coefficients of variation for the Apo B assay were 4,37% and 5,5% respectively.

## APPENDIX A-16 : DETERMINATION OF GLYCOSYLATED HAEMOGLOBIN LEVELS

Glycosylated haemoglobin ($HbA_1$) levels were quantitated by a micro-chromatographic procedure using a commercial kit (Helena Laboratories, Texas, USA).

### A-16.1 Principle

The method used to measure $HbA_1$ 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 ($HbA_1$) are less positively charged than haemoglobin A.

Therefore the $HbA_1$ 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 $HbA_1$ eluate and the diluted total haemolysate are read on a spectrophotometer and the percentage $HbA_1$ calculated.

A-16.2 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 $HbA_1$ Control

The lyophilized $HbA_1$ control was reconstituted with 100 µℓ of redistilled water.

A-16.3 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 µℓ of the haemolysate reagent to 20 µℓ 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 µℓ of the haemolysate was loaded onto the quick column and a further 100 µℓ 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 eluation 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-16.4 Calculation

The percent $HbA_1$ for each sample was calculated as follows:

$$HbA_1\ (\%) = \frac{\text{Absorbance F.F. tube}}{5(\text{Absorbance T.F. tube})} \times \frac{100}{1}$$

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

A-16.5 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 $HbA_1$ assay were 3,9% and 7,2% respectively.

# 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 Students t test (paired and unpaired).

Non-parametric data was analysed by the Mann Whitney U test (corrected for ties) and the Wilcoxon sign rank test (Siegel, 1956).

Because of the wide scatter of growth hormone (GH) responses and the fact that the GH response curves of the patients with NIDDY and the reference subjects cross over, the responses were analysed by a two way analysis of variance (Winer, 1971).

Pearson's correlation coefficients (r) were calculated to quantitate the correlation between relevant variables; for non parametric data log transformation ($Log_{10}$) of data was undertaken prior to computing the correlation coefficient.

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

Computations were undertaken on a Hewlett-Packard 9815A calculator and a Tektronix 4051 computer.

Data in this thesis is expressed as $\overline{X} \pm$ S.E. unless stated otherwise.

# APPENDIX C

## CONSENT

Informed consent was obtained from all participants (patients and reference subjects) in this study. In addition, the provocative tests (100 g oral glucose tolerance test and the intravenous insulin tolerance test) were approved by the Ethics Committee of the Faculty of Medicine, University of Natal.

# APPENDIX D

## UNITS USED

In the present study the S1 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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