INSULIN RECEPTOR BINDING IN HYPERTENSION AND NON-INSULIN DEPENDENT DIABETES MELLITUS

BY

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Summary

Human erythrocyte and mouse adipocyte insulin receptor binding assays have been used to quantify insulin receptor binding in mild hypertensive patients with and without non-insulin dependent diabetes and obese mice. In normal male volunteers erythrocyte insulin receptor binding was not affected by a 12 hr overnight fast. Treatment of mildly hypertensive non-diabetic patients with bendrofluazide increased erythrocyte insulin receptor concentration after only one month without producing a significant deterioration in glucose tolerance. In mildly hypertensive diabetic patients, bendrofluazide treatment caused a significant aggravation in glucose intolerance after 4 months with little effect on insulin receptor binding. This may have been the result of hypokalaemia since bendrofluazide with potassium supplementation significantly increased the insulin receptor concentration in this group. Treatment with nifedipine neither precipitated glucose intolerance in non-diabetic patients nor aggravated the glucose intolerance of diabetic patients. Indeed, there was a paradoxical increase in serum insulin levels in both groups of patients. Guarem treatment of non-insulin dependent diabetics lowered plasma glucose and insulin levels and marginally improved glucose tolerance without influencing erythrocyte insulin receptor binding. High doses of guarem lowered the plasma glucose, insulin and body weight of obese mice. Only a high dose of guarem significantly increased adipocyte insulin receptor affinity which became evident after 48 hours. In obese mice starvation probably caused the improvement in glycaemia observed in response to guarem treatment. Adipocyte insulin receptor concentration and both basal and insulin stimulated rates of glucose oxidation were significantly reduced in obese mice compared with lean suggesting that defects in adipose tissue insulin receptor binding and insulin action may be primary aetiological factors in the insulin resistance of obese mice. Differences in insulin receptor concentration and affinity but not insulin action have been demonstrated in abdominal and subcutaneous fat from obese mice.

Key words. Insulin receptors, Diabetes, Hypertension, Obese mice, Guar gum

This thesis is dedicated to:

My family, my wife and
my lovely daughters (Zina, Banan and Rabab)

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CHAPTER ONE

General Introduction

Insulin is a polypeptide hormone synthesised and secreted by the B-cells of the endocrine pancreas. It plays a central role in the regulation of carbohydrate, fat and protein metabolism. Since it's discovery, considerable efforts have been directed towards determining the mechanism of action of this hormone. Although much remains unknown, a great deal has been learned about the first step in insulin action - the binding to specific receptors on the plasma membrane of target cells (1,2). The accumulation of information on insulin receptor binding has extended our understanding of certain metabolic diseases. This is especially true for diseases characterised by insulin resistance such as non-insulin dependent diabetes.

Insulin resistance is a state in which a given concentration of insulin produces a subnormal biological response. Over the past 10 years, studies on insulin receptors and insulin-resistant disease states have advanced in parallel. Thus, the quantitation of insulin receptor binding on target tissues has improved our understanding of the mechanisms responsible for the production of insulin resistance. In a complementary fashion, studies of the manner in which insulin receptor binding may be altered in disease have led to a greater understanding of the molecular mechanism of insulin action (3).

For the purpose of this thesis, the term insulin receptor will refer to that receptor which shows a very high degree of specificity for insulin and which mediates the effects of insulin on intermediary metabolism. Our knowledge of insulin receptor structure, function and binding kinetics has been used as a basis for considering insulin receptor status both in health and in disease states during drug

therapy. Diabetes mellitus is characterised by a myriad of secondary complications, one of which is essential hypertension (4). Essential hypertension is a multi-factorial condition in which genetics, constitutional, metabolic, hormonal, haemodynamic, and psychological factors play a role with varying degrees of importance. The most common type of hypertension in man is primary or essential hypertension and this category comprises about 95% of patients with hypertensive disease (5). Hypertension is considered to develop as a result of disturbance of the body's blood pressure regulating system. Many problems have been created over the years from the variable criteria used to record the blood pressure (e.g. korotkoff phases IV or V for diastolic) (6) and the many definitions of hypertension. The accepted diagnosis of hypertension involves a systolic pressure of >160 mm Hg and a diastolic pressure of >95 mm Hg (4). The level of blood pressure is determined by the diameter of the small arteries and arterioles (resistance vessels) and the cardiac output. The latter can change as a result of inotropic effects on the heart itself; alterations in blood volume and body electrolyte composition and also through effects on the venous capacity. During normal circulatory homeostasis the heart, kidney, vascular diameter and the veins are influenced by a variety of factors including the autonomic nervous system, various circulating hormones such as catecholamines and a number of "local hormones" such as prostaglandins (7). In man it is still not established whether essential hypertension is a single disorder or the product of several contributing disorders. On haemodynamic grounds, there are at least two identifiable groups, one with disturbances in "volume factors" (cardiac out) and one with disturbances in "constrictor factors"

(8). These disturbances could be the result of alterations in either autonomic or hormonal function (9). Both volume (cardiac output) and constrictor factors can exert distinctive long term effects on blood pressure, these are often most obvious in the early stages of the development of hypertension (10). In essential hypertension, the rise in blood pressure results from constriction of the small arteries and arterioles, which leads to an increased resistance to blood flow. This increase in peripheral resistance may theoretically result either from the presence of increased stimuli leading to excess vasoconstriction or from intrinsic abnormalities within the resitance vessels leading to an increased response to normal stimuli or a combination of the two mechanisms (11). Most forms of hypertension, both in man and in experimental animals, are associated with an increase in peripheral vascular resistance. It has still not been resolved, however, whether this increase is due to an alteration in the structure of resistance vessels or to an increase in the contraction of smooth muscle (12). Increased contractility could be due to increased sympathetic nervous acitivity, alterations in circulating humoral factors having a direct primary effect on a variety of components including vascular sympathetic nerve endings and vascular smooth muscle itself. There is substantial conflicting evidence in support of both these possibilities (13).

An early phase of essential hypertension in which increases in blood pressure are associated with elevated heart rate and cardiac output, has been suggested to be due to an increased activity of the sympathetic nervous system (14). The positive correlation between degrees of glucose intolerance and mild hypertension (15) suggests that diabetes may not be the cause of hypertension <u>per se</u>, but that

both might be manifestations of a single defect, perhaps of central nervous origin (16). The sympathetic nervous system plays an essential role in the regulation of arterial pressure (16). Goldstein has reported raised levels of adrenaline in hypertensive patients (17).

The role of the renin-angiotensin system in hypertension has received considerable attention (18) and it has been established that in some hypertensive patients there may be evidence of hyperactivity of the renin-angiotensin system (19). Increased activity of the renin-angiotensin system is often considered to be the dominant factor in hypertension secondary to unilateral renal artery stenosis, although normal values of plasma renin activity have been reported (18). The close inter-relationship between the renin-angiotensin system and the sympathetic nervous system suggests the possibility of a neurogenic component in renovascular hypertension in man (16).

It is now generally agreed that human essential hypertension is a genetic trait, but the mode and mechanism of heredity remains unclear. Human population studies suggest that blood pressure is multifactorially determined, with 33 to 45% of variations attributed to genetic factors (20). Animal models (The Japanese and New Zealand strains of spontaneously hypertensive rats) have confirmed the importance of genetic factors in hypertension and its complications indicating various possible modes of inheritance (20).

The relationship between hypertension and diabetes mellitus (non-insulin dependent diabetes).

Diabetes mellitus is one of the World's greatest health problems. It is estimated that more than 40 million people world-wide suffer from the disease. Diabetes is associated with a 2-3 times higher mortality, a 2-3 times higher frequency of blindness and gangrene and amputations are necessitated 20 times more often than in the rest of the population (21). Although many excellent studies have focused upon the epidemiology of human diabetes and the occurrence of spontaneous diabetes in animals (particularly small rodents) and much work has been done on the metabolic effects of insulin, the aetiology of the disease remains poorly understood. There are many interesting features of diabetes that have no precise explanation. One of these is the fact that diabetes tends to run in families and at certain times and certain localities mini-epidemics of the disease have occurred. The notion that some forms of diabetes may appear subsequent to viral infections has appeared from investigations in many parts of the world (22). In addition it has been noted time and time again that diabetes is more common in obese than in non-obese individuals. There is increasing evidence to suggest that diabetes ia a heterogeneous multifactorial syndrome resulting from many causes, each requiring intensive and distinct investigations to elucidate it's nature.

In recognition of the intensive search for the aetiology of the various forms of diabetes, a newer and more useful classification of the disorder has emerged. The previously used term juvenile-onset diabetes, or youth-onset diabetes, has now been renamed insulin-

dependent diabetes mellitus (IDDM) or Type I diabetes. The previously known adult-onset diabetes, or maturity-onset diabetes, has now been redesignated non-insulin-dependent diabetes mellitus (NIDDM) or Type II diabetes. There are other forms of diabetes based on fasting blood glucose and glucose tolerance determination i.e. impaired glucose tolerance (IGT), Gestational diabetes and Spontaneous diabetes.

Early diagnosis and early treatment is a prerequisite for reducing the frequency of diabetic complications. Diabetes mellitus is characterised by chronically increased levels of glucose in the blood and impaired glucose tolerance (21). Both states are secondary to a defective insulin action due either to a shortage of insulin or to factors which antagonize the effect of insulin, thereby disturbing the metabolism of carbohydrates, lipids, and proteins. The recognition and diagnosis of the syndromes is based upon blood glucose measurements under strictly defined conditions. If the fasting blood glucose level is found to be 7 mmol/l or higher, there is a strong indication of diabetes mellitus. When the level is between 5.5 and 7 mmol/l, there is a strong indication of diabetes mellitus in patients under 40 years of age, a relatively strong indication in patients from 40 to 60 years of age, but of uncertain value in patients older than 60. If the level is less than 5.5 mmol/l, the indication is against the presence of diabetes mellitus. However, in patients under 40 years of age, a value between 5.0 and 5.5 mmol/l should be regarded with some suspicion (21).

Non-insulin dependent diabetes is the most common form of diabetes mellitus. About 80% of the diabetic population belong to

this category (23). Non-insulin dependent diabetes has a genetic basis which seems to be stronger than for insulin dependent diabetes and the former seems to be primed for the development of the disease at birth because of genetic susceptibility. The concordance of non-insulin dependent diabetes in twins is about 90% (23) and in the first years the disease is asymptomatic. The symptomatic phase of the disease often develops after the age of 40 due to precipitation by environmental factors. A special form of non-insulin dependent diabetes may develop in young subjects because of an autosomal dominant inheritance (24). Patients with non-insulin dependent diabetes are not dependent upon insulin for the prevention of ketonuria and not prone to ketosis. However, some patients may require insulin for the correction of hyperglycaemia and may develop ketosis under special circumstances such as severe stress and fasting. About 80% of non-insulin dependent diabetes are obese. Subjects with normal fasting plasma glucose levels and glucose values two hours after the intake of a 75g glucose load, between normals and diabetics are said to have IGT. About 1-5% per year of subjects with IGT develops non-insulin dependent diabetes and this suggests that most diabetics pass through a state of impaired glucose tolerance before they become hyper-glycaemic (23). Therefore, the treatment of subjects with IGT has been aimed at preventing the development of diabetes mellitus (23). In most non-insulin dependent diabetes both abnormal insulin secretion and insulin resistance have been described. The relative significance of insulin deficiency and insulin resistance for the development of non-insulin dependent diabetes remains unclear. Furthermore, the question as to which of the two abnormalities is the primary remains to be answered. The

concentration of glucose in plasma is the product of two processes, the rate of endogenous glucose production and the rate of glucose removal by all the tissues of the body. In the fasting state, in normal patients, the rates of the two processes are equal and the plasma glucose concentration remains constant. In non-insulin dependent diabetes the fasting plasma glucose level is constantly increased due to an elevated release of glucose from the liver and a reduced peripheral glucose utilisation (27). The characteristic defect in insulin secretion in both lean and obese non-insulin dependent diabetics is a reduced early (first 10 minutes) insulin release both after intravenous and oral glucose stimulation (28). In obese subjects the total amount of insulin secreted usually is normal or increased, whereas most lean diabetics are really insulin deficient (28, 29). All subjects whether lean or obese with non-insulin dependent diabetes show evidence of insulin resistance. This means that the effect of insulin in these subjects is lower than expected. The insulin resistance in non-insulin dependent diabetes has been shown to be located at the cellular level in at least adipocytes and monocytes (30). Plasma concentrations of counter regulatory hormones such as growth hormone is normal and no antibodies to insulin have been demonstrated (31).

The prevalence of hypertension among diabetic patients has been a controversial subject for 70 years and estimates have ranged from less han 10% to 80% in different diabetic populations (4). When simultaneous controls were first employed in clinical studies they were frequently not matched for body weight which has since been shown to have a significant effect on blood pressure (32). Inadequate cuff sizes for obese patients (33) and varying postures

during measurement have also compounded the problem, both in terms of absolute values and comparability between studies.

Early studies by Major showed that the majority of elderly diabetic patients had significantly greater systolic blood pressures than normal or hospital based control subjects (34). Adams reported a sixfold increase the occurance of systolic hypertension in both sexes above the age of 40 years, but the difference was not significant after correction for obesity (35). Freedman, Moulton and Spencer studying a hospital diabetic clinic population (36) showed an increased prevalence of diastolic hypertension only in those patients over 60 years. In another study of 662 diabetic patients, with carefully matched controls, Pell and D'Allonzo (37) found that the prevalence of hypertension (>150/94 mm Hg) was 54% greater among diabetic patients than among control subjects. This was not explained by differences in body weight. In men aged 40 years and over, a positive correlation was found between systolic and diastolic pressure and blood glucose (15) which was independent of body weight. In the Framingham study, the mean systolic blood pressure was slightly higher in diabetic subjects than in age- and sex-matched controls, the difference being greater in women (38). A more recent study, which included subjects aged 50-80 years demonstrated a clear association between diabetes and hypertension in both sexes and at all ages. An adjustment for obesity, however, decreased the association (39). Few of these studies have differentiated between insulin-dependent and non-insulin-dependent diabetes. It appears that, in both insulin-dependent and non-insulin-dependent diabetes, there is an increased number of patients with systolic hypertension. In non-insulin dependent diabetes much of this is clearly

related to the obesity seen in many of the patients, but even when this factor is taken into account a significant increase in prevalence is observed, at least in patients above 50-60 years of age. Evidence of any increased frequency of diastolic hypertension before old age has not been totally convincing. In insulin-dependent diabetes there appears to be a small increase in systolic blood pressure from early in the disease. Definite diastolic hypertension (>95mm Hg) in this group is generally associated with the presence of renal involvement while there appears to be a marked increase in systolic hypertension above the age of 45-50 years (4).

Most physicians appear to consider hypertension as a complication of clinically manifest diabetes. However, it might also be a result of biochemical and physiological changes already present in the prediabetic stage. Diabetic vascular complications can appear in people without manifest diabetes, but with lowered glucose tolerance which is a pathological base for the development of hypertension (40). Hypertension, together with overweight and a family history of diabetes, might represent risk factors for the development of diabetes. Siperstein has suggested that alterations such as thickening in the capillary basement membrane may precede insulin deficiency and that the microangiopathy itself may interfere with the secretion of insulin (41).

In non-insulin dependent diabetes there are considerable experimental data to suggest that abnormalities in body sodium may be involved. De Fronzo has studied the role of insulin in sodium reabsorption by the kidney and shown significant sodium retention with only small increases in plasma insulin (30 mU/l) (42). It seems possible that the basal hyperinsulinism seen in non-insulin dependent

diabetes (especially with obesity) might lead to an increased exchangeable sodium, as reported by some in essential hypertension (4). To support this suggestion there is evidence that exchangeable sodium is increased by about 10% in normotensive and hypertensive diabetic subjects compared with normal age and weight matched controls (43). This "obligatory" retention of sodium by insulin should not be confused with the possible role of dietary sodium in hypertension (4).

The renin-angiotensin system has also been considered as a possible contributing factor to the pathogenesis of hypertension in diabetics (40). Christlieb and co-workers (18) have studied diabetics with hypertension but without renal disease, as well as patients with hypertension and diabetic nephropathy. Comparison of the plasma renin activity (PRA) in normal patients and in patients with essential hypertension showed that diabetics without complications had normal PRA values. In diabetics with hypertension but without nephropathy, the same distribution of PRA values was found as seen in patients with essential hypertension. When diabetes is complicated by hypertension and nephropathy, the PRA values are low. However, the PRA has been variously reported as normal (18) or high (44). Several workers have shown an increased vascular sensitivity to angiotensin II in diabetic patients (43,45). Increased vascular sensitivity to plasma catecholamines has also been described in diabetic patients with hypertension (43,45). It has been suggested that an increased production of aldosterone, cortisol or growth-hormone might contribute to the aetiology of diabetes and hypertension (40).

The clinical treatment of hypertension.

The clinical treatment of hypertension is based on an understanding of the pathophysiological mechanisms underlying hypertension, the type of diabetes, the presence of complications and a detailed knowledge of the mechanism of action of the various groups of anti-hypertensive drugs available. The aim is always to treat the patient with either a single drug or a combination of drugs which will have the maximum benefit with respect to lowering the blood pressure, yet have the minimum of side effects (46). There are many drugs available for the treatment of hypertension. This makes it easier to vary a patient's therapy if the treatment is not effective or it is producing side effects. Dustan and colleagues (47) have shown that relatively few hypertensive patients can sustain their reduction in blood pressure without sustained medication. Currently, in clinical practice, the management of mild and moderate hypertension with or without diabetes is being reevaluated. Most patients with mild hypertension whose diastolic pressure is above 95 mm Hg are likely to benefit from hypertensive drug treatment (48). There is an increasing uneasiness that many of the millions of patients taking antihypertensive drugs with known and unknown long-term side effects may be doing so inappropriately (49). Finnerty has shown, in a large series of patients that with careful management the total dosage or the number of antihypertensive drugs used can be reduced without impairing the antihypertensive effect and with considerable reduction in the adverse symptoms of medication (50). In many cases, a non-pharmacological approach may eliminate the need for antihypertensive drugs (47). Repeated physical exercise (physical training) has been

shown to cause a fall in blood pressure in normotensive and mild hypertensive individuals whether obese or not. This is followed by other haemodynamic changes characteristic of reduced sympathetic nervous system activity (51). It has been postulated that both physical activity and a low calorie diet, regardless of weight loss, may improve insulin sensitivity, lower insulin concentrations, decrease catecholamine responsiveness (52) and thus lower peripheral resistance (53).

Dietary fibre may also be of use in the treatment of hypertension. Studies have shown that vegetarian groups have lower mean blood pressures than matched control groups (54) and this might be related to their increased fibre intake. It has also been shown that many people in non-industrialised societies whose diets provide more fibre than most western diets, maintain lower blood pressures throughout their lives, than people in western societies (55). Two recent studies of dietary regimens including high cereal intake, have reported a lowering of the blood pressure in both diabetic (56) and non-diabetic (57) hypersensitive subjects. Although the evidence available is incomplete, increasing fibre intake does appear to produce a small reduction in the blood pressure, but the mechanism for this effect is unknown. Several hypotheses have been suggested. The increased intake of dietary fibre may stimulate different input to the brain by altering the intestinal transit time, lowering colonic intraluminal pressure and decreasing the residence time of faeces in the colon, which thus alters the central regulation of blood pressure (58). Also the absorption of dietary fat is slower when consumed with dietary fibre and a change to a high fibre diet is associated with a concomitant increase in potassium intake (59). The

anti-natriuretic potential of insulin has also generated interest in this context (60) since high fibre diets are generally accompanied by lower postprandial serum insulin concentrations (61) which might facilitate sodium excretion.

Weight reduction seems to be the treatment of choice in obese hypertensive patients with or without diabetes. It must be considered as only part of the total treatment and be given together with sodium restriction and physical activity. Weight reduction leads to an improved state of training and a reduction in hyperinsulinaemia and insulin resistance (62). A reduction in plasma insulin concentrations has been shown to be associated with a parallel fall in blood pressure (63) and insulin has been shown to facilitate sodium reabsorption in the distal tubules of the kidney (42). It seems possible, therefore, that the effect of physical training in lowering the blood pressure might be mediated via a decrease in the sodium retention. This thesis is unlikely to be true for dietary manipulation because invariably sodium excretion is not changed (54). However, a reduction in dietary salt intake has been shown to be associated with a significant fall in blood pressure (64).

The present treatment of hypertensive diabetic patients may be far from satisfactory because of the multiple adverse effects of antihypertensive drug therapy (4,65). Recently there has been renewed interest in the role of diet as an alternative to drug therapy in the treatment of hypertensive diabetic patients (66). And dietary regimen made in the treatment of the hypertensive diabetic subject must take into account the current dietary recommendations for diabetics (67), which are intended to improve glycaemic control

and combat other common problems, e.g. hyperlipidaemia, among these patients (68). Diets containing relatively low sodium (64), low fat (69), and high potassium (70) have all been claimed to significantly lower the blood pressure. Combinations of these individual measures have been reported to decrease blood pressure in both non-diabetic (71) and diabetic (72) hypertensive subjects and may also reduce other coronary risk factors (73). Special consideration regarding sodium restriction must be given to the diabetic since there is consistent evidence to suggest that exchangeable sodium is increased by approximately 10% in diabetic subjects regardless of blood pressure status, compared to normal age-matched and weight-matched non-diabetic controls (43). Furthermore, this abnormality can be reversed by diuretic agents such as bendrofluazide and is accompanied by a highly significant reduction in blood pressure (43). Potassium supplementation combined with sodium restriction has been suggested to have a synergistic effect in hypertension (74).

Drugs used in the treatment of hypertension.

Drugs used to treat hypertension can be divided into five distinct groups, those that act directly on the vasomotor centre within the central nervous system, such as clonidine, those that reduce cardiac output such as β-adrenergic blocking drugs, those that act on the kidney to increase sodium and water secretion such as bendrofluazide, those that inhibit the function of the peripheral sympathetic nervous system such as labetalol, and those having a direct relaxant effect on vascular smooth muscle such as hydrallazine (46).

For the initial treatment of mild hypertension various authors advocate a diuretic - for example bendrofluazide, hydrochlorothiazide (75) or indapamide (76). Thiazide diuretics have been used for many years and remain the most widely used treatment for hypertension (77) and are probably the best therapy if sodium excess is accepted as a significant factor. Thiazide diuretics are cheap and effective antihypertensive drugs. They are, however, reported to precipitate and aggravate diabetes mellitus (65,78), but their role in provoking glucose intolerance in previously glucose-tolerant subjects remains controversial. Changes in metabolic parameters such as plasma potassium (79), plasma glucose (80), serum cholesterol, triglyceride levels (81,82) and serum uric acid (83) have to be weighed against the benefits of treatment, which may be modest in patients with mild hypertension. Potassium depletion in man is most commonly due to continuous treatment with diuretics. Most patients taking diuretics are given potassium prophylactically.

Thiazide diuretic therapy for hypertension is widely used in both insulin- and non-insulin dependent diabetic patients. A recent comparison between the effect of nutrition (high fibre, low fat, low sodium diet) and the diuretic bendrofluazide in diabetic subjects with mild hypertension (66) showed that after 3 months treatment, both diet and bendrofluazide caused a significant decrease in systolic and diastolic blood pressure. Both groups of patients lost weight and bendrofluazide tended to elevate the serum levels of cholesterol and triglycerides. These observations contrast with the results of other studies that have showed no change in biochemistry after thiazide therapy for one (84) and six years (85). In a later study deterioration in glycaemic control was observed after both six

(80) and fourteen years (65) of therapy. The mechanisms by which thiazides impair carbohydrate metabolism are unknown but potassium depletion may be important (86). Indeed a recent study has reported that potassium supplementation may be protective against impaired glucose tolerance caused by thiazides (87). Some importance has been attached to the administration of low doses of diuretics, because with increasing dose, the number and degree of unwanted biochemical-side effects increase without a corresponding increase in antihypertensive action (85,88). Diuretic therapy appears to increase the hypotensive effects of other antihypertensive therapy. Therefore, it seems reasonable to assume that a low sodium intake would either increase the hypotensive effect of a drug regimen or decrease the amount of drug required (76).

The use of β-adrenergic blocking agents (beta-blockers) to control mild hypertension in diabetic subjects remains controversial. There is evidence to suggest that these drugs may reduce the rate of recovery from hypoglycaemia (89) and their effect on overall control in non-insulin dependent diabetes appears to be small (90). In clinical practice β-adrenergic blocking agents do not seem to increase or aggravate hypoglycaemic reactions in insulin treated patients. Some clinicians defer the start of drug treatment, and subsequently use a "step-care method", beginning with a diuretic alone and avoiding complicated multiple-drug regimens (91). Opinions are divided about recommending a step wise treatment scheme in all cases. More rapid control of blood pressure may possibly be achieved by administering a combination of a diuretic and a β-adrenergic blocking agent from the beginning of treatment (92). Which of these two regimens is preferable is not clear even in the non-diabetic population (93).

Calcium antagonists, already widely used in the treatment of angina pectoris, have also been found to be active in lowering high blood pressure and are increasingly prescribed for hypertension (94). Calcium antagonists comprise several existing and some new drugs with markedly different chemical structures. Nifedipine and verapamil have been used successfully as antihypertensive agents (95). Various derivatives of nifedipine, such as nicardipine, nitrendipine, nisoldipine, felidipine and other substances having a similar mode of action, are currently undergoing clinical trials. So far, all these drugs have the disadvantage of relatively short duration of action and must be given at least twice, preferably three times daily (76). Few studies have been conducted concerning the effect of nifedipine on glucose tolerance and insulin release. Results to date have been controversial (96,97), and it remains to be established whether nifedipine aggravates glucose tolerance. It has been indicated that when nifedipine is given at the recommended anti-anginal dose of 30mg daily there is no significant effect on glucose tolerance in normal individuals (98). It has also been shown that nifedipine increases fasting plasma glucose concentrations and induces appreciable glucose intolerance associated with a delayed insulin response (96). These results, however, do not exclude a significant effect of conventional doses of nifedipine in diabetic patients, who have a limited capacity to secrete insulin (97). It appears that the antihypertensive effect of calcium antogonists is not accompanied generally by salt and fluid retention. The acute administration of nifedipine results in a distinct fall in blood pressure in patients with essential hypertension and in patients with diabetes and hypertension but not in normotensive subjects. This

observation suggests that calcium antagonists might be the first choice for the treatment of older patients with essential hypertension (94). In all cases care should be excised against starting the drug treatment of mild hypertension prematurely (99), and the frequency of hypertension among the diabetic population suggests that adequate detection and treatment of high blood pressure may be paramount in the prevention or retardation of the development of nephropathy, retinopathy and large vessel disease.

The action of insulin at target tissues.

Over the past 30 years evidence has come from a variety of sources that the first step in the action of insulin is binding to specific receptor sites on the plasma membrane of the target cell (1,100). This marks the first link in a chain of cellular events which culminate in the biological action of insulin. Direct studies of insulin receptor interaction were attempted as early as 1953 (101), however, these were fraught with problems, particularly related to the preparation of biologically active tracer and defining biologically specific binding (102). These early problems were alleviated when Freychet, Roth and Neville (103) and Cuatrecasas (104) showed that insulin could be labelled with iodine and remain biologically active. Using this preparation they could define hormone binding that had the property of biological specificity. Since this early work insulin receptors have been demonstrated on many cells of vertebrate species (105). The subsequent surge in this field of study has been admirably charted in a succession of excellent reviews (1-3, 106-109).

The available experimental data (110) suggests that the insulin receptor has two fundamental functions: firstly, as a discriminator which recognises the hormone from other molecules to which the cells are exposed through binding insulin with a high affinity. Secondly, this interaction results in activation of cellular biological processes such as transport and metabolism (postreceptor effects). Clearly, alterations in either post-receptor events responsible for the mediation of insulin action or in insulin binding to its receptor, are possible mechanisms by which alterations in insulin action could be initiated.

The insulin receptor:

i - Distribution and physical properties.

That insulin is able to mediate its biological effects without entering the cell has been known since the middle of the late 1960's (111,112) and it is now well established that insulin receptors are located predominantly within the plasma membranes (103) of many different target organ cells (113). The major sites of action for insulin are liver, adipose tissue and skeletal muscle and insulin receptors have been characterised on the plasma membranes of all these organ systems (31,114,115). Insulin receptors have also been found in other cells such as placental cells (116), monocytes (117), lymphocytes (118), granulocytes (119), fibroblasts (120) and erythrocytes (121). Intracellular binding sites have been identified on nuclei of human lymphocytes (122), the Golgi apparatus of rat hepatocytes (123) and on the surface of mouse muscle mitochondria (124). In various studies estimates of the number of insulin

receptor sites suggest considerable variation between cell types. For example, expressed per um^2 of plasma membrane, estimates range from 14 sites in human erythocytes, 24 in cultured human lymphocytes, 63 in rat hepatocytes (121) and 5-9 in human adipocytes (125).

The insulin receptor is highly specific for the insulin molecule. Proinsulin, the biological potency of which is about 5% that of insulin, binds to insulin receptors of liver and lung with about 5% of the affinity of insulin, suggesting that the differences in potency may be due entirely to different affinities for the receptor (126). The affinity of synthetic human insulin for the human insulin receptor is virtually equivalent to that of purified porcine insulin, which differs from human insulin only by the substitution of one amino acid and has in vivo potency equivalent to that of human insulin (126). Somatomedin C possesses structural and functional homology with insulin and is now considered to be identical to the insulin like growth factor IGF-1. This homology suggested that their respective receptors might also share structural homology and this now appears to be true. The insulin receptor possesses considerable homology with the IGF-1 receptor but not with the IGF-11 receptor (127). So this suggests that measurements of insulin binding should take into account the possibility of IGF-1 binding to insulin receptors. There is also evidence that low affinity insulin receptors have an ability to bind IGF-1 to some extent (126).

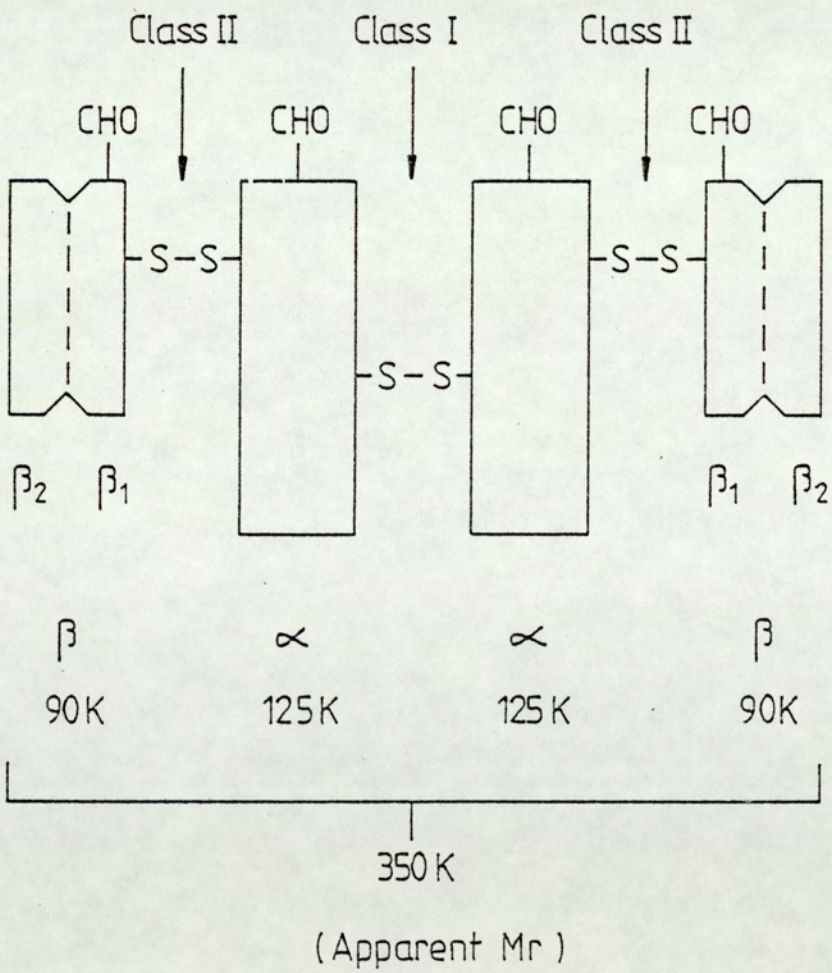
Insulin receptors have a half-life measured in hours. In addition to this rapid turnover under basal conditions, the affinity and concentration of insulin receptors are subject to dynamic regulation by many signals emanating from inside and outside the cell

(2,128). Several physical and chemical factors are important regulators of insulin binding in vitro. Insulin binding is very sensitive to alteration in pH and temperature with maximum binding occurring optimally between pH 7.4 and 8.2 (129,130) and between 10 to 20°C in most systems (131). In addition, high temperature may bring about considerable degradation of ^{125}I-insulin (132,133). It has been shown that the hormone-receptor complex is internalised by the cell more rapidly and to a greater extent at 37°C than at lower temperatures (134). Many other factors may also affect insulin receptor binding, for example, albumin concentration (135), divalent cations Ca^{2+} and Mg^{2+} (121), ATP concentration (136), CAMP (137) and the insulin concentration (138).

ii - Insulin receptor structure.

The insulin receptor appears to consist of a minimum of four glycoprotein sub-units, Figure 1. These sub-units form a large globular complex with a molecular radius of about 7 nm and an apparent Mr of 300-350 K daltons (139,140). There are two larger sub-units (α, molecular weights of 125 K daltons each) which are linked together by one or more class I disulphide bonds, and two smaller sub-units (β-molecular weights of 90 K daltons each) which are class II disulphides linked to the α sub-units. This sub-unit composition is designated the $(\alpha\beta)_2$ complex. There may also be further sub-units which are not covalently linked to the complex. Oligosaccharde side chains appear to be attached to both α and β sub-units (141). Indeed, a sialylated glycosidic moiety has been shown to participate in the interaction with insulin, involving D -

Figure 1. Proposed subunit organisation of the insulin receptor, after Czech, Massague and Pilch (146).

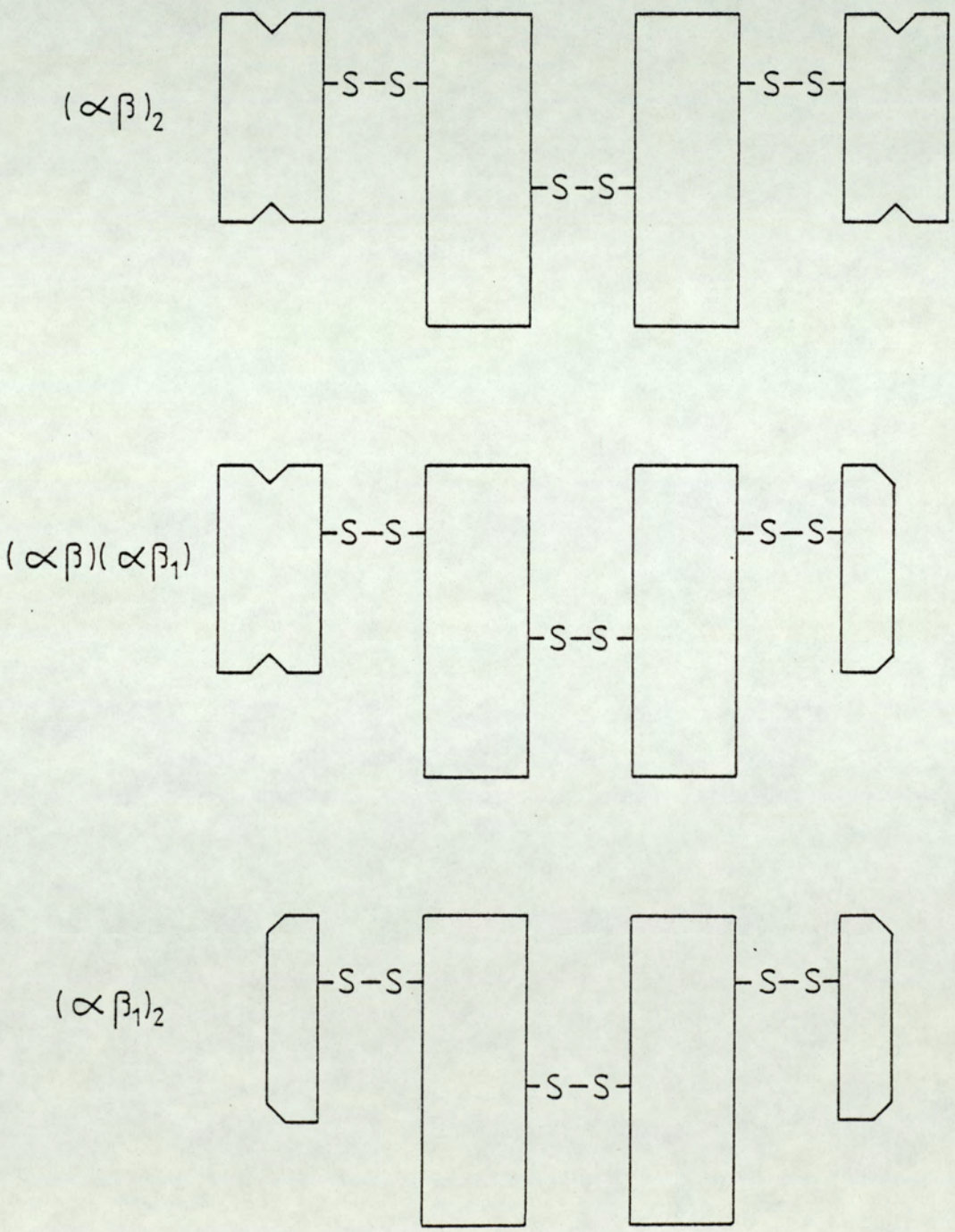


galactose, N-acetyl-D-glucosamine and D-mannose residues (142). The exact location of the insulin binding site on the receptor complex, however, has not yet been established. Neither has the precise role of the disulphide-linked oligomers of the α and β sub-units. Several investigators have identified an active receptor moiety with a molecular radius of about 4 nm, suggesting that the high molecular weight receptor complex (Mr 300-350 K daltons), Figure 1, may comprise two active components (143 -145). Consistent with these observations, selective reduction of class I S-S bonds dissociates the $(\alpha\beta)_2$ receptor complex into two symmetrical $(\alpha\beta)$ halves, each of which will continue to bind insulin (146). Separation of the two α-S-S-α halves of the receptor complex does not appear to impair the binding of insulin or the linking of insulin action to its intracellular effects, such as glucose oxidation (126). Proteolytic fragmentation of the β sub-units of the $(\alpha\beta)_2$ receptor complex yields two additional forms of the insulin receptor, $(\alpha\beta)(\alpha\beta_1)$ and $(\alpha\beta_1)_2$, as shown in Figure 2 (140,146). Partially purified membranes from several insulin sensitive tissues have been shown to contain all three receptor forms: $(\alpha\beta)_2$, $(\alpha\beta)$ $(\alpha\beta_1)$ and $(\alpha\beta_1)_2$ (146). Thus, different forms of the insulin receptor may exist on the same cell type and this may account in part for the apparent discrepancy over the size of insulin receptor sub-units (147). In addition different cells may exhibit variations in insulin receptor structure (148).

The mechanism of insulin action.

At the target cell, the action of insulin is thought to include several biochemical steps: binding of insulin to it's specific

Figure 2. Proteolytic fragmentation of $(\alpha\beta)_2$ insulin receptor complex into two additional forms of the insulin receptor, $(\alpha\beta)(\alpha\beta_1)$ and $(\alpha\beta_1)_2$ (140).



receptor in the plasma membrane at the cell surface, translation of the insulin-receptor interaction into a transmembrane signal, generation of intracellular messengers and finally changes in activity of transport systems and enzymes, which result in the biological effects of insulin (110). In mammals, the biological actions of insulin are concerned primarily with the control of rapid adjustments of intermediary metabolism, to accommodate fluctuations in nutrient supply and energy demand (149). The immediate effects of insulin involve alterations in the cellular metabolism of glucose and lipids through changes in the activities of pre-existing enzymes and membrane transporters. Insulin rapidly promotes glucose uptake into muscle and fat cells, where in the former, glycogen production is greatly enhanced and in the latter, glucose is converted mainly to glycerol and fatty acid moieties of triacylglycerol. Lipolysis in fat cells, particularly that enhanced by other hormones, is depressed, while in the liver insulin initiates a reduction in gluconeogenesis and causes an increase in glycogen storage and triacylglycerol synthesis. The hypoglycaemic action of insulin is reflected at the cellular level either by an increased glucose uptake mediated by an enhanced capacity of the specific carrier system (150) or recruitment of carrier molecules from intracellular sites (151,152).

A complete understanding of the mechanism of insulin action at the cellular level has yet to be provided. Although insulin is best known for its promotion of glucose metabolism it exerts a wide variety of effects at the cellular level (153). Thus in addition to stimulating glucose and amino acid transport, insulin can also activate or inactivate cytoplasmic and membrane enzymes, alter the

rate of synthesis of protein and DNA and influence the processes of cell growth and differentiation (3). These multiple effects vary widely with respect to dose response and time course. Some effects, such as the stimulation of glucose transport and glucose oxidation, occur within minutes or less if the cells are exposed toinsulin concentrations of less than 10^{-9} mol/l (154,155,156). At the other extreme, actions on DNA synthesis and cell growth require hours to days and generally involve higher concentrations of the hormone 10^{-7} mol/l (157). These growth promoting effects appear to be mediated by a class of plasma membrane receptors distinctly independent from receptors mediating the effect of insulin on intermediary metabolism. In addition, it has recently been reported that insulin might act directly on the nucleus, inducing enhanced mRNA synthesis (158), thus questioning the assertion that all the actions of insulin are initiated by insulin interaction with its specific membrane receptors (159). It is apparent that the biological actions of insulin are brought about by the altered activity of enzymes controlling key metabolic pathways and the regulation of many if not all of the intracellular activities is accompanied by changes in the phosphorylation of the affected enzymes, usually but not always, dephosphorylation (160). A chemical mediator of insulin action, released into the cytoplasm after exposure of target cells to insulin was first proposed in 1972 (161). By 1979 an insulin mediator substance had been identified that specifically inhibited CAMP dependent protein kinase, stimulated glycogen synthase phosphoprotein phosphatase (162) and activated mitochondrial pyruvate dehydrogenase (163) by stimulating pyruvate dehydrogenase phosphatase (164). This insulin mediator was subsequently identified as an

oligoglycopeptide with a molecular weight of 1000-1500 daltons (162). By 1980, two peptides had been separated from the mediator cell extract which had opposing effects on protein phosphorylation (165) although it was not known if one represented a metabolic product of the other or a separate entity (166). It was, however, suggested that the presence of two or perhaps multiple mediators would provide a mechanism by which insulin could induce both the phosphorylation and dephosphorylation of intracellular proteins (167,168). It has also been suggested that insulin could activate glucose transport by increasing the translocation of hexose carrier proteins from intracellular membranes to the plasma membrane and this would explain the insulin stimulation of glucose transport (169).

Currently the primary action of insulin is considered to be an acceleration of glucose transport into the cells of skeletal muscle and adipose tissue (170,171). There are tissues such as brain and liver where insulin has little effect on hexose transport. The intracellular messenger for insulin can also be generated by the addition of concavalin A, anti-insulin receptor antibody (172) or trypsin (166,173) to adipocyte plasma membrane, and this observation along with the blocking of insulin action by proteolytic inhibitors, has lead to the suggestion that insulin acts via a proteolytic mechanism (166). A general hypothesis has therefore been put forward (166,168,174) to suggest that insulin binding facilitates a proteolytic reaction at the plasma membrane involving an arginine-specific serine protease and an endogenous membrane substrate, followed by the internalisation of free rather than vesicular bound mediator(s) with subsequent alterations in the phosphorylation of key enzymes. Present estimates indicate that the mediator(s) is/are

present at 100-1000 times in excess of the insulin receptor concentration and it appears therefore unlikely that proteolysis of the receptor itself generates the mediator(s) (166). Thus, in essence, insulin is a cofactor for the membrane-bound protease which it might activate by initially cross linking the receptor (166). The sensitivity of the mediator(s) to proteolytic enzymes remains to be resolved.

In fat cells, insulin action has been proposed to be mediated through changes in the states of phosphorylation of certain proteins (notably ATP-citrate lyase and uncharacterised proteins of sub-unit molecular weights of 61, 35, and 22 K daltons) and catalysed by a mediator (possibly a protein kinase) released from the plasma membrane after insulin-receptor interaction (160), Figure 3. It has been observed that the insulin receptor is itself phosphorylated in intact cells (175) and in solubilised rat liver membranes (176) after exposure to insulin due to the phosphorylation of a tryrosine residue (176). Indeed it has recently been reported that the insulin receptor itself is a tyrosine-specific protein kinase which phosphorylates the β sub-unit of the insulin receptor after insulin binding to the α sub-unit of the receptor (177,178). The simplest reconciliation of the available data is that insulin receptor binding activates a regulatory cascade involving a tyrosine protein kinase that results in the activation of serine-threonine protein kinase and/or a protease that generates several messengers. A recent summary of some of the intracellular events influenced by insulin binding is shown in Figure 4. Since the only known activity of the insulin receptor is to function as a protein kinase, this hypothesis is an attractive one, but whether it is true remains to be

Figure 3. Proposed mechanism of insulin binding in fat cells (160).

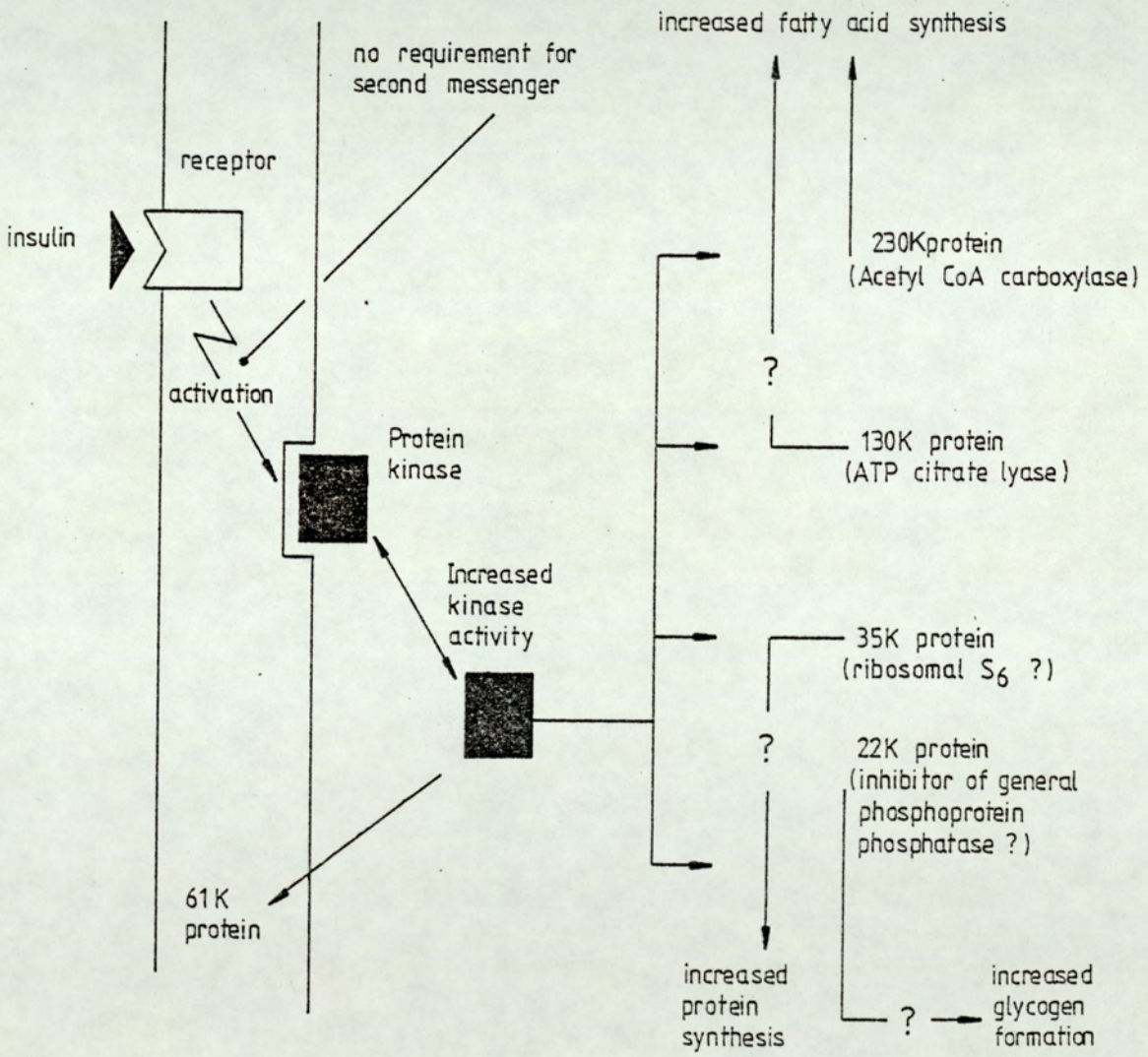
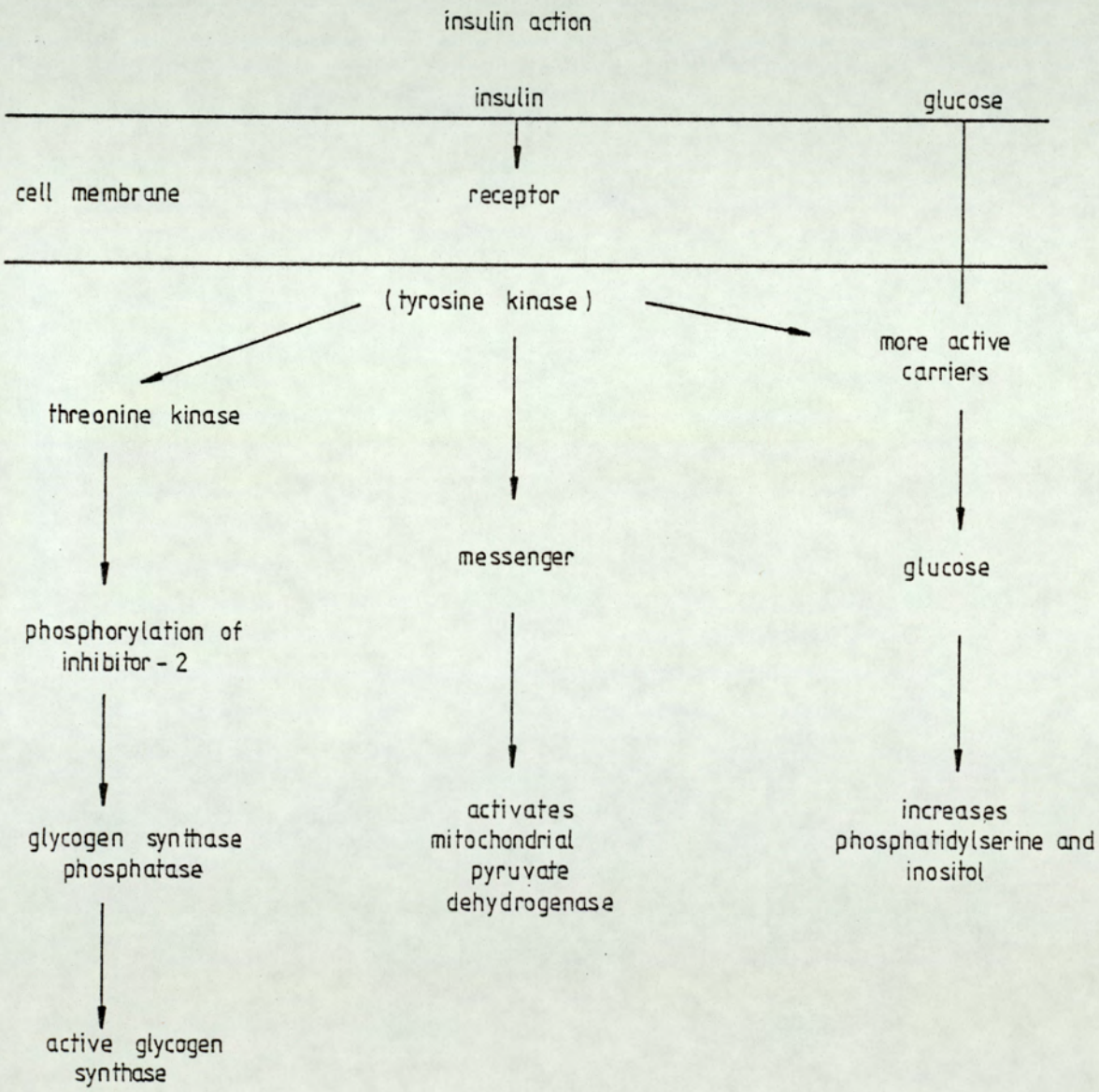


Figure 4. Insulin action at the cellular level with emphasis on the regulation of glycogen synthase, pyruvate dehydrogenase, glucose transport, and phospholipid accumulation (179).



established (179) and in the long run it may be wise to consider multiple mechansisms for the mediation of the action of insulin.

Insulin insensitivity and insulin unresponsiveness.

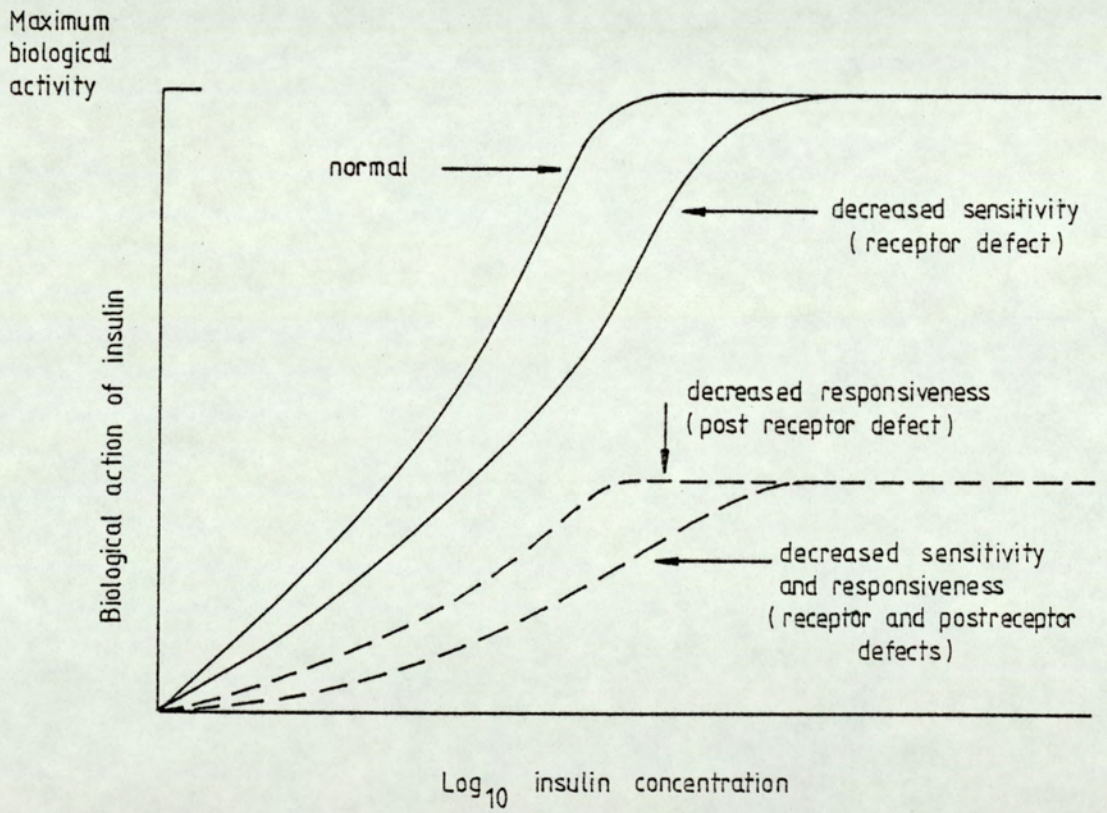
Variations in the biological effect of insulin may either be due to a change in the sensitivity to the hormone (as manifested by a shift in the insulin dose response curve to the right), a change in the maximal response to insulin or to a combination of altered sensitivity and maximum responsiveness to insulin (180). Target tissue defects contributing to insulin resitance may reside in the receptor or in postreceptor pathways, Table 1. Since a maximum biological effect is generated when only a small proprtion (normally 10-30 per cent) of the insulin receptor sites is occupied, a defect which is predominantly at the insulin receptor should impair the biological action of insulin at sub-maximally effective insulin concentrations, but a normal biological action should be achieved at maximally effective insulin concentrations (assuming receptor loss is not greater than 70-90 per cent). When this is so, the dose-response curve is displaced to the right, and this is designated insulin insensitivity, Figure 5.

If the postreceptor pathways are defective, a proportional reduction in the biological action of insulin is anticipated at all insulin concentrations. Such a defect would prohibit a normal maximum biological action, even at inordinately raised insulin concentrations. Thus the dose-response curve is proportionately reduced at all insulin concentrations, and thus this is designated insulin unresponsiveness, Figure 5.

Table 1. Possible mechanisms which may influence insulin action (257).

Prereceptor	Receptor	Postreceptor
Altered insulin biosynthesis (eg. defective insulin molecule or hyperproinsulinaemia) Binding of insulin to insulin antibodies Altered insulin degration Altered concentrations of insulin counterregulatory hormones	Changes in insulin receptor number or affinity	Changes in coupling mechanisms, transport functions, second messengers or intracellular enzyme activities

Figure 5. Graphical illustration of insulin action in terms of insulin sensitivity and maximal insulin responsiveness.



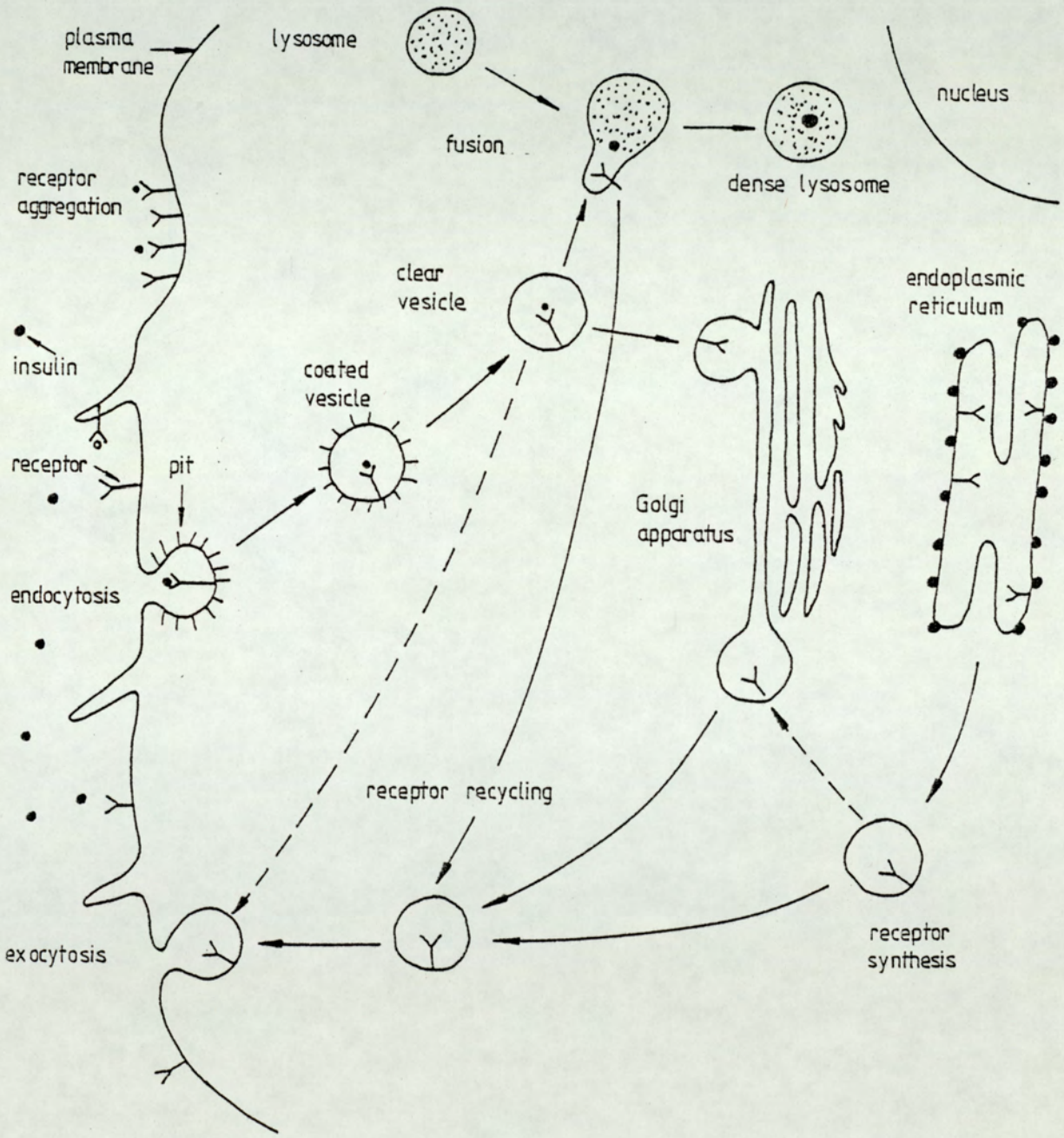
The coexisistence of a receptor defect and a postreceptor defect compounds the effects of insulin insensitivity and insulin unresponsiveness. Accordingly, the dose-response curve is displaced to the right, and the normal maximum biological action is not achieved, Figure 5.

Insulin receptor internalisation, degradation, recycling and biosynthesis.

When a target cell is exposed to insulin there follows an aggregation of occupied receptors into specific pitted regions of the plasma membrane (181). This receptor aggregation appears to be a dynamic process mediated by the binding of insulin to diffusely located receptors (182). Aggregates of receptors are then taken into the cell (internalisation) by endocytosis (106,134). In a steady state binding situation, as much as 40% of the insulin associated with a target cell may be localised within the cell. The fate of the aggregated receptor complexes is not entirely clear, although the lysosomal degradation of the receptor appears to represent an end point of the binding sites functional role. It appears that not all of the internalised receptors are degraded and consequently many are recycled and reinstated back into the plasma membrane (183,184) possibly by an exocytotic like mechanism as summarised in Figure 6.

Insulin bound to receptors has been demonstrated to be the substrate for cell mediated insulin degradation and the recyling and degradation of receptors in most (133,185,186) but not all (187) cells. The final lysosomal degradation of internalised insulin-receptor complexes provides for the receptor mediated degradation of

Figure 6. Schematic drawing of the main morphological events underlying binding, internalization, degradation, recycling and biosynthesis of insulin receptors in a target cell.



the insulin molecule. A soluble enzyme which rapidly degrades insulin by proteolysis has been found in many animal tissues such as rat liver (188) and pig skeletal muscle (189), and some human tissues such as erythrocytes (190) and skeletal muscle (191). Reductive disulphide cleavage of insulin by a glutathione-insulin transhydrogenase activity and an insulin specific protease has been implicated in the degradation of the insulin molecule itself (186). Degradation of receptor bound insulin has not been shown in either human monocytes or erythrocytes when incubated at 15°C (117,121,192). Evidence has accumulated that both insulin action and degradation might be mediated through the binding of insulin to its specific receptors on the plasma cell membrane as the initial step (130,192), although the precise nature of this relationship remains to be elucidated.

The binding of insulin to plasma membrane receptors not only accelerates the process of internalisation, but also stimulates the production of new receptors (106). Biosynthesis of new insulin receptors takes place at the rough endoplasmic reticulum; receptors concentrate in the Golgi region and transfer to the plasma membrane by exocytosis of Golgi vesicles (193). This transfer and insertion of new receptors into the plasma membrane is likely to be an active process requiring microtubule involvement (194). Recycled receptors probably transfer to the plasma membrane in the same manner.

Down-regulation.

Insulin in addition to the induction of changes in receptor affinity, can also alter the number of insulin receptors, although the latter is a more chronic effect (138,196). In general, raised concentrations of insulin are associated with a reduced number of

insulin receptors. This insulin-induced receptor loss or diminution in insulin binding capacity (113) is termed "down-regulation". At high insulin concentrations there is no apparent alteration in the rate of receptor synthesis or degradation (198) and although the Scatchard plot indicates a reduced receptor concentration, the number of receptors that can be extracted with detergent is unchanged. This suggests that down-regulation may be achieved by the process of internalisation described previously. In direct support of this view, Marshall and Olefsky (199) have demonstrated a correlation between receptor loss and insulin internalisation. Furthermore, insulin induced receptor loss is usually accompanied by a reduced ability of residual receptors to internalise insulin, indicating the existence of a feedback control mechanism. Using agents with insulin like acitivity which act distally to the receptor, such as spermine and vitamin K5, Caro and Amatruda (200) have been able to demonstrate receptor loss. At present there is no evidence that insulin receptor biosynthesis is altered by down-regulation. It would, therefore, appear that insulin receptors on the cell surface are regulated purely by the rate of internalisation, the rate of degradation of internalised receptors and the rate of recycling of non-degraded receptors. Since receptor degradation and recyling depend on the initial internalisation event, it would appear that receptor occupancy is the initial rate controlling step i.e. in the presence of a high insulin concentration receptor occupancy is high, initiating down-regulation (195).

The spare receptor concept.

It is well established that target cells with a normal sensitivity to insulin show a maximum biological response to insulin when only a fraction of the total number of insulin receptors are occupied, this fraction may vary from as little as 10% to 30% (201-204). The unoccupied receptors are known as "spare receptors". The term "spare" is unfortunate since it might give the incorrect impression that these receptors are not functional and can be lost without affecting biological activity. It has been argued that all receptors are potentially available for binding and the 10-30% of receptors which bind insulin represent the consequence of a random statistical event. If the aggregation of insulin receptors is required to produce a biological action of insulin, it is possible that the unoccupied receptors exist in a less receptive state and are less readily occupied.

Cell types used to study insulin receptors.

Studies of both kinetic and equilibrium insulin binding have normally been performed on either isolated intact cells or plasma membrane preparations although binding to subcellular organelles, pieces of tissue and intact organs have also been determined. Muscle cells, hepatocytes and adipocytes are quantitatively the most important cell types upon which insulin acts to regulate glucose homeostasis. Ideally, studies of insulin receptor status *in vivo* should examine each of these cell types but there are practical limitations especially in clinical studies, where muscle, liver and

fat biopsies are difficult to obtain. Hence, insulin target tissues have been studied extensively in animals and correlated with the human studies. Isolated cells are metabolically active and thus the distinct advantage of allowing both the biological effects and the binding of insulin to be determined simultaneously. Animal studies have indicated general similarities between the regulation of insulin receptors in adipocytes, hepatocytes and muscle cells (205-208), but comparative studies of these cell types in man are still awaited. Assessments of insulin receptor activity in man have relied mostly on the use of readily available circulating monocytes (209) and erytrocytes (210). The properties of monocyte insulin receptors have been shown to mirror closely the properties of adipocyte insulin receptors in a number of conditions in man (30,192,196). The insulin receptors of erythrocytes respond in a similar mannner to those of monocytes, although changes in the binding characteristics of erythrocyte receptors may occur more slowly (121,211,212). This may reflect in part the incapacity of erythrocytes to undertake de novo protein biosynthesis, which is anticipated to compromise receptor turn-over. Up-regulation of insulin receptors may be particularly vulnerable and may depend to some extent on the recruitment of new cells into the erythrocyte pool. Within erythrocyte preparation, reticulocytes show greater specific insulin binding than erythrocytes, and binding decreases exponentially with erythrocyte age, due to a decline in the concentration of receptor sites (213-215). The fat cell preparation is homogeneous compared with the composite cell suspension used in insulin receptor studies of monocytes and erythrocytes. However, fat cells from a given region in any one person vary considerably in size (130). This dissimilarity together with

regional differences in adipocyte size make adipose tissue heterogenous (130,216). Studies of insulin binding to monocytes are also difficult because they require relatively large volumes of blood (50-120 ml) (117). Thus at the present time the estimation of insulin receptor binding on freshly isolated monocytes is not convenient for clinical studies in diabetics, since additional blood will also be required for biochemical profiles and oral glucose tolerance testing. Both adipocytes (130) and erythrocytes (217) have low nonspecific binding and so binding assays using these cells have a high precision.

Despite the general similarities between insulin receptor activity in different cell types, sufficient differences have been observed between cell types (206,211) to encourage further investigation. Hence, the extrapolation of receptor data beyond the cell type studied should be undertaken only with extreme caution.

Insulin receptor binding and its assessment.

The assays for insulin receptor binding use methods that are conceptually similar to those of the competitive protein-binding assays originally described by Yalow and Berson 1960 (218) and now widely employed for the radioimmunoassay of many hormones. Modification of these assays for the quantitative determination of various parameters of hormone receptor interaction (118,121,130, 209,219) involves the optimally controlled incubation of replicate aliquots of the receptor population (e.g. isolated cells or plasma membranes) with a constant concentration of high specific activity tracer, ^{125}I-monoiodoinsulin, in the presence of varying

concentrations of unlabelled insulin for a given time at a specific temperature and pH. At steady state the tracer and unlabelled insulin are bound in proportion to their molar ratios. At this point the ^{125}I-labelled insulin bound to the preparation is rapidly separated from the free insulin either by centrifugation (through oil or buffer) or filtration. After any required washing steps, the radioactivity associated with the bound fraction is determined and can be used to derive the fraction of total insulin, which is bound, provided the tracer iodo-insulin behaves like the unlabelled insulin. Using this technique for the measurement of ^{125}I-labelled insulin interaction with intact cells, the data obtained has been considered to be the sum of at least two processes: binding to a saturable component and binding to a non-saturable component. The former is thought to represent binding to a specific receptor and the latter to represent the adsorption of insulin to the cell membrane or cellular uptake of whole or fragmented molecules of ^{125}I-insulin. In the receptor literature the non-saturable component is conventionally termed the non-specific binding (NS) and is determined in the presence of excess native insulin. All binding data is corrected for non-specific binding at all insulin concentrations used. Binding data can be subjected to a variety of graphical analyses which have been reviewed elsewhere (221). It is appreciated that changes in receptor status may occur during the preparation and incubation period required for steady state binding in the assay procedure. Insulin receptor binding is highly dependent upon temperature and sub-physiological temperatures are generally used to achieve steady state binding during receptor assays (30,221). Thus the observed condition of the receptors may not reflect precisely their conditions immediately prior to assay.

Analysis of insulin receptor binding data.

Generally it is sufficient to construct a competition (displacement) curve for the analysis of steady state binding data (196), and it is the only plot of many that are currently used to display raw, untransformed data. For the creation of the binding curve the percentage of bound ^{125}I-insulin is plotted as a function of the total insulin concentration, Figure 7. The plot is typically sigmoidal and can be used for the assessment of receptor affinity (222). Although this plot closely represents the actual experimental results (196) the limited amount of information gained from such plots and the fact that tracer hormone and binding site concentrations and the dissociation constant are often unknown, render the plot of limited use for the quantitative interpretation of steady state binding data and are thus often used in conjunction with other methods of analysis.

Insulin receptor interaction is generally considered as a simple, bimolecular reversible reaction described by the law of mass action and its characterisation has been well documented (224). Estimation of the concentration and affinity of the receptor sites is most commonly based on the method of Scatchard (225) in which the ratio of the concentrations of bound to free insulin is plotted against the concentration of bound insulin. The Scatchard plot for insulin receptor binding is in general curvilinear with an upward concavity, Figure 8. The curvilinearity of Scatchard plot of insulin binding data are thought to represent particular properties of the insulin-receptor interaction. Interpretation can be based on a receptor population heterogeneous with respect to affinity

Figure 7. Competition - inhibition binding curve of ^{125}I - insulin binding to a receptor population.

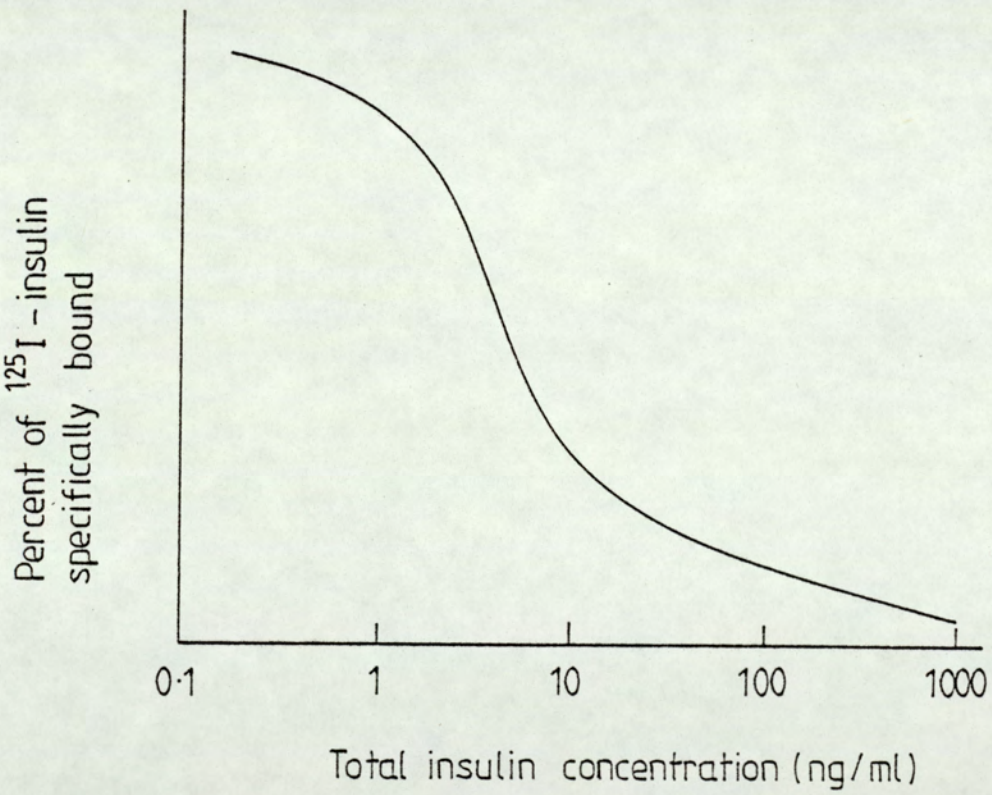
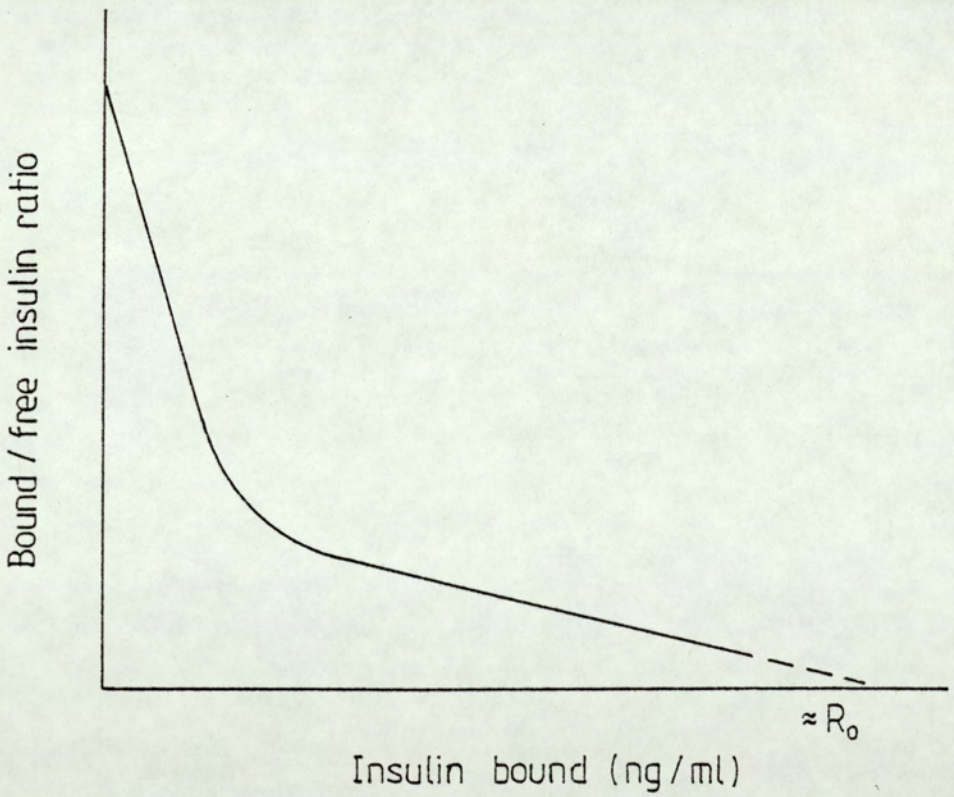


Figure 8. A typical curvilinear Scatchard plot for insulin binding to a receptor population. R_o is derived by extrapolation of the curve to the abscissa intercept and represents the maximum binding capacity.



(132,226,227). The nonlinear Scatchard plot has been interpreted to represent two or more classes of independent binding sites with different receptor affinity and receptor concentration. Some investigators have proposed a two site model (i.e. a high affinity, low capacity site and a low affinity, high capacity site) where only the high affinity, low capacity site has physiological significance (228), Figure 9. In contrast, other workers have carried out kinetic experiments as well as equilibrium studies with different insulin analogues suggesting that the nonlinear Scatchard plot can also be explained by a homogenous class of receptors displaying negative cooperative site-site interactions. This implies that the affinity of receptors is not fixed and the affinity of binding sites for insulin decreases with increasing levels of binding as the occupancy of the receptors increases, Figure 10 (229,230). At low physiological concentrations of insulin most receptors are empty and in their highest state of affinity. With increasing receptor occupancy by insulin the affinity of the receptors gradually decreases to their lowest affinity in the filled state. Consistent with this interpretation De Meyts and Roth (230) proposed the use of an average affinity profile to analyse steady state binding data, Figure 11. The profile indicates two inter-convertible sites: a high affinity site with a binding constant $\overline{K}_e$ and a low fractional occupancy $\overline{Y}_e$: and a low affinity site with a binding constant $\overline{K}_f$ and a high fractional occupancy $\overline{Y}_f$. Receptors adopt the high affinity state at low insulin concentrations and convert to the low affinity state as insulin concentrations increase. Extensive kinetic data have been reported in support of negative cooperativity, and the last 8 residues of the B chain have been identified as especially important

Figure 9. Scatchard plot revealing two classes of insulin receptors with differing but fixed affinities, one of high affinity (k_1), low capacity $(R_o)_1$ and one of low affinity (k_2), high capacity $(R_o)_2$. R_o represents the combined binding capacity of both classes of receptors.

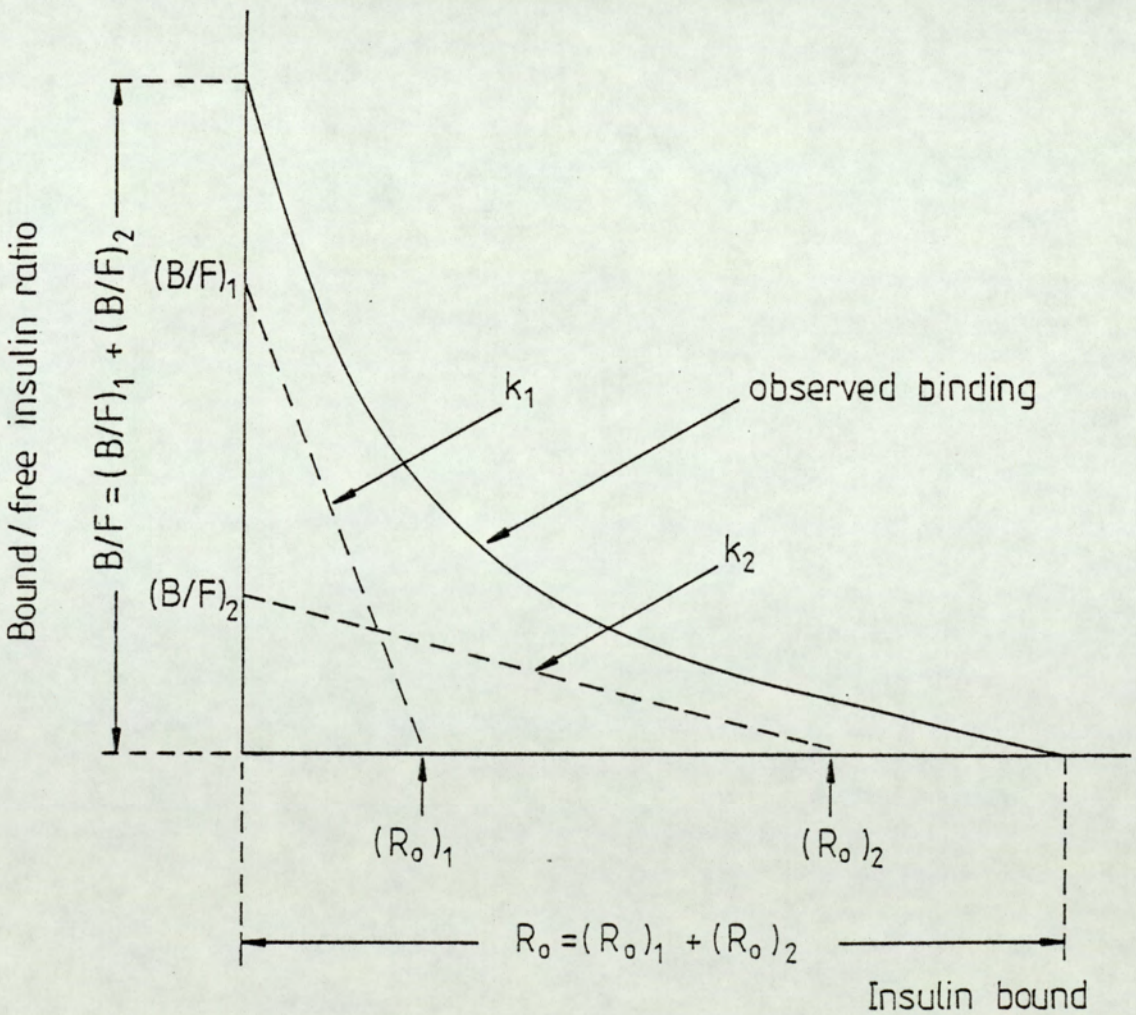


Figure 10. Scatchard plot revealing a single class of receptor sites with negative cooperativity. When occupancy increases the average affinity decreases from a limiting high affinity, $\bar{K}_e$, "empty sites" affinity, to a limiting low affinity, $\bar{K}_f$, "filled sites" affinity, as represented by the straight line rotating around the abscissa intercept of the curve. R_o represents the maximum binding capacity of receptor population.

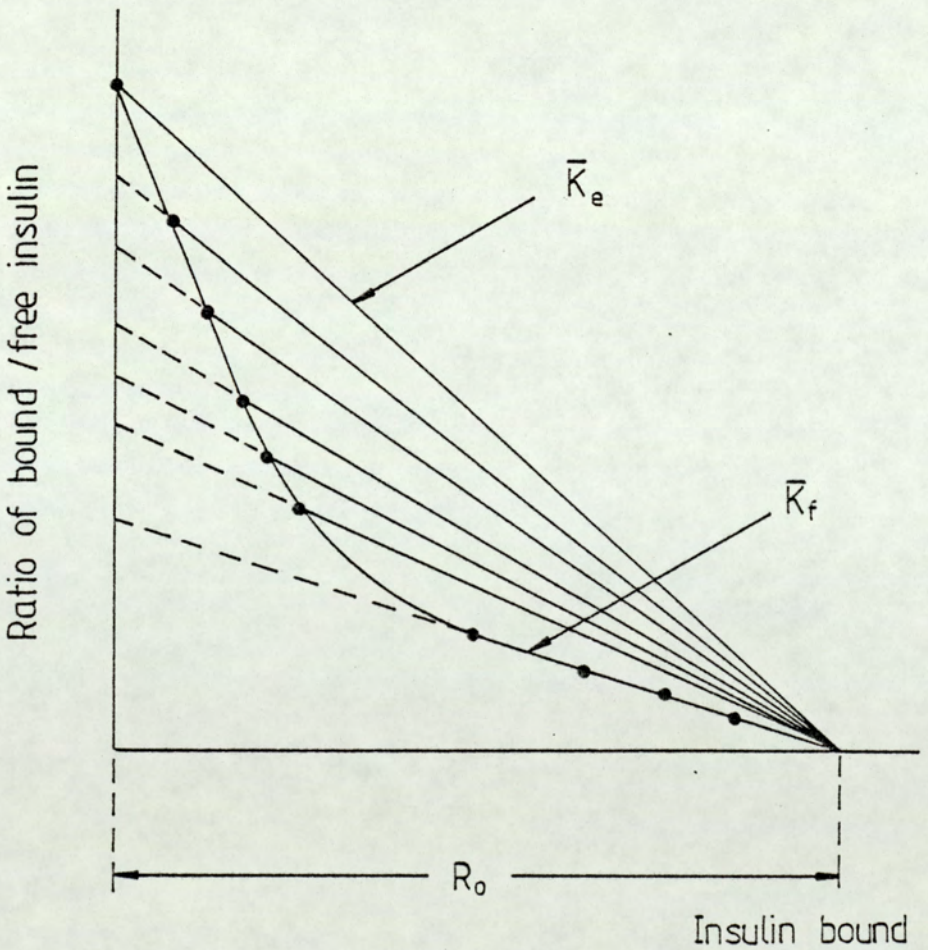


Figure 11. The average affinity profile of ^{125}I-insulin binding to an insulin receptor population. $\bar{K}$, represents the average affinity, calculated as $B/F\ /R_o - B$ and is plotted as a function of $\log_{10} \bar{Y} = \log_{10} (B/R_o)$.

$\bar{Y} = 0$ when all sites are empty and

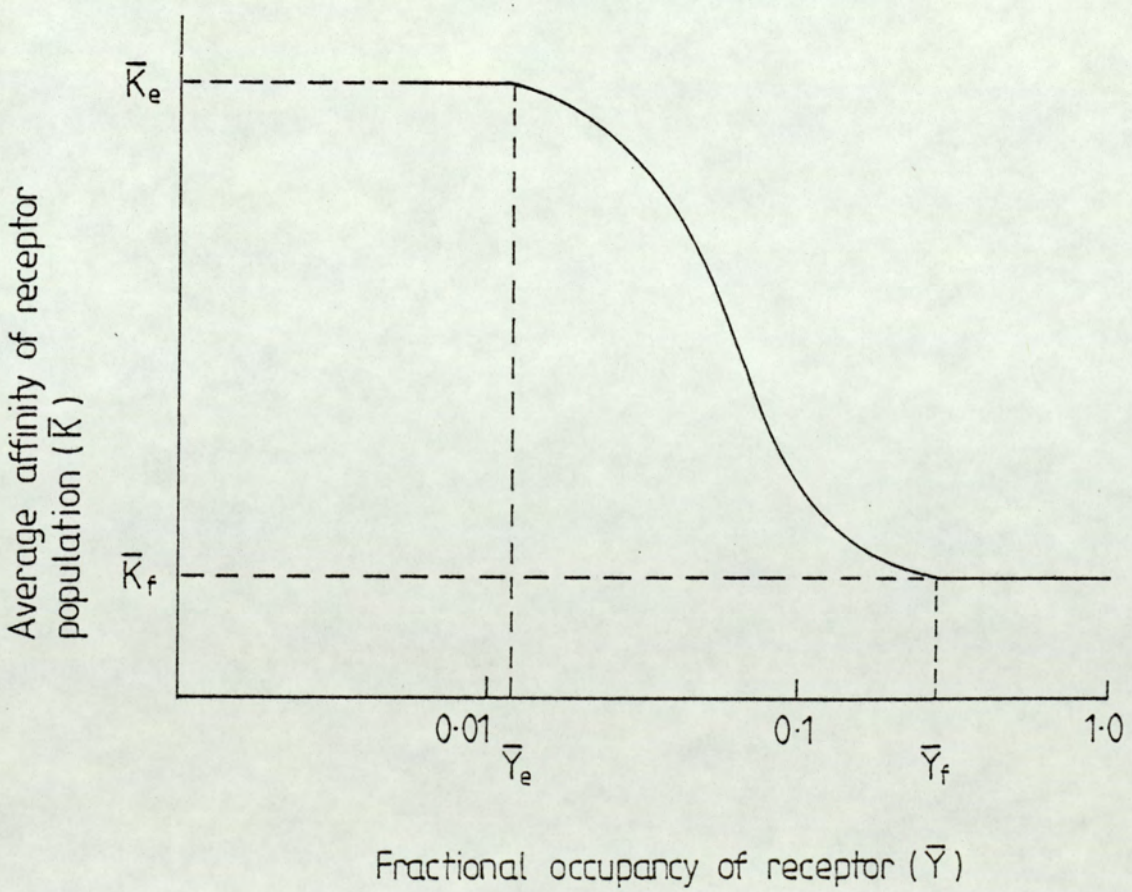
$\bar{Y} = 1$ when all sites are occupied.

$\bar{K}_e$ = affinity of the "empty sites" conformation,

$\bar{K}_f$ = affinity of the "filled sites" conformation,

$\bar{Y}_e$ = threshold for measurable site-site interactions, and

$\bar{Y}_f$ = fractional occupancy at which site-site interactions are maximal (all sites in $\bar{K}_f$).



in this respect (229,231). These residues plus the C- and N-terminal regions of the A chain are essential for the full metabolic potency of insulin.

The concept of negative cooperativity has not found universal acceptance. Herzberg and colleagues (232) have demonstrated a complete block of the high affinity site by concavalin A without any effect upon the low affinity site. In addition, intact hepatocytes degrade ^{125}I-insulin into fragments which show lowered binding (233,235) and if this degradation is taken into account a linear Scatchard plot results. Using cultured lymphocytes, Sonne and Gliemann (187) failed to confirm insulin fragmentation.

One mechanistic explanation for the cooperative model proposes interactions between (or conformational changes in) the receptor sub-units induced by increasing insulin concentrations (229,234). The altered sub-unit structure reduces the affinity of the receptor and induces a fast dissociating state. De Meyts, Bianco and Roth (229) have drawn an analogy with the interaction between haemoglobin and oxygen. At low oxygen levels the haemoglobin molecule has a low affinity for oxygen, and oxygen dissociates rapidly from the haemoglobin molecule. At high oxygen levels the affinity of haemoglobin is high and dissociation is slow. Thus, when availability is low, haemoglobin releases oxygen more quickly. The insulin receptor is envisaged to behave in the opposite way. At low insulin concentrations the receptor has a high affinity for insulin and slow dissociation rate. At high insulin concentrations the affinity is low and the dissociation rate is fast. Thus, negative cooperativity might serve as a buffer system which protects the cell from high insulin concentration, but retains sensitivity to low insulin concentrations.

An alternative account of negative cooperativity suggests the movement of receptors within the fluid mosaic of the plasma membrane (236,237). Aggregation (or clustering) of insulin receptors in the presence of rising concentrations of insulin has been demonstrated experimentally (181,238). Levitzki (234) proposed that the receptors might exist in clusters prior to occupancy and conformational changes induced by binding result in a less receptive state which could be transmitted to other receptors in the cluster. It has also been suggested that insulin binding enables the newly occupied receptors to aggregate unoccupied receptors.

Studies on insulin receptor binding in health and disease.

In healthy individuals, insulin receptor binding shows a diurnal variation (239) and is influenced by diet (240,241), exercise (242), age (243), pregnancy (244), menstrual cycle (245), sex (243) and sampling site (246).

Regional differences in tissue insulin binding.

Significant regional differences have been observed in the *in vitro* basal metabolism of adipose tissue from obese women (246). In addition regional differences in insulin receptor binding and action have been compared in subcutaneous and abdominal fat from obese women (125). Insulin receptor number was found to be higher in femoral than in abdominal adipocytes while the apparent insulin receptor affinity was higher in abdominal fat. The mean dose response relationships for the antilipolytic effect of insulin were almost

identical in both regions while the mean insulin dose-response curves for glucose oxidation differed and the responsiveness was enhanced 2-fold in femoral fat. In non-obese patients a higher insulin receptor affinity has been demonstrated in subcutaneous compared with omental adipocytes, but no difference in receptor concentration has been reported. Subcutaneous fat has been shown to be more responsive to the antilipoytic action of insulin than omental fat (247).

Insulin receptor binding in diabetes mellitus.

Heterogenous defects of insulin receptor function occur in diabetes. Not all diabetics exhibit insulin receptor defect, but where such defects exist they appear to be associated with insulin resistance manifest by the co-existance of hyperglycaemia and hyper-insulinaemia. Available data suggest that most non-obese subjects with impaired glucose tolerance (IGT) and all non-obese non-insulin dependent diabetics with raised basal glucose concentrations exhibit insulin resistance (248-251). In general, the severity of insulin resistance in patients with IGT and non-insulin dependent diabetic subjects increases with the magnitude of the basal hyperinsulinaemia, but there is no correlation between insulin resistance and the insulin response to a glucose challenge.

Obesity is common among subjects with IGT and non-insulin dependent diabetes and when present, exacerbates the insulin resistance in these subjects (196,251).

Insulin receptors in patients with non-insulin dependent diabetes mellitus.

Using the euglycaemic and hyperglycaemic clamp techniques Kolterman and colleagues (251) observed that the dose-response curve for the biological action of insulin (Figure 5, page 35) was displaced to the right in obese and non-obese non-insulin dependent diabetes. In addition to this reduction in insulin sensitivity, most patients with non-insulin dependent diabetes showed a decrease in the maximum biological action of insulin. This degree of insulin unresponsiveness, implying a postreceptor defect, followed closely the level of basal hyperglycaemia in the more severely hyperglycaemic patients. In these severely hyperglycaemic non-insulin dependent diabetic patients the findings suggested a postreceptor defect as the predominant abnormality. A continuum of defects is therefore apparent among patients with non-insulin dependent diabetes. Patients with mild insulin resistance suffer only from insulin insensitivity, while patients with more severe insulin resistance display a postreceptor defect in addition to the receptor defect. A receptor defect in non-insulin dependent diabetes mellitus has been identified as a decrease in receptor site concentration, while receptor affinity is normal.

In common with obese non-diabetic subjects and subjects with IGT, non-insulin dependent diabetics show an impaired suppression of hepatic glucose output at submaximal insulin concentrations, but not at maximal insulin concentrations (251). A receptor defect in hepatocytes, but not a postreceptor defect, is therefore inferred. While obesity *per se* causes a reduced concentration of insulin

receptor sites and aggravates insulin resistance in IGT subjects and mild non-insulin dependent diabetics, in severely insulin resistant non-insulin dependent diabetics an additive effect of obesity is not observed. This suggests that the receptor concentration is already markedly compromised in severe non-insulin dependent diabetes, and obesity exerts no further effect (251).

Animal models of diabetes.

Many spontaneous syndromes of inappropriate hyperglycaemia have been described. Insulin receptor status has been examined in the obese-diabetic (ob/ob) mouse, which exhibits moderate hyperglycaemia, marked hyperinsulinaemia and severe insulin resistance. Consistent with obesity and non-insulin dependent diabetes in humans, this model shows a reduced concentration of insulin receptor sites and a normal receptor affinity in a variety of cell types (207). A postreceptor defect has also been identified (208), and may precede the onset of the receptor defect in these mice (252). Other models of spontaneous hyperglycaemia and hyperinsulinaemia, such as New Zealand Obese (NZO) mice and diabetic KK mice, show a reduced concentration of insulin receptor sites in liver plasma membranes (253,254), however, insulin binding to hepatocytes from Zucker fatty (fa/fa) rats is only increased above that of lean rats in the fasting state (255).

Aims of the Study.

The majority of clinical investigations are preceeded by a 12 hour or overnight fast in order to provide a stable baseline for profile determinations and subsequent therapy. With this in mind a preliminary study was initiated to evaluate the effect of overnight fasting on the erythrocyte insulin receptor binding status of normal, age and weight matched volunteers.

The normal situation confirmed, attention was then focussed on the most common clinical condition showing insulin resistance impaired glucose tolerance and impaired insulin receptor binding status, that of non-insulin dependent diabetes mellitus. There is growing evidence from diabetic clinics and the diabetic literature to indicate that the treatment of non-insulin dependent diabetes is often complicated by the presence of a mild to severe essential hypertension. The clinical remedy is to treat these patients with either thiazide diuretics, potassium sparing if indicated or calcium antagonists. Contentious and sometimes conflicting reports in the literature have suggested that the prolonged treatment of hypertensive patients with thiazides and certain calcium antagonists can lead to the precipitation of glucose intolerance and additionally with thiazides, potassium depletion.

On this basis, clinical investigations were conducted to examine the effects of a commonly used thiazide, bendofluazide with and without potassium supplementation on the oral glucose tolerance, fasting glucose and insulin levels, erythrocyte insulin receptor binding status and biochemical and haematological profiles of mildly hypertensive patients with and without diet controlled non-insulin dependent diabetes mellitus. The study will provide information on the role of cellular insulin receptor binding in the mechanism of glucose intolerance induced by thiazides and the potential aggravation of the glucose intolerance seen in non-insulin dependent diabetes mellitus.

Calcium antagonists such as nifedipine are also of potential use in the treatment of mild hypertension and yet here too there is some indication that they may impair normal glucose tolerance, perhaps by

interfering with the release of insulin. In this respect the treatment of hypertension with nifedipine might be contra-indicated in non-insulin dependent diabetics whose endogenous insulin secretory capacity and ability to maintain near normal glucose tolerance may be compromised. In this respect a further clinical study was initiated to examine the effect of long term nifedipine treatment, a short term of withdrawal and then subsequent retreatment on the oral glucose tolerance, serum glucose and insulin of mildly hypertensive patients with and without non-insulin dependent diabetes mellitus. This study should provide important information regarding the validity and usefulness of calcium antagonists in the treatment of hypertension associated with non-insulin dependent diabetes.

We now know that insulin receptors are far from being inert structures and that both the concentration and affinity of insulin receptor sites are subject to continual regulation by a multitude of acute and chronic influences. The evaluation of insulin receptor binding status in non-insulin dependent diabetes mellitus should emphasise the heterogenecity of the disorder. A reduced concentration of insulin receptors can be offered to account for any decrease in insulin sensitivity, but as the severity of insulin resistance increases in these diabetics, especially if they are also obese, the possibility of a postreceptor defect becomes evident. It may well be that the correction of the altered insulin receptor status of non-insulin dependent diabetics is commensurate with the establishment of good metabolic control. However, in other situations where a defect of insulin action has tentatively been identified, such as non-insulin dependent diabetes with obesity the relationship of the expected change in receptor binding and

postreceptor events to the development of insulin resistance remains to be confirmed.

Currently, great store has been placed in the possible treatment and control of non-insulin dependent diabetes with guar gum. Evidence has been presented to suggest that guar gum treatment may improve glucose tolerance and lower both postprandial glucose and insulin levels. With this in mind a clinical study was initiated to investigate the possible therapeutic usefulness and patient acceptability of guarem (a commercial form of guar gum) in the treatment of non compliant, poorly controlled non-insulin dependent diabetics. The study involved the measurement of oral glucose tolerance, plasma glucose and insulin, insulin receptor status and a biochemical profile. The presence of a well established colony of genetically obese diabetic mice (ob/ob) in the department, an animal model of obesity and non-insulin dependent diabetes, allowed a parallel animal study involving the effect of guarem treatment on the body weight, plasma glucose, plasma insulin and insulin receptor binding status of obese mice. In this study a more classical insulin sensitive cell, the adipocyte was used for insulin receptor binding studies. This was followed by a comparative study, using age matched lean and obese mice, to investigate the impact of obesity on adipocyte insulin senstivity and insulin receptor binding status.

Unlike erythrocytes, adipocytes vary in size and degree of insulin sensitivity. Regional differences in adipose tissue sensitivity have been documented for obese human patients but not for animal models of obesity. With this in mind the concluding work of this thesis has involved investigation of possible regional variations in insulin receptor binding, basal and insulin stimulated

glucose oxidation and ^{125}I-insulin degradation using isolated adipocytes and fat segments from the lower abdominal and upper femoral subcutaneous regions of obese mice.

CHAPTER TWO

General Materials and Methods

General chemicals

The following chemicals were used in the studies presented in this thesis. N-2-hydroxyethylpiperazine-N^1-ethanesulphonic acid (HEPES), N-tris[hydroxymethyl] methyl-2-aminoethanesulphonic acid; 2-(2-hydroxy-1, 1-bis[hydroxymethyl]-ethyl amino ethanesulphonic acid) (TES), collagenase Type II prepared from Clostridium Histolyticum No. C-6885, Lot 92 F-6834, 230 units/mg, and bacitracin were purchased from Sigma Chemical Co. Ltd., Poole, Dorset. Bovine serum albumin fraction V (Lot numbers 330 and 335, insulin free) was purchased from Miles Laboratories Ltd., Slough, Bucks. Monocomponent porcine insulin (Lot number 58311160) was purchased from Novo Industria A/S, Copenhagen, Denmark. Na ^{125}I (IMS-30) and D-(U-C^{14}) glucose (specific activity, 270 µCi/µmol) were purchased from Amersham International, Amersham, Bucks. ^{125}I monoiodoinsulin with the labelled iodine at tyrosine 14 (specific activity, 249 µCi/µg) was a generous gift from the Novo Research Institute. Ba $^{14}Co3$ (specific activity, 0.27 µCi/mg) from NEN Boston Mass, U.S.A. Ficoll-Hypaque and Sephadex G50 were obtained from Pharmacia Fine Chemicals, Hounslow, Middlesex. Di-n-butyl phthalate (weight per ml at 20°C 1.042 to 1.045), Dinonyl phthalate (weight per ml at 20°C 0.967 to 0.970) and Repelcote (2 percent v/v solution of dimethyl dichloro-silane in carbon tetrachloride) for siliconizing glassware were all purchased from BDH Chemicals Ltd., Poole, Dorset. Silicone oil (density 0.99 gm/ml, Dow Corning 200/50CS) was purchased from Hopkins and Williams, Chadwell Heath, Essex. LP3 radioimmunoassay tubes were obtained from Luckhams Ltd., Burgess Hill, Sussex. Microfuge tubes and other microfuge accessories were purchased from Beckman Instruments Ltd., High Wycombe, Bucks.

All other reagents were of analytical grade and purchased from British Drug Houses Ltd., Poole, Dorset. Double distilled water was used in all experiments.

General details of experimental animals and human volunteers.

A) Experimental animals:

Overnight fasted male and female genetically obese diabetic (ob/ob) mice and their homozygous lean (+/+) littermates from the Aston colony aged between 18-24 weeks and weighing between 40-120g together with 300-450g male Wistar rats were used in the present studies. All animals were housed in an air conditioned room at 22 ± 2°C and maintained on a standard pellet diet (Mouse breeding diet, Heygate and Sons Ltd., Northampton, U.K.) and tap water ad libitum.

B) Human volunteers:

As far as possible age and weight matched normal male volunteers and poorly controlled non-insulin dependent diabetics of both sexes with and without mild hypertension were recruited into clinical studies. All female recruits were postmenopausal to eliminate effects of the menstrual cycle. In all clinical studies the human volunteers acted as their own controls. The criteria used for patient selection has been fully described in each section.

Radioiodination of insulin.

A high specific activity tracer is an essential ingredient of insulin receptor binding studies because the concentration of hormone

must be kept within the physiological concentration for that hormone. For insulin this is often as low as 10^{-10} mol/l. In addition the labelled hormone must retain the full biological activity and receptor binding properties of the native insulin. Radioiodine is particularly convenient for the preparation of labelled protein tracers as it can be readily substituted into the tyrosine residues of proteins and peptides. Two convenient gamma-emitting radioisotopes of iodine are widely available, ^{125}I and ^{131}I and these offer advantages over the common β-emitting isotopes ^{3}H and ^{14}C for the production of hormones of high specific activity. Theoretically, monoiodoinsulin labelled with ^{131}I would be of a higher specific activity than the equivalent ^{125}I preparation. However, ^{131}I is available at only about 20% isotopic abundance, ^{127}I being a major contaminant. On the other hand ^{125}I can be obtained at an isotopic abundance approaching 100%, so the latter produces a high specific activity tracer and is therefore used preferentially. Other factors favouring this choice of isotope include the longer half life of ^{125}I compared to ^{131}I (60vs. 8 days) and the greater counting efficiency in most gamma counters of ^{125}I decay products.

The incorporation of one radioiodine atom per molecule of protein is the minimum conceivable substitution for the production of labelled hormone. At any level of substitution there may be problems of reduced biological activity and immunoreactivity particularly if the labelled residues are closely associated with the binding region(s) of the hormone. The probability of hormone damage due to radiation also increases. An absence of diiodination is essential in labelled insulin preparations, particulary those to be used in receptor studies, due to a much reduced binding affinity towards

these molecules and the possible production of monoiodinated products of unknown biological fate (258). Iodination damage generally appears as alterations in structure and hence immunoreactivity caused by the substitution of iodine for hydrogen atoms in the protein. Structural alterations in the protein can be caused by exposure to high levels of radiation during or after iodination (218). The reagents frequently used in the iodination reaction may cause specific chemical damage to the antigen. In the case of the chloramine-T method proteins are exposed to an oxidising agent, chloramine-T and a reducing agent, sodium metabisulphite. Oxidising agents may cause oxidation of sulphydryl groups of proteins and oxidative damage of disulphide bonds. Reducing agents may effect reductive cleavage of disulphide bridges, however chemical damage of this sort does not appear to be a general problem with the chloramine-T method since only low concentrations of oxidising and reducing agents are used and exposure is only for a short period of time. Chemical damage possibly caused by impurities that may be present in radioiodide solutions and radioiodide obtained from two different sources and used in parallel experiments to label the same batch of protein, has been shown, in a few cases, to give labelling with marked differences in iodination damage (259). Some iodine may also react with histidine (260), tryptophan (261) and/or sulphydryl groups (262) but tyrosine is the principal amino-acid involved. For the production of labelled hormones of high specific activity without the use of large amounts of radioiodide small quantities of protein need to be used. Methods developed as a consequence of this include the use of iodine monochloride (263), chemical oxidants (264), electrolytic (265) and enzymatic iodination (266) although no one

method has proved to be completely satisfactory and reliable.

When radiolabelled materials are used to study biological processes, the tracer compound is often tacitly assumed to be indistinguishable from the parent compound. Scatchard analysis of hormone receptor binding and kinetic analysis of hormone degradation require that the radioactive ligand or substrate be identical to it's native, nonradioactive competitor (267). In the case of insulin, much of what is known concerning receptor binding and the metabolic fate of the hormone stems from studies using iodinated insulin. Since the iodination reaction may occur at either of four tyrosyl amino acid residues of the insulin molecule (located at positions 14 and 19 of the A-chain and at positions 16 and 26 of the B-chain), conventionally used ^{125}I insulin preparations are a mixture of monoiodoinsulin and diiodoinsulin isomers, even monoiodinated insulin is likely to be a heterogeneous mixture of monoiodospecies. However, this type of preparation has been extensively used for ligand binding studies to the insulin receptor. In addition, the conditions under which the iodination is performed have been shown to affect the relative distribution of iodine on the four tyrosine residues of insulin (270). No method specifically iodinates only one of these four residues. Thus, even monoiodinated materials prepared in different laboratories could potentially possess markedly different properties. The fact that most iodoinsulin preparations used until recently have differed with respect to purity, homogeneity, specific activity and distribution of ^{125}I makes comparison of insulin binding data between laboratories difficult (103,271). It is well known that different tyrosine residues in a protein show different degrees of reactivity in the iodination process, depending on the micro-

environment of each individual side chain of the protein (272). Also those residues on the surface of the molecules are iodinated more rapidly than inner residues and may also be important in the binding and biological activity of insulin. It has been shown that in the case of insulin one tyrosine residue, A14, is iodinated preferentially (273). Procedures employed today in most laboratories involve the use of the chemical oxidant chloramine-T or the enzymatic method using lactoperoxidase. Both methods are relatively easily carried out and produce very little diiodoinsulin. At the level of substitution routinely achieved approximately 80% of the radioactivity is associated with the A14 tyrosine. The remaining activity is accounted for chiefly by the iodination of the A19 tyrosine with very little iodination of the B16 and B26 residues (273). ^{125}I-tyrosine A14 insulin is known to retain the full binding and biological activity of native insulin (268), this residue is distant from the biologically active site of the molecule and hence the monoiodinated insulin molecule retains full biological activity, as judged by its ability to stimulate glucose oxidation in isolated fat cells (274). ^{125}I-tyrosine A19 insulin exhibits less than one half of the binding activity of native insulin in adipocytes, hepatocytes and IM-9-lymphocytes (269). The reduced affinity of ^{125}I-tyrosine A 19 may reflect changes in the binding interface of the insulin molecule, since the A19 amino acid residue is considered part of the putative binding region (275). The extent of A19 labelling is dependent on the percentage incorporation of ^{125}I into insulin during the iodination procedure. Providing the incorporation of ^{125}I into insulin from iodination to iodination is within reasonable limits (50%) the level of iodinated tyrosines other than A14 will be

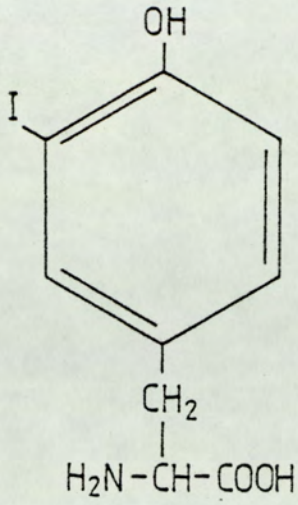
similar. The level of ^{125}I incorporation in the present study was fairly consistent, reaching a mean of 60.72 ± 1.52% (mean ± SEM, n=14). Recent studies have suggested that the B26 isomer has higher binding affinity and the B16 isomer has similar binding affinity to the A14 isomer in extrahepatic tissues (eg. adipocytes, cultured human fibroblasts, IM-9-lymphocytes and human placental membranes). However, both B26 and B16 isomers exhibit a lower binding affinity in hepatic tissues when compared with the A14 monoiodo-insulin isomer (276). To ensure the use of pure A14 labelled hormone, techniques are now available for its rapid separation from other labelled products by purification on DEAE cellulose, by HPLC (277) or polyacrylamide gel electrophoresis (278).

The chloramine-T method first described by Hunter and Greenwood (279) for the labelling of human growth hormone has been introduced in our laboratory for the routine production of ^{125}I-monoiodinated insulin of suitable purity, integrity and specific activity in sufficient quantities for use as a tracer (274). Roth and coworkers (1) formulated two generally applicable methods that reproducibly yielded monoiodo ^{125}I labelled hormones of high specific radioactivity suitable for hormone-receptor studies. One approach, applied successfully to insulin (274) and oxytocin (280) was to label a small number (10% or less) of the molecules with one iodine atom, followed by separation of the uniodinated molecules from the monoiodo-hormone. The final product ("carrier-free" monoiodohormone) have one iodine atom per molecule. The second approach (stoichiometric iodination) has been applied to insulin (281,282) and growth hormone (283), and involves the direct introduction of an average of 0.2 to 0.8 atoms of iodine, per hormone molecule. The

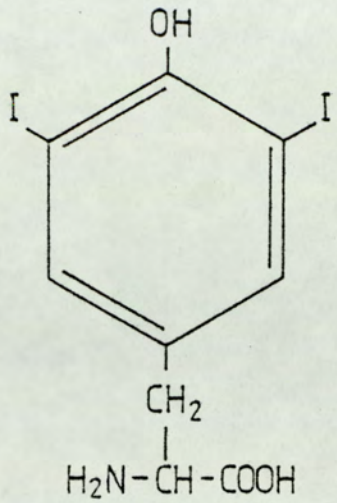
monoiodination is most suited for use with hormones of low molecular weight, the stoichiometric iodination method is most suited to hormones of higher molecular weight. The ^{125}I-insulin produced by either method is about the same quality as regards specific activity (281). In fact, "carrier-free" monoiodinsulin prepared by Sodoyez and colleagues (271) and stoichiometrically labelled insulin prepared by De Meyts (220) has been compared in an interlaboratory experiment and found indistinguishable in terms of receptor binding studies when using either lymphocytes or rat liver membranes.

The chloramine-T method for the iodination of insulin.

Chloramine-T is the sodium salt of the N-monochloro derivative of p-toluene sulphonamide. It breaks down in aqueous solution slowly forming hypochlorous acid and is consequently a mild oxidising agent. Under mildly alkaline conditions at pH 7.5 in the presence of chloramine-T, Na^{125}I is oxidised forming cationic iodine, $^{125+}$I. Tyrosine molecules at this pH will only be very slightly ionized due to the pk of the phenolic side chain of tyrosine being greater than 10. The iodination reaction probably proceeds through this small proportion of ionized groups, the iodine atoms being substituted at the ortho position to the hydroxyl group on the phenolic ring of tyrosine. The structures of mono and diiodotyrosine are shown as below:



monoiodotyrosine



diiodotyrosine

The amount of chloramine-T used in the reaction is dependent upon the amount of insulin to be iodinated and the particular batch of $Na^{125}I$. Some workers have shown that larger amounts of oxidant are required for carrier-free radioiodide than for the equivalent amount of $Na^{127}I$ (284). Greenwood and Hunter (285) have reported that the amount of chloramine-T required for optimum iodination is for all practical purposes independent of the iodine concentration. Excess chloramine-T is reduced by the addition of sodium metabisulphite and free iodine is reduced to iodide. The quantity of sodium metabisulphite added is limited to twice that of chloramine-T. De Meyts (220) has used the same ratio of chloramine-T to sodium metabisulphite, although Stentz and colleagues (277) reported that the controlled iodination of insulin with chloramine-T without the use of sodium metabisulphite provides a reliable procedure for the preparation of monoiodinated insulin. These workers also reported that the use of sodium metabisulphite resulted in variable yields of iodinated insulin,

variability in the amount of labelled tyrosine A14 insulin produced and multiple species of labelled insulin when separated by gel filtration. Albumin is also included in the reaction volume to act as a carrier for the labelled insulin. Albumin also reduces the adsorption of reaction products to the reaction vial and the Sephadex column (271). The concentration of chloramine-T is kept low to minimise damage to insulin. The reaction time is short (15-20 seconds) to minimise the exposure of insulin to chloramine-T.

Preparation of reagents for iodination.

1. Phosphate buffer (0.5 and 0.05 mol/l) (appendix 1 page 290).

2. Column eluant: consisted of 0.5% BSA and 0.1% sodium azide (to prevent bacterial contamination of the column) dissolved in 0.05 mol/l phosphate buffer and adjusted to pH 7.4.

3. Column primer: 20 ml of 2.5% BSA in 0.05 mol/l phosphate buffer, adjusted to pH 7.4.

4. Porcine insulin (0.25 mg/ml). 0.25 mg of porcine insulin was dissolved in 1 ml of 0.05 mol/l phosphate buffer, pH 7.4. 40 µl of this solution was aliquoted into each of 25 microfuge tubes (polystyrene, 500 µl capacity), frozen and stored at -15°C. A fresh tube of insulin was used for each iodination.

5. Chloramine-T and sodium metabisulphite: 25 mg of chloramine-T and 50mg of sodium metabisulphite were weighed out into separate LP3 tubes and wrapped with foil to exclude the effect of light (220).

25 mg of chloramine-T was diluted with 100 ml of 0.05 mol/l phosphate buffer, pH 7.4 and mixed well to provide a concentration of 0.25 mg/ml. 50 mg of sodium metabisulphite was diluted with 100 ml of 0.05 mol/l phosphate buffer, pH 7.4 to provide a concentration of 0.5 mg/ml.

6. Na ^{125}I (1 mci) 10 µl of Na ^{125}I was supplied by Amersham in a reactivial. The chloramine-T reaction had a pH optimum of pH 7.4 and yields were reduced below pH 6.5 and above pH 8.5 (286). The Na ^{125}I was obtained in dilute sodium hydroxide solution (pH 7-11) and therefore the pH had to be adjusted to 7.4 by the addition of 100 µl of 0.5 mol/l phosphate buffer.

Procedure for the radioiodination of insulin.

Iodinations were carried out one or two days after Na ^{125}I batch synthesis. Label older than 3-4 days tended to yield preparations containing an increased percentage of damaged insulin (>10%). Although the reason for this was not clear, it may have been due to an increase in the amount of irradiation products.

Iodination was carried out in the standard conical glass vials supplied by Amersham. All solutions were added using automatic pipettes with disposable tips and thus uncapping and capping of the reaction vessel was required several times during the iodination procedure. Each reaction was carried out in a fume cupboard behind a quarter of an inch lead shielding. The quantities of insulin and chloramine-T used in the reaction depended on the age of the Na ^{125}I. The 10 µl aliquot of Na ^{125}I employed for the iodination of insulin had a calculated activity of 1 mci on the given activity

reference date but batches were actually synthesised 15 days prior to this date. Thus the activity of the ^{125}I was in excess of 1 mci when used in the iodination procedure. Generally the Na ^{125}I preparations were used 13 or 12 days before the activity reference date. Therefore the concentration of reactants especially insulin and chloramine-T had to be adjusted in accordance with the specific activity of the $Na^{125}I$ preparation.

Protocol for the iodination of insulin using Na ^{125}I.

Reactions were generally carried out 13 days before the ^{125}I-activity reference date (day two after batch synthesis).

The following additions were made to the reaction vial containing 10 µl of $Na^{125}I$ (1.162 mci).

100 µl of 0.5 mol/l phosphate buffer,

11.62 µl of porcine insulin (2.905 µg) taken from the stock standard solution (40 µl).

and 23.24 µl of chloramine-T (5.81 µg).

The vial was inverted twice to gently mix the contents. Then after 20 seconds 20µl of sodium metabisulphite (10 µg) was added followed by two gentle inversions of the reaction vial. After a further 15 seconds 200 µl of 0.05 mol/l of phosphate buffer was added containing 2.5% BSA.

The following adjustments were made for iodinations carried out 12 days before the activity reference date:

10 µl $Na^{125}I$ (1.149 mci - approximately 1% decay from day 13),

11.49 µl porcine insulin (2.873 µg),

and 22.98 µl chloramine-T (5.746 µg).

The labelled insulin was then separated from the unreacted iodide and damaged insulin by gel filtration on Sephadex G-50 (fine). The column was preflushed with 8 ml of 0.05 mol/l phosphate buffer pH 7.4 (containing 2.5 % BSA) to purge the column of material remaining from previous iodinations and to prevent the adsorption of the freshly prepared labelled insulin to the Sephadex and the column itself. A 10 µl aliquot was removed from the reaction vial immediately after the reaction for the assessment of the percentage ^{125}I incorporation into insulin. The remaining reaction mixture was transferred to the top of the Sephadex column. The reaction vial was washed with a further 180 µl of 0.05 mol/l phosphate buffer (containing 2.5% BSA), and this too was transferred to the top of the column. The combination of reaction mixture and wash was allowed to permeate into the column and eluted at a flow rate of approximately 1 ml/minute. Sixty-four, 1 minute fractions were collected in LP3 tubes on a Serva Linear II Fraction Collector. The ^{125}I-activity in each tube was counted for 3 seconds on a gamma counter (ICN, Tracerlab, with a counting efficiency of 57%).

Four well defined peaks could be identified in the elution profile (activity versus fraction number), Figure 12. Peak I represented a high molecular weight fraction probably containing aggregates of the insulin molecule (274) and was contained within fractions 14-18. Peak II was assumed to represent monoiodinated insulin (these tubes contained the maximum activity associated with insulin) and fell typically in the region of fractions 26-31. Peak III probably represented free unreacted ^{125}I mixed with damaged ^{125}I-insulin and was located in fractions 34-38. Peak IV probably represented free unreacted ^{125}I located within fractions 39-42. Four

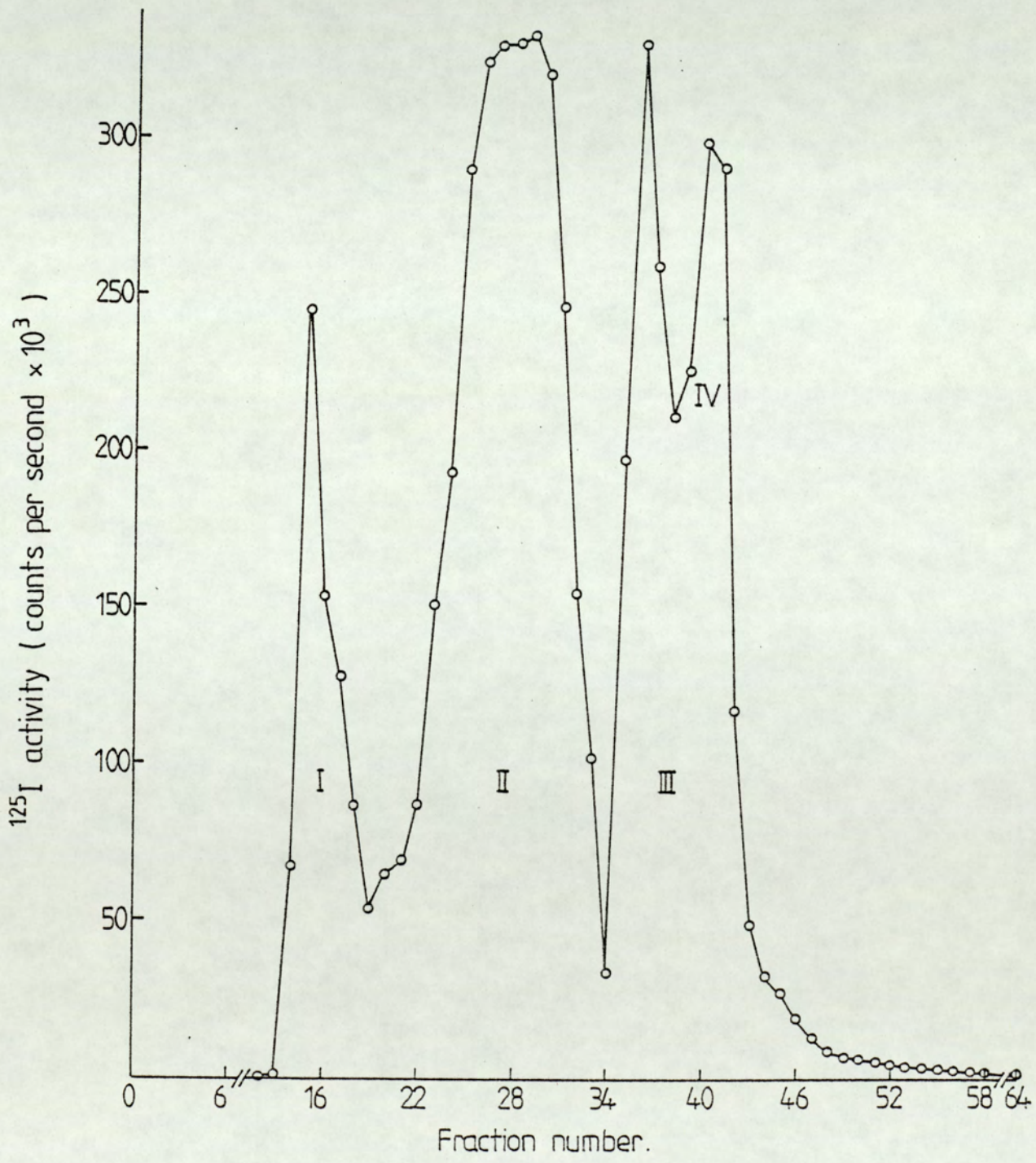
Figure 12. Typical elution profile of labelled fractions obtained by chloramine-T iodination of porcine insulin and seperation on Sephadex G-50 (fine). Flow rate = 1 ml / minute.

Peak I High molecular weight fraction containing aggregates of insulin (fractions 14 - 18).

Peak II ^{125}I-insulin (fractions 26 - 31).

Peak III Free unreacted ^{125}I and damaged ^{125}I-insulin (fractions 34 - 38).

Peak IV Free unreacted ^{125}I (fractions 39 - 42).



to five fractions representing the apex of peak II (monoiodinated insulin) were pooled in a single vial. A 10 µl aliquot was taken from this vial for assessment of the percentage of damaged insulin and 50 µl aliquots of the remainder were dispersed into LP3 tubes and stored at -20°C. A typical iodination generated about 60 tubes each containing 50 µl aliquots of ^{125}I-insulin (2 µCi/tube), with a specific activity of 242.8 ± 6.34 µCi/ug (mean ± SEM, n=14). Fractions corresponding to the other three peaks (peaks I, III and IV) were discarded after taking 10 µl aliquots from them to measure the percentage of tracer damage.

Assessment of the specific activity and integrity of ^{125}I-insulin preparations.

The 10 µl aliquot taken from the reaction vial immediately after the iodination reaction was used to assess the percentage incorporation of ^{125}I into insulin and the specific activity of the preparation. To the 10 µl aliquot was added 0.5 ml of eluant and 0.5 ml of 10% trichloracetic acid (TCA). The albumin formed a visible precipitate which coprecipitated the undamaged intact ^{125}I-insulin. After counting the radioactivity of this sample, followed by centrifugation for 10 minutes (2000 rpm, MSE MISTRAL 4L) the supernatant containing ^{125}I activity, predominantly as free ^{125}I was aspirated and the ^{125}I associated with the precipitate determined from counting the radioactivity of the precipitate. Thus the percentage incorporation of ^{125}I into intact, undamaged insulin was calculated as follows:

$$= \frac{\text{Radioactivity count of the precipitate after centrifugation}}{\text{Radioactivity count of total mixture before centrifugation}} \times 100$$

Since the total amount of ^{125}I and insulin present in the reaction mixture and the percentage radioactivity incorporated into insulin was known, the specific activity could be calculated from the following formula:

$$\text{Specific activity } (\mu Ci/\mu g) = \frac{\text{Percentage incorporation of } ^{125}I\text{-insulin X amount of } ^{125}I \text{ present in the reaction mixture } (\mu Ci)}{\text{amount of insulin present in the reaction mixture } (\mu g)}$$

In the present study the average percentage incorporation of ^{125}I into insulin was found to be 60.72 ± 1.52% (mean ± SEM, n=14), Table 2. These data corresponded to an incorporation level of 0.5 - 0.6 molecules of iodine per molecule of insulin and suggested the production of monoiodinated insulin. Specific activities of over 350 µCi/µg indicated di-or tri-iodination (287). The presence of only one iodine atom per insulin molecule ensured a minimal effect on insulin activity (288). It is generally accepted that the biological activity of iodinated insulin preparations decreases as the average level of iodine incorporation increases above approximately 1 mole of iodine per 1 mole of insulin monomer (289,290). A high fraction of the labelled molecules may show either a reduced affinity for cell receptors (291) or an increased affinity for non specific sites.

Assessment of iodination damage to ^{125}I-insulin.

In preliminary studies the ^{125}I-insulin was assessed for iodination damage to the insulin molecule using trichloracetic acid precipitability (TCA) and adsorption to dextran coated charcoal. TCA precipitability was determined by the addition of 0.5 ml of eluant and 0.5 ml of 10% TCA to a 10 µl aliquot taken from the pooled peak II

Table 2. Percentage incorporation of ^{125}I-insulin, percentage of undamaged insulin and the specific activity of labelled insulin.

Iodination characteristics	Number of iodinations (n)	Mean ± SEM	Coefficient of variation (cv) %
Percentage incorporation of ^{125}I-insulin	14	60.72 ± 1.52	9.37
Percentage of undamaged insulin	14	95.81 ± 0.62	2.44
Specific activity (µCi/ µg)	14	242.8 ± 6.34	9.77

tubes containing ^{125}I-insulin. After centrifugation (10 minutes, 2000rpm), the supernatant was aspirated and the activity of the precipitate counted. Charcoal adsorption was assessed in the same way by the addition of 25 µl of the pooled ^{125}I-insulin preparation to 400 µl of 0.5 mol/l phosphate buffer, pH 7.4, containing 10 mg of dextran coated charcoal (293). After mixing and centrifugation (10 minutes, 2000 rpm), the supernatant was aspirated and the precipitate ^{125}I-activity determined.

Labelled insulin was used in receptor assays when TCA precipitability was greater than 95% and charcoal binding was greater than 90% (293). Samples pooled from peak II satisfied these criteria. Subsequently, TCA precipitability was routinely used for the assessment of damage and was usually 95% (average 95.8 ± 0.62%, mean ± SEM, n=14) but never below 92% (Table 2) and in good agreement with values obtained by other investigators (274,294).

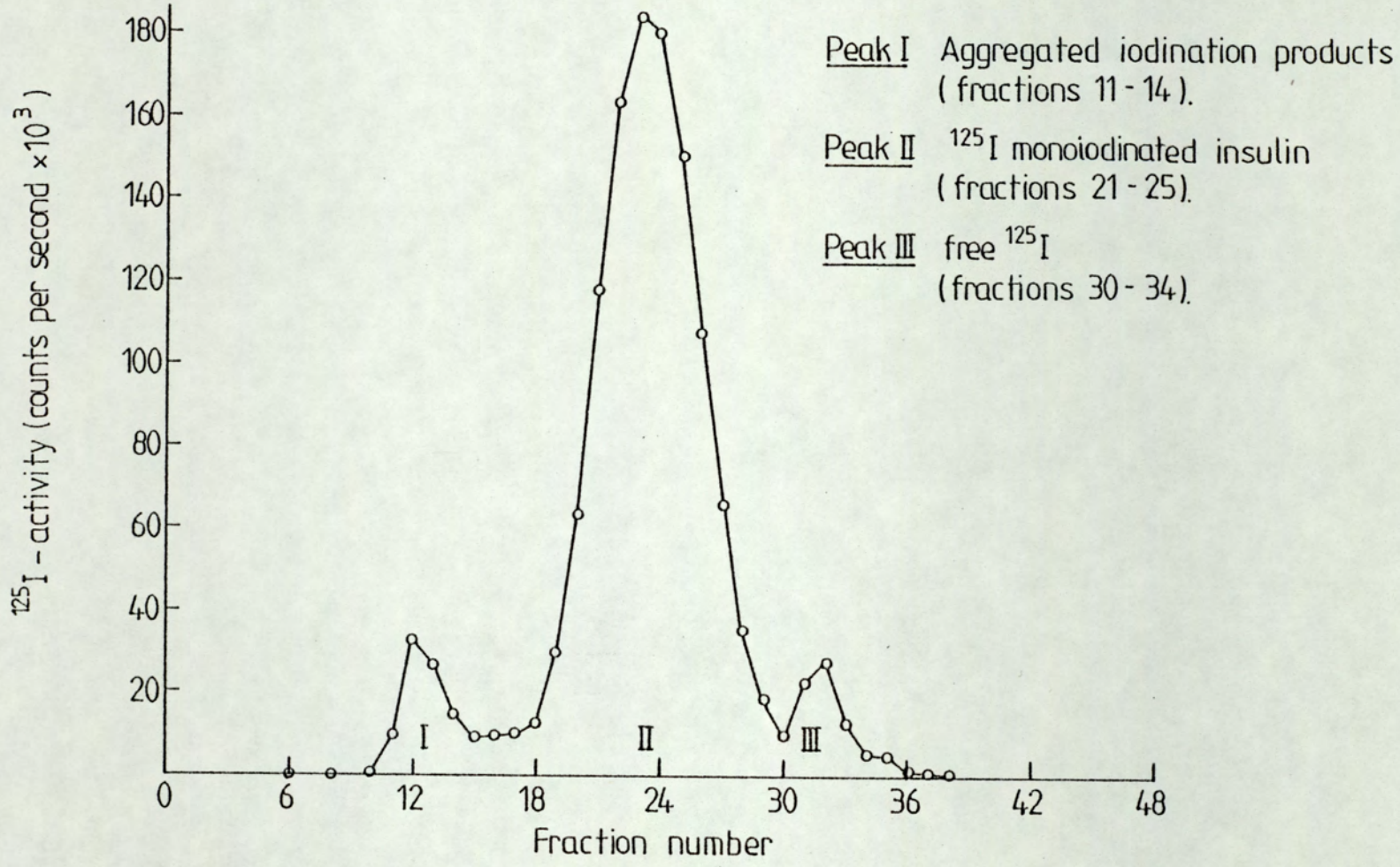
As mentioned earlier a cause of damage to the insulin molecule, that could easily be avoided, was the age of the Na^{125}I supplied by Amersham. If the iodide was 3 days old or less, then TCA precipitability was routinely greater than 95%. However, if the tracer was 1 week old, TCA precipitability was reduced to approximately 92% and after 2 weeks to about 87%. The reason for this deterioration is not understood, but it may be that the concentration of irradiation products is higher in the older iodide, causing damage to the tracer produced. Freezing the ^{125}I-insulin had no apparent effect on TCA precipitability, but once thawed was never refrozen for further use. Iodination damage of stored ^{125}I-insulin, as assessed by TCA precipitability generallly increased by about 3% (percentage of undamaged decreased from 95.8 ± 0.62 to 92.7 ± 0.37

mean ± SEM, n=14) after two weeks, possibly due to self irradiation (295). The specific activity of the ^{125}I-insulin on the day of use was calculated from the original specific activity of the ^{125}I-insulin assuming a decrease of 1% per day. Aliquots of the ^{125}I-insulin preparation were used for both insulin radioimmunoassay and insulin receptor binding studies on isolated human erythrocytes and isolated adipocytes from mice and rats.

Repurification of ^{125}I-insulin.

Simple purification of the ^{125}I-insulin was carried out 2 weeks after synthesis when the iodination damage of stored ^{125}I-insulin had generally increased by about 3%. Repurification was carried out to retain the activity of the ^{125}I-insulin for an additional two weeks by decreasing the percentage of damaged ^{125}I-insulin. Purification was carried out by gel filtration on Sephadex G-50 (fine). The interior of the column was pretreated with 15 ml of 0.05 mol/l phosphate buffer, pH 7.4, containing 2.5% BSA to remove contaminated material remaining from previous iodinations and to prevent the adsorption of ^{125}I-insulin to the Sephadex beads and the column itself. 1-2 ml of the original ^{125}I-insulin was transferred gently to the column and eluted in 64 1 ml fractions. The counting of repurified ^{125}I-insulin activity was carried out as described previously (page 73). Three peaks were obtained in the elution profile (activity versus fraction number), Figure 13. Tubes containing the maximum radioactivity associated with repurified monoiodoinsulin could be identified with fractions 21-25. The peak representing aggregated iodination products of high molecular weight

Figure 13. Typical elution profile of labelled insulin fractions after repurification on Sephadex G-50 (fine). Flow rate = 1 ml/minute.



were represented by fractions 11-14 and the peak probably representing the free, separated ^{125}I appeared in fractions 30-34, Figure 13. Four fractions from peak II were pooled and after thorough gentle mixing distributed into a number of aliquots (each aliquot [100-200 µl] typically contained 2 µCi/tube) and stored at -20°C until required. 10 µl aliquots were taken from the original pooled ^{125}I-insulin fraction (before adding to the column) and from each of the three peaks of the repurification for assessment of the amount of damaged ^{125}I-insulin, Table 3.

Determination of the specific activity and iodination damage of repurified ^{125}I-insulin.

The specific activity of the purified preparation was calculated from the original specific activity of ^{125}I-insulin assuming a 1% reduction in the amount of specific activity per day for 2 weeks.

The repurified ^{125}I-insulin was assessed for iodination damage to the insulin molecule using TCA precipitability as described previously (page 76). Iodination damage to ^{125}I tended to increase in the labelled insulin with time during storage and was probably the result of self irradiation damage (295). The concentration of immuno-chemically active insulin in the mono-^{125}I-tyrosine A14 insulin preparation decreased as a function of time because the disintegration of the ^{125}I nucleus was assumed to cause an immuno-chemical inactivation of the parent insulin molecule. Linde and colleagues (296) have shown that older ^{125}I-insulin preparations may contain relatively more unlabelled immunoreactive insulin than new ones. This may cause an apparent decrease in the binding of labelled

Table 3. The percentage of undamaged insulin in the the different peaks of the original iodination and the repurification profiles (data from Figures 12 and 13).

	n	Percentage of undamaged insulin mean ± SEM	Coefficient of variation (cv) %
Original iodination:			
Peak I	7	68.7 ± 1.81	6.99
Peak II	14	95.81 ± 0.62	2.44
Peak III	7	16.6 ± 2.62	41.6
Peak IV	7	16.8 ± 2.89	45.51
Repurification of ^{125}I-insulin:			
Peak I	5	84.26 ± 3.95	10.47
Peak II	5	98.66 ± 1.34	0.61
Peak III	5	79.8 ± 1.5	4.19

insulin to the receptor or antibody. In those studies ^{125}I-insulin prepared by the chloramine-T method showed 15% tracer damage after 2 months. In the present work chloramine-T iodination resulted in about 4.2% damaged insulin which increased by approximately 3% over two weeks storage. The percentage of undamaged insulin increased from 92.7 ± 0.37 (mean ± SEM, n=14) to 98.66 ± 1.34 (mean ± SEM, n=5) after repurification. The repurification of ^{125}I-insulin was both effective and economical and provided sufficient ^{125}I-insulin for both radioimmunoassay and radioreceptor assays over a period of 4 weeks.

Insulin receptor binding studies.

The preparation of cells and tissues.

A) The preparation of erythrocytes.

Gavin and colleagues (294) were the first to demonstrate that erythrocytes have specific insulin binding sites. These cells bound ^{125}I-insulin, and the bound hormone was displaced by nanogram quantities of unlabelled insulin. Gambhir, Archer and Carter (210) reported that freshly isolated circulating human erythrocytes had highly specific insulin receptors and possessed properties that made them ideal cells for clinical hormonal radioreceptor studies. The membrane characteristic of these cells were similar to those of other circulating human cells (297) and there was substantial evidence to suggest that the insulin receptor status of these cells might reflect the receptor activity of the major target tissues for insulin i.e. fat, muscle and liver (30,211). Although glucose metabolism in the

erythrocyte is not thought to be dependent upon insulin (257), erythrocytes are readily available in large quantities. A few milliliters (15 ml) are sufficient to carry out a complete radioreceptor assay and they are obtained as a relatively homogenous cell suspension without extensive purification (121). It has been shown that blood samples may be stored at 4°C for up to 36 hours without affecting the insulin binding to erythrocytes (243). In the present study the erythrocyte was chosen as the receptor model for clinical studies involving normal and diabetic patients with and without hypertension where additional blood was required for glucose tolerance testing and determination of biochemical and haematological profiles.

The method used for the isolation of erythrocytes was essentially that described by Gambhir and colleagues (121). 15 ml of blood was obtained by venipuncture of the median basilic vein and transferred to a heparinised blood tube (orange top). 7 ml of blood was generally transferred into each of two 15 ml plastic centrifuge tubes. Following centrifugation (10 minutes, 400xg, 20°C), the resulting plasma layers were aspirated. Both red blood cell pellets were gently mixed with one part of physiological saline (0.9 g/100 ml) and layered onto 3 ml of Ficoll-Hypaque mixture. (Ficoll 9% and Hypaque 33.9%, 2.4:1 volume/volume) in two 15 ml plastic tubes as descrtibed by Boyum (298). After 20 minutes centrifugation (400xg) at 20°C the erythrocytes were separated from the other cell components on a density basis and the saline, mononuclear cells, Ficoll-Hypaque, granulocyte phases and the upper layer of the erythrocyte phase aspirated off. Each of the resulting erythrocyte pellets were resuspended in one equal volume of saline and the above

procedure repeated. Each resulting erythrocyte pellet was then resuspended in two volumes of buffer G supplemented with 1% BSA and 0.08% bacitracin (Appendix 2 page 290) to equilibrate the cells. The contents of each tube was centrifuged for 10 minutes (400xg) at 20°C. The supernatant buffer was aspirated and the cell pellets in the two tubes combined and resuspended in an appropriate volume of buffer G resulting in a suspension containing $4.4x10^9$ cells per millilitre. The total erythrocyte concentration was determined using a coulter counter [Coulter Electronic Ltd./ Model ZBI (100 µm aperture)]. The counting procedure was repeated four times and the background counts for saline alone subtracted. More than 95% of the erythrocytes were viable, as determined by trypan blue exclusion (299).

Erythrocyte insulin binding decreases exponentially with erythrocyte age due to a decline in the concentration of receptor sites (213, 215) and this factor should be taken into account when using erythrocytes for insulin receptor binding studies. In addition erythrocyte samples contain small numbers of reticulocytes and granulocytes which have a higher binding capacity than erythrocytes (121,300). It was assumed that the small concentration of these cells present would not contribute significantly to the total insulin binding of the erythrocyte preparation (117, 213). On this basis studies involving physical separation of reticulocytes from the erythrocyte sample and binding studies on erythrocytes of different ages were not attempted.

B) The preparation of adipocytes

In addition to erythrocytes it was considered important to study insulin receptor binding in a tissue which is known to be an important site of insulin action (219). The adipocyte is such a model and over the last decade adipocytes have been used to measure both insulin binding and insulin action in man (130, 301) and animals (302, 303). In the present studies it did not prove possible to obtain adipocytes from human volunteers. Local clinicians considered the biopsy of adipose tissue as described by Pedersen (257) to be too invasive. Fortunately, animal studies have indicated a general agreement between the regulation of insulin receptors in adipocytes (205, 206) from man and animals. In the present studies insulin binding was carried out using rat epididymal fat pads and both subcutaneous and abdominal adipocytes and fat segments from lean and obese mice.

The procedures adopted for the isolation of adipocytes were essentially those described by Rodbell (303) and Pedersen and coworkers (130). These techniques have been shown to generate a high yield of cells with greater than 97% viability (130). Only plastic or siliconised glassware was used for the preparation and incubation of the isolated fat cells.

i - Procedure for the isolation of rat adipocytes (only used for preliminary studies and are described here for reference purposes)

Anesthetized rats (diethyl ether) were stunned by a blow on the head and decapitated. The epididymal fat pads were quickly removed and rinsed in 0.85% warm saline (37°C). Thin distal portions from each pad were cut into small fragments weighing 20-30 mg and, blood vessels, connective tissue and clotted blood removed. Fragments of adipose tissue (1g) were inserted into a siliconised 25 ml conical flask containing 3 ml of Krebs bicarbonate buffer, pH 7.4 (Appendix 3, page 291). Collagenase (3 mg/ml) and bovine serum ablumin, fraction V (40 mg/ml). Each flask was capped with nescofilm (Osaka, Japan), placed in a shaking water bath (100 cycles/minute) and gassed continuously (C02-02, 5 : 95, v/v) for 1 hour at 37°C. After this time the tissue was dispersed into small fragments and fat cells were liberated from the tissue fragments by gentle teasing with a glass rod. The separation of the adipocytes was associated with an increased turbidity of the incubation medium. Separated adipocytes were then filtered through a 250 µm nylon mesh to remove connective tissue and centrifuged in a sterile polystyrene tube (15 ml, Corning TM, C25310) for 1 minute at 200 rpm. Adipocytes floated to the surface of the centrifuge tube while the stromal vascular cells sedimented and were removed by aspiration. The separated adipocytes were washed at room temperature in 10 ml of Tris buffer, pH 7.45 (Appendix 3, page 291) and centrifuged for 1 minute at 200 rpm. The washing procedure was repeated four times and after the final rinse, the adipocytes were resuspended in an appropriate volume of Tris

buffer which resulted in a suspension containing 4-5 x 10^5 cells per millilitre. These cells were retained at room temperature prior to use in experimental work.

Estimation of adipocyte diameter and concentration

1 ml of the isolated adipocyte suspension was transferred to a 30 ml plastic scintillation vial containing 1 drop of 1% methylene blue. After staining for 10 minutes, successive 5 µl aliquots of the stirred suspension were transferred with the aid of a Gilson pipette to the cavity of an inverted siliconised microscope slide. The cells in the hanging drop located themselves in the cavity and were examined under the microscope (Vickers Instruments, England). At a substage magnification of 100 x, using an eye piece micrometer, the calibration slide was adjusted so that the unit marks had a constant interval of 12 µm. The free adipocytes floating on the surface of the medium, were identified by their spherical shape, stained nuclei and cytoplasm. These details readily distinguished adipocytes from larger lipid droplets. The adipocyte suspension was diluted to give a concentration of about 100 cells per 5 µl aliquot. Adipocytes from the same population were brought into the caliper field with systematic movement of the microscopic stage control knobs. The cells were aligned on the caliper scale, the equatorial plane of each cell was brought into focus, and the fat cell diameters between 12-144 µm were recorded in classes with mid-points of successive 12 µm multiples to provide a frequency distribution of diameter in 12 categories of size. The sizing and counting of 100-150 fat cells was accomplished in about 15 minutes. The surface area (πD^2) and

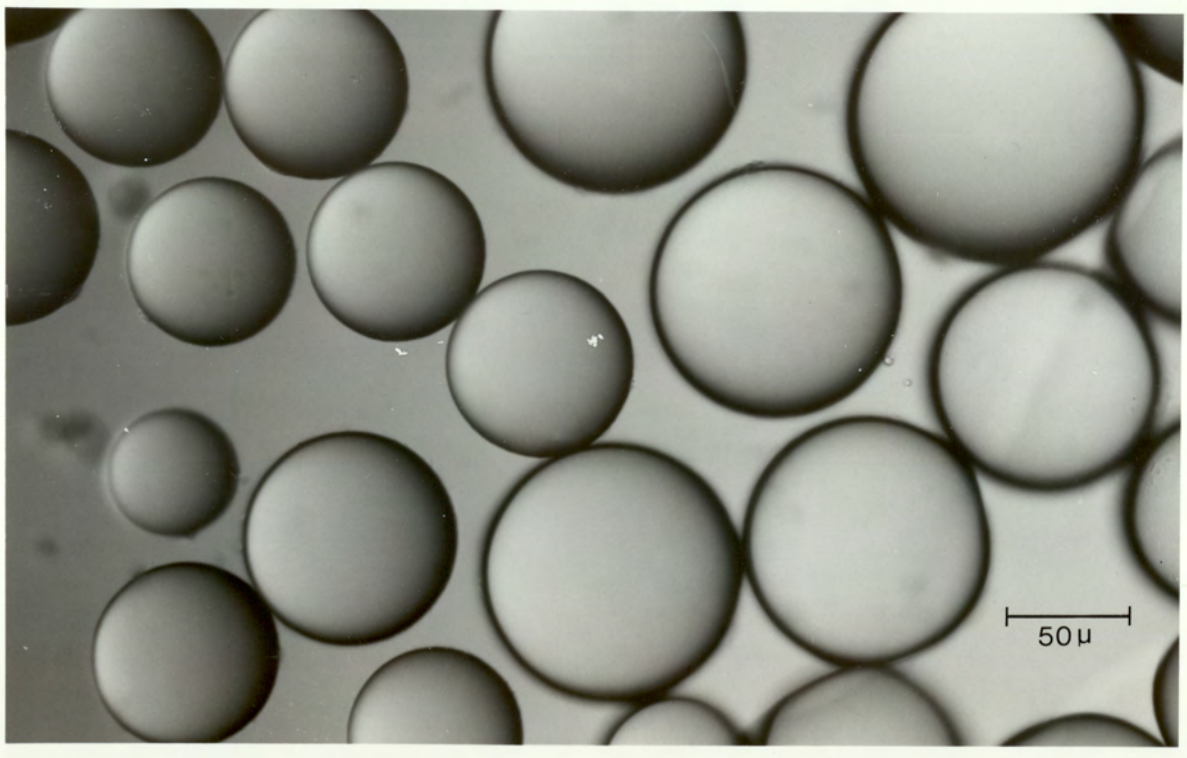
volume ($\pi D^3/6$) were calculated for every measured cell diameter and all cells were assumed to be spherical. The precision of the counting and sizing procedure was evaluated by repeating the measurement of adipocyte number and diameter in four replicate samples from the same cell batch. The adipocyte suspensions were adjusted to a lipocrit of 5%. Since the mean diameter of the adipocytes was found to be about 60 µm a cell suspension giving a lipocrit of 5 to 7% corresponded to approximately 5×10^5 cells/ml (304).

ii - Procedure for the isolation of adipocytes from lean and obese mice.

Mice were killed by a blow on the head and subsequent cervical dislocation. The skin and hair on the femoral and abdominal regions was dampened with water to facilitate easy cutting and folding back. Subcutaneous adipose tissue was excised from the upper lateral part of the femoral region. Using this procedure about 10 g of subcutaneous adipose tissue could be obtained from each femoral region, or about 20 g per obese mouse. The abdominal wall was then opened to expose the two lower abdominal fat pads and a 10 g portion of adipose tissue was removed from each fat pad. In lean mice fat was only available from the lower abdominal adipose tissue depot. Adipocytes were isolated according to the method of Pedersen and colleagues (130). Adipose tissue samples irrespective of origin were placed in round petridishes (Sterilin 7 cm diameter, Teddington, Middlesex TW 118 QZ) containing warm glucose/saline (37°C). Each piece of adipose tissue was chopped into fragments weighing 20-30 mg and all blood vessels, connective tissue and clotted blood was

removed. The chopped fragments were washed three times in warm glucose/saline (37°C) in a tea strainer. 1 g of adipose fragments was transferred to a siliconised 25 ml conical flask containing 2 ml of Hepes buffer (Appendix 4, page 292) supplemented with BSA (25 mg/ml), glucose (5 mmol/l) and collagenase (0.5 mg/ml). Each flask was shaken in a water bath (100 cycles/minutes) for 60 minutes at 37°C. Collagenase digestion resulted in the liberation of adipocytes from the adipose fragments into the incubation medium. Adipocytes were also teased free with a plastic paddle and the remaining stroma removed by passing the digest through a tea strainer. Residual adipocytes were collected in a high sided plastic dish. The total cell suspension was filtered through two layers of nylon gauze, one layer of nylon mesh (tights) and finally collected in a sterile polystyrene centrifuge tube (A Corning TM 15 ml, C25310). After a short centrifugation (10 seconds at 1000 rpm) the adipocytes were allowed to float to the surface. Fat droplets tended to float more rapidly to the surface than adipocytes and were aspirated. The infranatant was removed by suction and the adipocytes washed by resuspension in an equal volume of Hepes supplemented with BSA (50 mg/ml), mixed gently and centrifuged at low speed (200 rpm) for 1 mimutes. The adipocyte preparation was washed four times at room temperature using this procedure and after the final rinse resuspended in the Hepes incubation buffer containing 50 mg/ml BSA and 5 mmol/l glucose, Plate 1. The final suspension contained 4-5 x 10^5 adipocytes/ml. Adipocyte number and diameter was estimated using two methods. The first method involved the use of an inverted drop in a cavity slide as described previously for rat adipocytes (page 88). The second method involved the construction of a

Plate 1. Typical isolated adipocytes from obese mice (320 × , magnification) .



rectangular frame (1 x 1 cm) of adhesive tape (about 0.2 mm in thickness), glued to a siliconised microscopic slide forming a "trough" for the reception of the cell suspension (305). With the tape frame facing downwards, a 5 µl aliquot of cell suspension containing 100-150 cells was applied from underneath into the middle of the trough, using an automatic pipette (Gilson) with it's tip held vertically. Application in this way prevented cell loss due to floatation within the pipette tip. A blue cell finder culture slide (Model Leiden 74 Microlab, Holland) was positioned on top of the tape trough and the cell number and diameter measured at 100 fold magnification using an eye piece micrometer. The lipocrit was adjusted to 5%.

C). The preparation of fat segments

Segments of fat (5 g) were taken from the femoral subcutaneous and lower abdominal regions of lean and obese mice and maintained in either warm saline (37°C) containing glucose (5 mmol/l) for studies on insulin degradation (page 112) or Krebs bicarbonate buffer, pH 7.4 (Appendix 3 page 291) for studies on the rate of glucose oxidation (page 250).

The insulin receptor binding assay

Binding assays were carried out by incubating cells for an optimum, predetermined length of time with a fixed amount of radioactivity labelled insulin in the presence of increasing concentrations of unlabelled insulin. Labelled and unlabelled insulin then compete for binding to the insulin receptor and the

binding of the tracer is progressively inhibited as the concentration of unlabelled insulin increases, Figure 7. When ^{125}I-insulin binds to cells two processes occur: a receptor binding and non-specific binding. The receptor binding is specific for insulin has a high affinity, is pH and temperature dependent, is reversible and has a rapid time course. Non-specific binding is thought to represent the adsorption of insulin to the cell membrane or the cellular uptake of whole or degraded molecules of labelled insulin (257). Non-specific binding of the labelled insulin must be taken into account and subtracted from values obtained for total binding to provide the level of specific binding to the insulin receptor. The incubation is allowed to reach a steady state, that is the rates of insulin-receptor association and dissociation are equivalent. At steady state the bound labelled insulin is separated from unbound (free) labelled insulin, generally by centrifuging the cell suspension through ice cold buffer or inert oil, depending on the cell type used. The optimum conditions for steady state binding vary between cell types. Several physical and chemical factors are important for the regulation of insulin binding *in vitro* e.g. time and temperature, Fig. 14, and pH, Figure 15 (220). The optimal binding of insulin to its receptor exhibits a sharp pH optimum of between 7.4 and 8.2 for most cells and tissues (129,130). Pedersen and colleagues (130) have shown a sharp pH optimum of 7.4 for human adipocytes. Insulin binding studies are generally carried out at subphysiological temperatures (10-20°C). Since there is an inverse correlation between insulin binding and temperature (131) which may be due to an enhanced rate of insulin degradation *in vitro* at higher temperatures (133) and/or an increased rate of hormone receptor internalisation

Figure 14. Effects of time and temperature on the binding of ^{125}I-insulin to human erythrocytes (121).

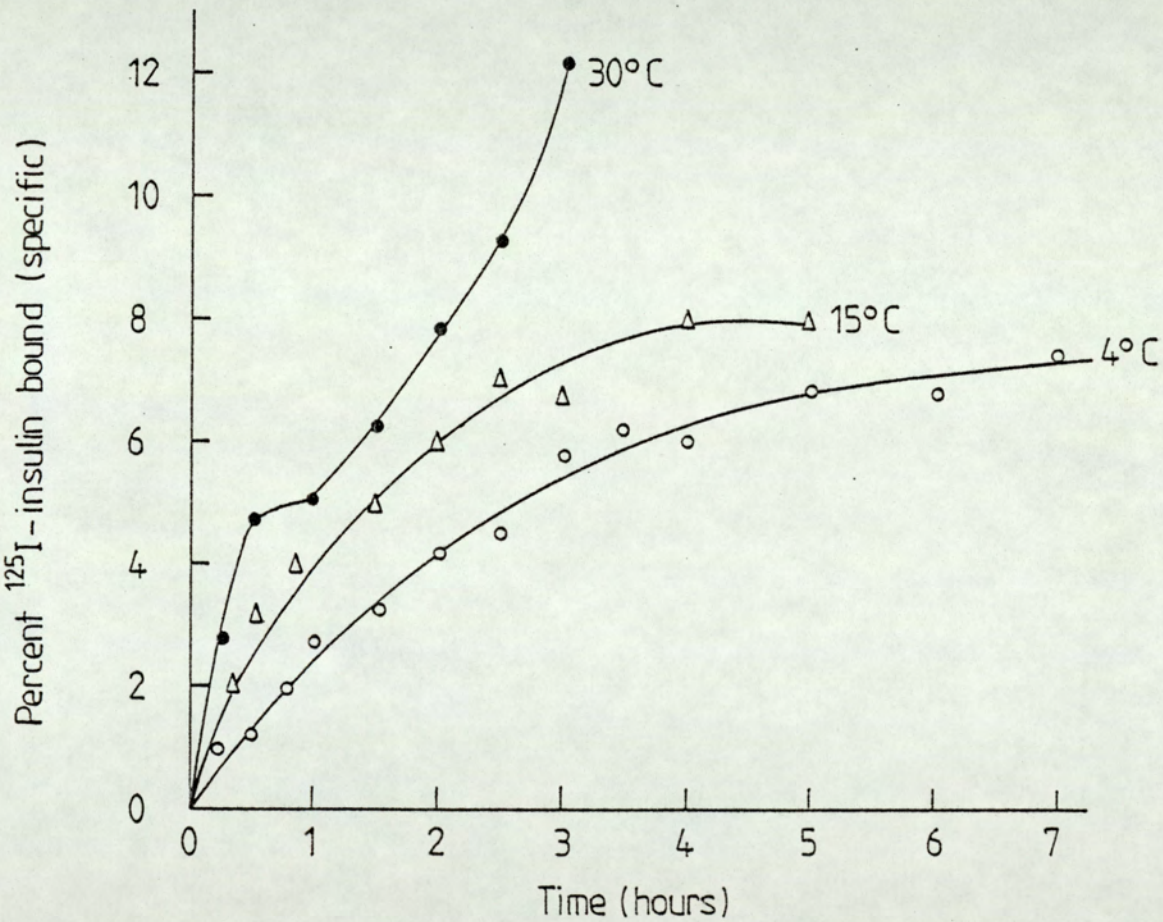
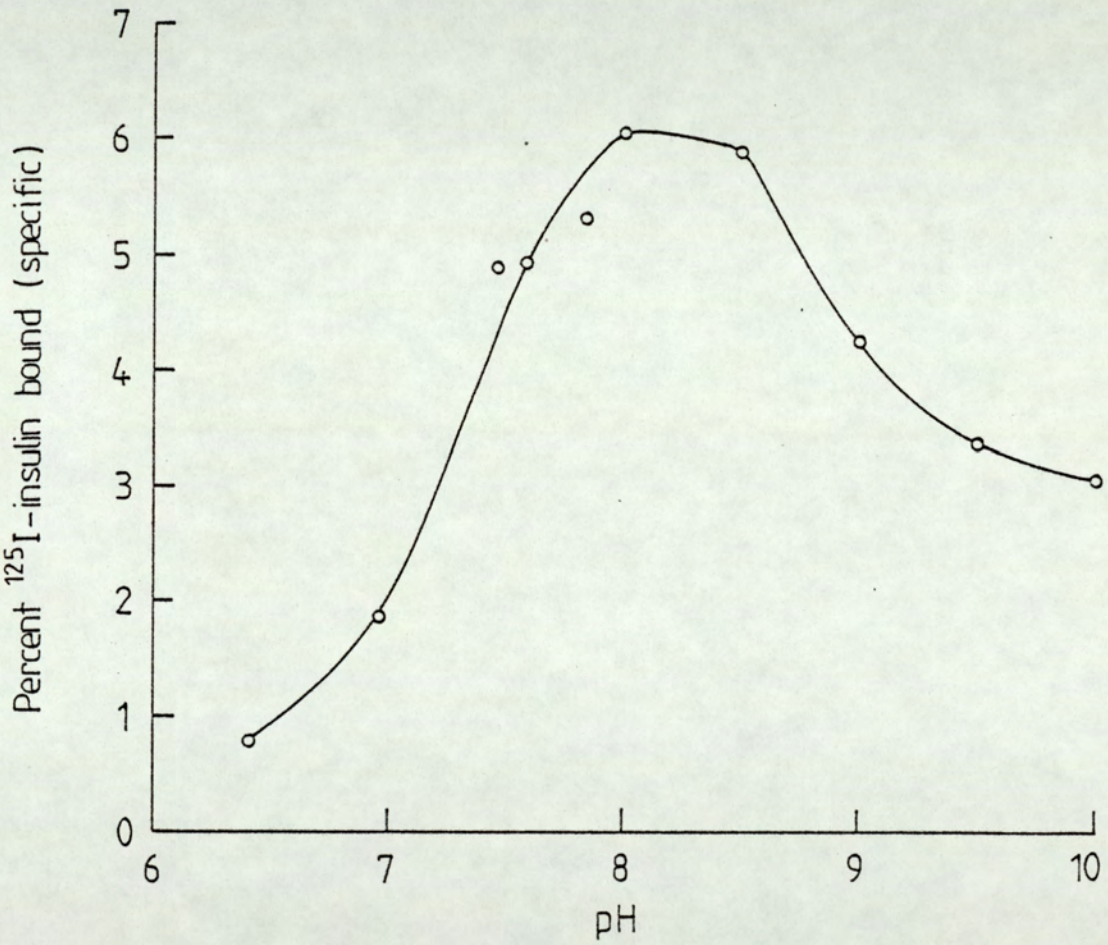


Figure 15. The effect of pH on ^{125}I-insulin binding to human erythrocytes, incubated for 2·5 hours at 15°C (121).



(134). The albumin concentration of the incubation medium may also influence insulin binding by affecting the rate of insulin degradation (135). Other factors affecting the binding of insulin to its receptor on a variety of tissues and species have been detailed by Ginsberg (306). The physical criteria for the incubation of cells must be constant between assays and also must be optimal for the cell or tissue used in order to obtain a reproducible radioreceptor assay. In studies using mice attempts have been made to examine insulin receptor status under normal physiological conditions i.e. free fed conditions. However, doing this presents certain problems because mice allowed food ad libitum will show greater variability in their insulin levels than fasted mice. As the level of circulating insulin affects receptor activity (307), any variation in plasma insulin concentration will be reflected in an alteration in the level of insulin binding. In order to minimize this variability receptor binding assays were carried out at the same time each day (10.00h) and circulating plasma insulin and glucose levels were monitored routinely. Essential details of the insulin binding assays used in the present work have been summarised in Table 4.

The erythrocyte insulin receptor binding assay.

The preparation of insulin standards :

A stock solution of porcine insulin was prepared contaning 0.01 mg of insulin/ml in buffer G. This solution was aliquoted and stored at -20°C. Serial dilution of this stock provided a range of insulin

Table 4. Incubation conditions used in insulin receptor binding assays with human erythrocytes and mouse and rat adipocytes.

Cell type	Source	Chemicals necessary for isolation	Incubation period (minutes)	Incubation pH	Incubation temperature (°C)	Buffer for incubation	Medium for separation of bound and free insulin
Human erythrocytes	Blood sample: (15 ml)	Ficoll-Hypaque	180	8.0	15	Buffer G (Appenix 2 page 290) + 1% BSA + 0.08% bacitracin	Cold buffer G + Cold Di-n-butyl phthalate
Mouse adipocytes	Subcutaneous or abdominal fat	Hepes buffer containing collagenase (0.5mg/ml)	45	7.4	37	Hepes buffer (Appendix 4 page 292) + 5% BSA + 5 mmol/l glucose	Chilled 0.154 mmol/l saline + Chilled silicon oil (density 0.99 g/ml)
Rat adipocytes	Epididymal fat pads	Krebs bicarbonate buffer containing collagenase 3mg/ml + 4% BSA	60	7.45	24 or 37	Tris buffer (Appendix 3 page 291) + 1% or 5% BSA + 0.08% bacitracin	Dinonyl phthalate

standard concentrations (0 - 10^4 ng/ml) for use in the binding assay. Freshly prepared standards were used twice and then discarded. A stock solution of insulin (1 mg/ml) in buffer G was used for the estimation of non-specific insulin binding (NS). 50 µl aliquots of this stock solution were dispensed into LP3 tubes and stored at -20°C. Two of these NS tubes were used in each assay.

The erythrocyte binding reaction :

The binding of ^{125}I insulin to prepared erythrocytes was carried out by the incubation of 400µl of erythrocyte suspension ($\simeq 1.76 \times 10^9$ cells in buffer G) with 50 µl of ^{125}I-insulin (0.04 ng or 10,000 cpm per tube, specific activity 242.8 $\pm$ 6.34 µCi/µg and 50 µl of either buffer G or unlabelled insulin standard in a final volume of 500 µl. Fourteen different concentrations of insulin were used, ranging from 0-10^5 ng/ml and 10^5 ng/ml represented the nonspecific binding. The reaction protocol is summarised in Appendix 5, page 293. The reactants were incubated for 3 hours at 15°C in duplicate and each tube was shaken gently every 30 minutes. At the end of the incubation period all tubes were placed in an ice bath to stop the reaction. A 100 µl aliquot of reaction mixture was transferred from each tube to a 400 µl plastic microfuge tube (ETH 26, Beckman Ltd., High Wycombe, Bucks.) containing 100 µl of buffer G supplemented with 1% BSA, 0.08% bacitracin and 100 µl of prechilled di-n-butyl phthalate. Each tube was capped and centrifuged for 3 minutes in a Beckman microfuge (9,000 g). The eythrocyte pellet was retained in the bottom of the tube while the unbound tracer remained in the buffer phase, the two being separated by the di-n-butyl phthalate

layer. The tip of each microfuge tube containing the erythrocyte pellet was removed with a heated scalpel, transferred to an LP3 tube and subsequently counted for five minutes in a gamma counter (Tracerlab Service Ltd., with 57% efficiency). A 100 µl aliquot of reaction mixture from any reaction tube in (duplicate) was taken for total radioactivity. Counts were adjusted for background activity. The radioactivity bound to the erythrocyte was determined using the following formula :

$$\text{Percent radioactivity bound} = \frac{\text{Radioactivity of erythrocyte pellet}}{\text{Total radioactivity (in 100µl of the incubated erythrocyte suspension)}} \times 100$$

The percentage of specific insulin bound at each concentration of unlabelled insulin was determined by subtracting the percentage of ^{125}I-insulin bound in the presence of 10^5 ng/ml of unlabelled insulin (NS) from the total percentage ^{125}I-insulin bound at each concentration of unlabelled insulin (219,302) (see Appendix 6, page 294).

<u>The assay of insulin binding to isolated rat adipocytes (only carried out for reference purposes).</u>

Serial dilution of stock porcine insulin(0.01 mg/ml) was carried out using Tris buffer, pH 7.45 to generate a range of insulin standards 0-10^4 ng/ml. A concentrated stock solution of 1 mg/ml insulin was used to genereate tubes for non-specific binding (10^5 ng/ml). The concentrated adipocyte suspension (prepared as described previously, page 87) was diluted with Tris buffer, pH 7.45 containing either 1 or 5% BSA and supplemented with 0.08% bacitracin. A 200 µl aliquot of stirred adipocyte suspension (4-5 x 10^5 cells/ml) was incubated with 25 µl of Tris buffer, pH 7.45 containing either 0.1 ng

(25,000 cpm) or 0.28 ng (70,000) of ^{125}I-insulin (specific activity 242.8 ± 6.34 µCi/µg) together with either 25 µl of Tris buffer or 25 µl of one of a range of unlabelled insulin concentrations (0-1 x 10^5 ng/ml) in a final volume of 250 µl. The reaction protocol has been summarised in Appendix 7 (page 297). Incubations were performed at both 24°C and 37°C over periods of between 15-120 minutes in 400 µl microfuge tubes in a shaking water bath (100 cycles/minute). Individual tubes were shaken manually every 15 minutes. The binding reaction was terminated after 15-120 minutes by the addition of 100 µl of dinonyl phthalate (310) and the tubes immediately centrifuged for 3 minutes. Three layers resulted adipocytes at the top, oil in the middle and buffer at the bottom. The tubes were cut horizontally with a cold sharp scalpel through the oil layer and both portions were transferred to LP3 tubes for counting. Assessment was made of both cell bound and free radioactivity. Duplicate 25 µl aliquots of the original labelled insulin was counted for total radioactivity. Radioactivity bound to adipocytes was determined from the following expression:

$$\text{The percent radioactivity bound} = \frac{\text{Radioactivity of adipocyte layer}}{\text{Total radioactivity (25 µl of the original labelled insulin)}} \times 100$$

The percentage of specifically bound insulin at each concentration of unlabelled insulin was determined by substracting the percentage ^{125}I-insulin bound in the presence of 10^5 ng/ml of unlabelled insulin (NS) from the total percentage ^{125}I-insulin bound at each concentration of unlabelled insulin (219,302). All binding studies were carried out in duplicate.

The incubation conditions for rat adipocyte insulin receptor binding are summarised in Table 4. This assay was used to establish

the adipocyte insulin receptor binding technique and was subsequently applied to studies involving obese mouse adipocytes.

The assay of insulin binding to isolated mouse adipocytes.

Adipocyte preparations were obtained from lean and obese mice as described previously (page 89). The concentrated mouse adipocyte suspension was diluted with Hepes buffer, pH 7.4 containing BSA (50 mg/ml) and glucose (5 mmol/l) to give a final concentration of 4-5 x 10^5 cells/ml. The adipocyte suspension was mixed gently and a 400 µl aliquote of cells taken from just under the surface. These cells were incubated in 15 ml polyethylene tubes with 50 µl of ^{125}I-insulin (containing a 0.08 ng ^{125}I-insulin, 20,000 cpm) and 50 µl of either Hepes buffer, pH 7.4 or unlabelled insulin standard (0-1 x 10^5 ng/ml) in a total volume of 500 µl. The reaction protocol is been shown in Appendix 8, (page 298). The binding reaction was carried out at 37°C in a shaking water bath (100 cycles/minute) to keep the cells in homogeneous suspension. The incubation was terminated after 45 mimutes by the addition of 9 ml of chilled (4°C) saline. 1 ml of chilled silicone oil was layered on to the surface of the saline and the tube was centrifuged for 3 minutes (3000 rpm) at 4°C. The cells located themselves at top of the oil and were taken up into the cut end of a yellow disposable pipette tip as described by Pedersen and colleagues (130). The radioactivity associated with adipocytes in the pipette tip was measured using an LP3 tube. Duplicate 100 µl aliquots of reaction mixture from any reaction tube were counted for total radioactivity. Specific adipocyte binding represented by the difference between the total and the non-specific binding was expressed as a fraction of the total radioactivity.

The analysis of insulin binding data.

In the present work binding data has been presented in four ways, each emphasising different quantitive aspects of the binding interaction.

A) The relationship between total insulin concentration and the total insulin specifically bound per cell.

The total amount of insulin specifically bound was expressed as a function of both receptor affinity and receptor concentration, Figure 16.

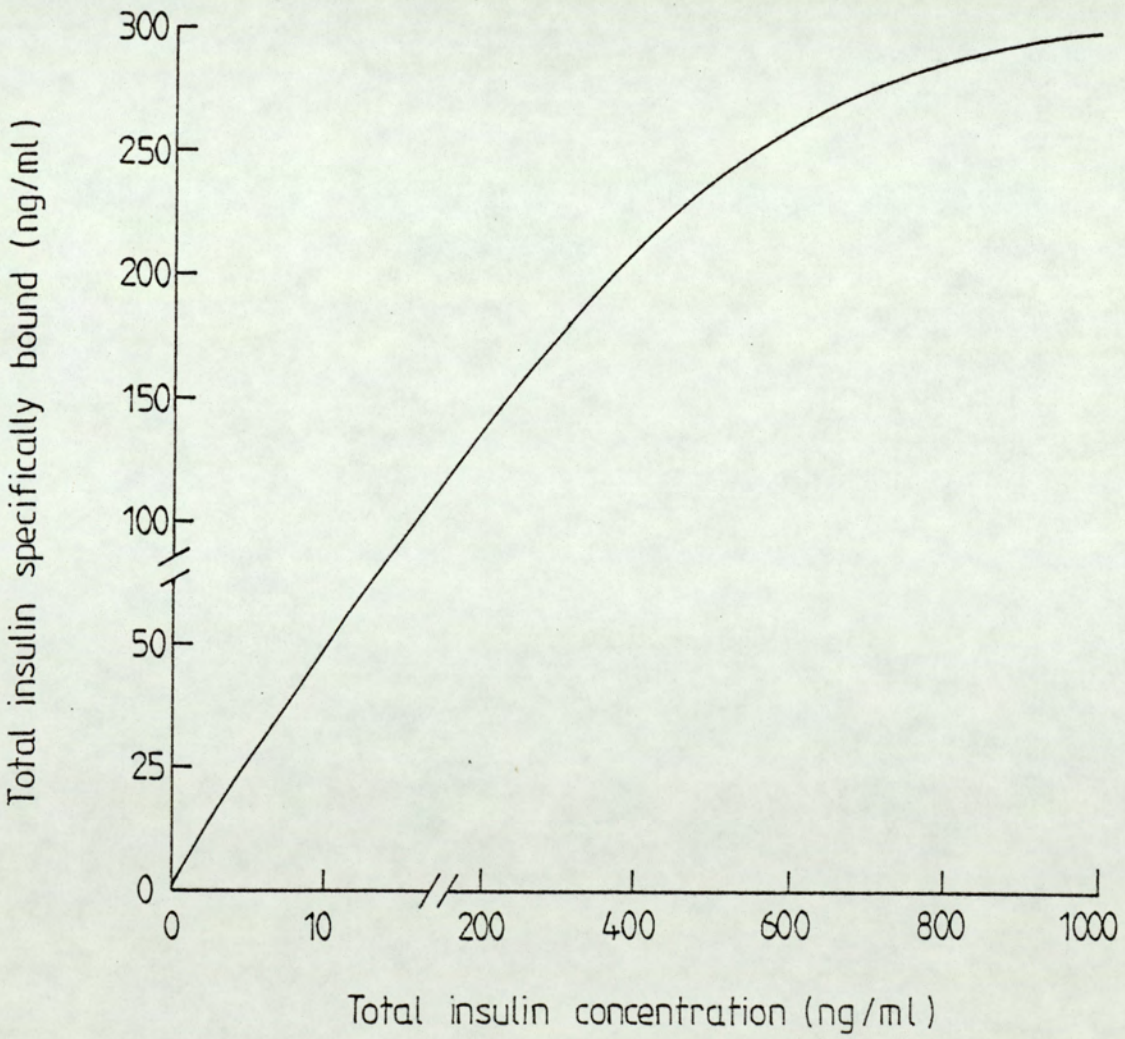
B) The competitive binding curve (competition curve).

In this case the percentage of the total ^{125}I-insulin specifically bound to receptor was plotted as function of the total insulin concentration, Figure 7. This expression most closely represented the sentiments of the actual experimental data (196).

C) The Scatchard plot:

The competitive binding of labelled and unlabelled insulin to human and animal cells was typically non-linear with an upward concavity when plotted according to Scatchard (110), Figure 8. The ratio of bound to free ^{125}I-insulin (B/F) was plotted as a function of the concentration of bound insulin (B). The curvilinearity of the Scatchard plot for insulin binding data was thought to represent a particular property of the insulin-receptor interaction and interpretation was based on heterogeneous receptor populations (311). The minimum model which best fitted the data consisted of two

Figure 16. The relation between the insulin concentration and the amount of insulin specifically bound.



classes of binding site with differing but fixed affinities, Figure 9, (118,132) a high affinity/low capacity binding site and a low affinity/high capacity binding site. The intercepts of the two slopes of the Scatchard plot on the x-axis give receptor number and the slopes of these lines represented the affinities of the receptor populations. The affinity constant ($\bar{K}_2$) of the low affinity binding site was given by the shallow part of the curvilinear plot, and the maximum binding capacity or the total concentration of receptor sites (Ro) was derived from the point where the linear extrapolation of the curve intersected the horizontal axis, Figure 9.

Thus the total number of insulin binding sites per cell was calculated using the following expression:

$$\text{Number of binding sites/cell} = \frac{\text{moles of insulin bound per litre} \times 6.022 \times 10^{23} \text{ *}}{\text{number of cells/litre}}$$

where * is Avagadro's number = number of molecules in one mole of insulin (220).

The total number of receptor sites per unit surface area [square micrometer (μm^2)] was estimated as follows:

$$= \frac{\text{number of sites per cell}}{\text{surface area } (\mu m^2)}$$

Similarly the high affinity constant ($\bar{K}_1$) and the binding capacity of the high affinity receptor (Ro_1) was estimated from the slope and intercept respectively of the steeper portion of the plot, Figure 9.

D) <u>The average affinity profile</u>

This analysis was based on the assumption that insulin receptor sites were not independent of one another, and the curvilinearity of the Scatchard plot represented a single set of homogenous binding

sites that underwent negatively cooperative site-site interactions such that the affinity of the receptors for insulin was inversely related to the fractional occupancy (229,312). That is, at low levels of insulin when very few receptor sites are occupied the receptors are in the high affinity state. As the level of insulin rises and occupancy increases, the receptors switch to a comformation that has a lower affinity for insulin. The proportion of receptors in each state being a function of the occupancy by the insulin monomer as well as environmental factors such as temperature and pH (230). The abscissa intercept (Ro) of a Scatchard plot based on the negative cooperativity interpretation of the curvilinearity is indicative of the total insulin binding capacity of the receptor population. The interpretation of the Scatchard plot for a single class of binding sites with negative cooperativity is shown in Figure 10. The abscissa intercept (Ro) remains as the molar concentration of binding sites or binding capacity. As occupancy approaches zero ($B \longrightarrow 0$) the B/F ratio approaches a limiting high value and all the sites will be in the high affinity conformation when B = 0. As the occupancy increases, the B/F ratio will thus fall along a straight line, joining the initial B/F to the abscissa intercept Ro. The negative slope of that line, termed $\bar{K}_e$, is equivalent to the affinity of the empty sites. When occupancy increases, the average affinity of all receptor sites decreases, due to site-site interactions. This changing affinity is represented by the successive lines of decreasing slope joining each point of the curve to the abscissa intercept, Figure 10. At each level of occupancy, the negative slope of the line joining the B/F ratio for that occupancy to Ro represents the average affinity (224) of the receptor sites at any level of

occupancy and is termed $\bar{K}$. This affinity is an average and does not represent any actual site binding constant, since the sites are now distributed between two conformations. When B approaches Ro, the average affinity reaches a limiting low value or $\bar{K}_f$. Assuming that all sites are then in the same low affinity conformation, $\bar{K}_f$ actually represents the affinity of the sites in the occupied conformation. Recently it has been shown that there may be a third receptor conformation of very low affinity and high capacity, known as $\bar{K}$ super (314). The physiological role of these states in insulin action has not yet been ratified, as the $\bar{K}_f$ and $\bar{K}$ super states occur at supraphysiological insulin concentrations. The average affinity profile express the relationship between the average affinity for insulin ($\bar{K}$) and the receptor occupancy $\bar{Y}$ (230), Figure 11. At any point i on the Scatchard curve the average affinity ($\bar{K}_i$) =

$$\bar{K}_i = \frac{(B/F)i}{Ro - Bi}$$

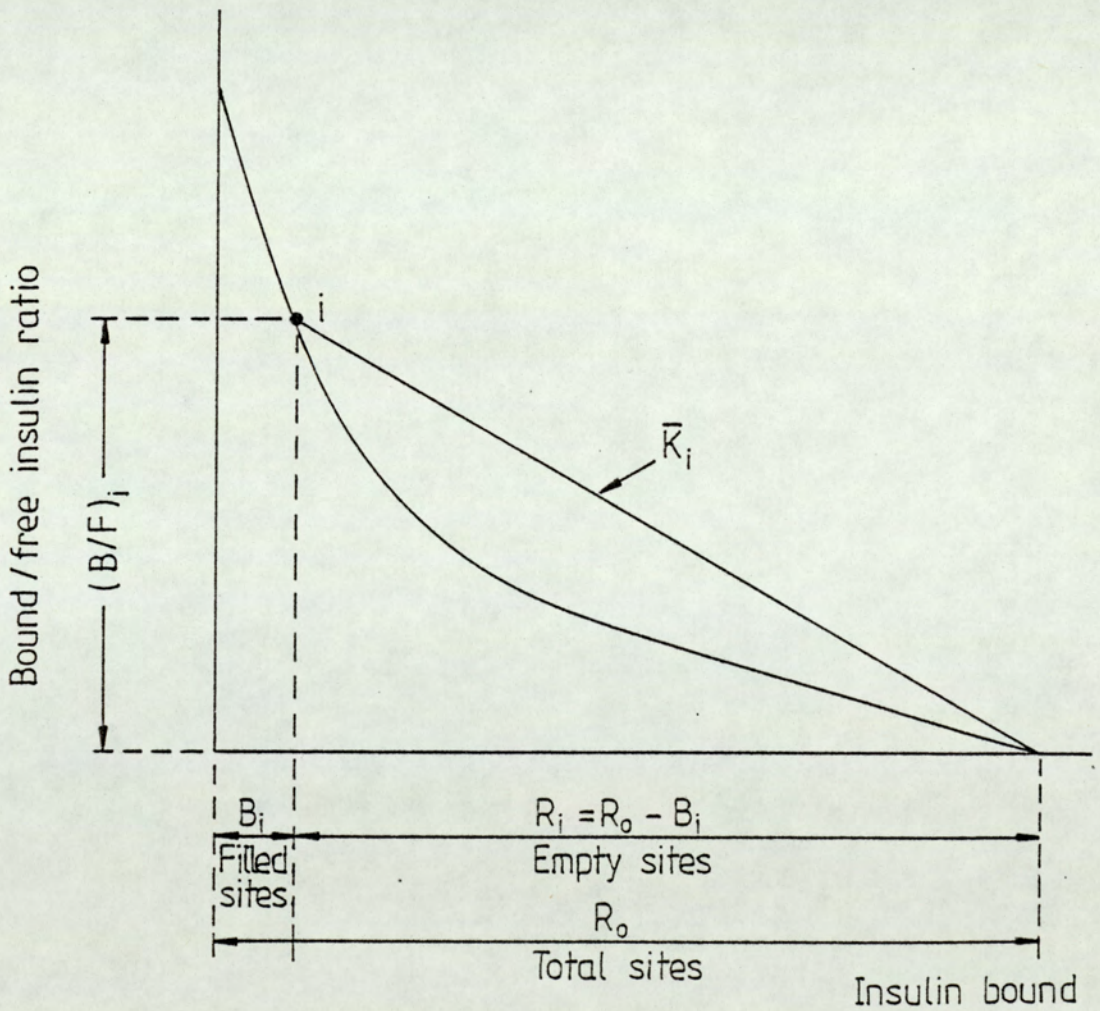
Where Bi = the concentration of bound insulin

(B/F)i = bound/free insulin at that point, Figure 17.

When the affinity $\bar{K}$ is plotted against the $\log_{10}$ of the percentage occupancy of receptor sites ($\bar{Y}$ x 100, calculated from B/Ro x 100), the plot displays the average affinity of the receptor at all levels of receptor occupancy and is referred to as the "average affinity profile" (230), Figure 11. In this analysis, the limiting high affinity state obtained at low levels of receptor occupancy, is designated $\bar{K}_e$, the limiting low affinity state, obtained at high levels of receptor occupancy, is $\bar{K}_f$.

In the present work using erythrocytes $\bar{K}_e$ has been defined as the value of $\bar{K}$ when the ^{125}I-insulin concentration is 0.08 ng/ml. Irrespective of how the nonlinear Scatchard plot is interpreted the

Figure 17. Calculation of the average affinity $\bar{K}$. At a given point on the curve, i, the level of bound insulin, B_i, corresponds to a given ratio of bound / free $= (B/F)_i$.
The value of the average affinity $\bar{K}$ for that occupancy is given by the negative slope $\bar{K}_i$, or $(B/F)_i / (R_o - B_i)$ (230).



total receptor concentration, Ro, can be obtained from the intercept on the abscissa whereas the estimated recptor affinity can be obtained by a variety of means. In addition, an alternative estimate of apparent insulin receptor binding affinity can be used and is represented by the concentration of insulin required to inhibit the receptor bound fraction by 50% (the 50% inhibition value). A low 50% inhibition value indicates a high apparent affinity (315). These binding values have been widely used as a rough estimate of receptor affinity at physiological insulin concentrations. Of note is the fact, however, that the 50% inhibition value is also a function of the insulin tracer concentration (315).

In the present study it was considered that a model based on a single class of insulin receptors exhibiting negative cooperativity would provide a useful means for the quantitative comparison of insulin binding data. However, some consideration was given to a model comprising two classes of insulin receptors of fixed but differing affinities by presenting insulin receptor number in terms of "high" and "low" affinity components.

Scatchard curves were fitted by dividing the plot into two sections, each of which were ideal for linear regression analysis. It must be emphasised, however, that such a procedure made no assumption concerning the model used to interpret the "curvilinearity" of Scatchard plots. The linear regression analysis allowed the determination of abscissa intercepts for the determination of receptor binding capacities. Calculation of both total and low affinity receptor concentration together with $\bar{K}_e$ and $\bar{K}_f$ required a knowledge of Ro which was obtained by extrapolation of the lower portion of the Scatchard plot to the abscissa.

When carrying out the erythrocyte binding assay, Gambhir and colleagues (210) indicated that although a full Scatchard plot could be produced, the assay was less sensitive to increasing insulin concentrations (above 100 ng/ml) making extrapoltion to the abscissa difficult and unreliable. However, in the present studies, the lower slope of the Scatchard plot was based upon 8 points and showed excellent linear correlation inferring accurate determination of both the slope and the abscissa intercept.

When data from adipocyte insulin binding assays were subjected to Scatchard analysis, the terminal points of the Scatchard plots were obtained with very high insulin concentrations and because non-specific binding was disproportionately high and difficult to determine at these high concentrations, it proved difficult to accurately define the precise point of the abscissa intercept. Hence reliable values for Ro and total receptor number were not easy to obtain. The proper denominator for the expression of adipocyte insulin receptor binding has yet to be universally confirmed. Binding may be expressed as a function of either the number of adipocytes, cell surface area, protein or nucleic acid content. Since changes to insulin receptor binding are assumed to be accompanied by parallel alterations in cellular insulin sensitivity it was decided that the cell surface area was the proper denominator to which adipocyte insulin binding should be related. Also adipocytes can be of different sizes especially when taken from different regions and different animal species.

Example calculations for the evaluation of erythrocyte and adipocyte insulin receptor binding data have been provided in Appendix 6, page 294.

The degradation of ^{125}I-insulin by human erythrocytes:

Erythrocytes from either normal volunteers or non-insulin dependent diabetics were separated from plasma as described previously (page 83) and their concentration in buffer G adjusted to 4.4 x 10^9 cell/ml. 400 µl aliquots from the erythrocyte suspension were incubated with 50 µl of ^{125}I-insulin (10,000 cpm) with and without 50 µl of unlabelled insulin standard (0 - 1 x 10^5 ng/ml) to give a final volume of 0.5 ml. Two additional tubes each containing 50 µl ^{125}I-insulin and 450 µl of buffer G (without erythrocytes) were included in the assay for the estimation of the degradation of intact unexposed ^{125}I-insulin. All tubes were incubated for 3 hours at 15°C and shaken by hand every 30 minutes. The reaction was terminated by placing the tubes in an ice bath. A 150 µl aliquot from each tube was inserted into a 400 µl microfuge tube containing 100 µl of buffer G layered over 100 µl of di-n-butyl phthalate. After centrifugation (9,000 rpm for 3 minutes), the erythrocytes were retained as a pellet at the bottom of the tube and separated from unbound tracer in buffer G by the layer of di-n-butyl phthalate. A 100 µl aliquot of unbound tracer was removed from each microfuge tube and transferred to an LP3 tube containing 0.5 ml of buffer G supplemented with 1% BSA. 0.5 ml of buffer G containing 10% TCA was added to each tube and the radioactivity of total mixture counted. Tubes were then centrifuged (2,000 rpm) for 10 minutes and the supernatant aspirated. The radioactivity associated with the precipitate was then counted. Insulin degrdation was determined by assessing the ability of ^{125}I-insulin remaining in the buffer to precipitate with 10% TCA. Insulin degradation was calculated as soluble radioactivity and expressed as the percentage of total radioactivity in the buffer.

The degradation of ^{125}I-insulin by isolated adipocytes:

Isolated adipocytes were prepared from lean and obese mice as described previously (page 89). Aliquots of adipocyte suspension were diluted with Hepes buffer pH 7.4, supplemented with BSA (50 mg/ml) and glucose (5 mmol/l) to give a final volume of 400 µl (final concentration of 4-5 x 10^5 cells/ml) and incubated in 15 ml polyethylene tubes with 50 µl of ^{125}I-insulin (20,000 cpm) either with or without a high concentration of unlabelled insulin (50 µl of 10^5 ng/ml insulin) to give a final volume of 0.5 ml. An identical set of tubes each containing 450 µl of Hepes buffer and 50 ul of ^{125}I-insulin but no adipocytes was also set up for the determination of the percentage degradation of intact unexposed ^{125}I-insulin. All tubes were placed in a shaking water bath (100 cycles/minute) for 1 hour at 37°C. 200 µl aliquots of the infranatant (buffer containing radioactive insulin) were transferred to LP3 tubes containing 0.5 ml of Hepes buffer, pH 7.4 supplemented with 4% BSA. To each tube was added 0.5 ml of Hepes buffer containing 12% TCA and the tubes were centrifuged at 2000 rpm for 10 minutes. The supernatant was removed and the radioactivity associated with the precipitate counted. Insulin degradation was monitored by assessing the ability of ^{125}I-insulin remaining in the incubation buffer to precipitate with 12% TCA (130). In this method TCA-precipitable radioactivity was considered to be associated with intact insulin and TCA-soluble radioactivity was taken to be degraded insulin.

The degradation of ^{125}I-insulin by isolated fat segments:

100 mg pieces of adipose tissue from lean and obese mice (page 92) were incubated for 2 hours at 37°C in 1 ml of Krebs bicarbonate buffer, pH 7.4 containing 4% BSA, glucose (5.6 mmol/l) and ^{125}I-insulin (10^5 cpm) in the absence and presence of unlabelled insulin (10^5 ng/ml). After incubation 200 µl of the infranatant was combined with 0.5 ml of Krebs buffer containing 4% BSA in an LP3 tube followed by the addition of 0.5 ml of Krebs buffer containing 15% TCA. The tubes were capped and the radioactivity associated with each tube counted. After centrifugation (2000 rpm for 10 minutes) and removal of the supernatant the radioactivity of the intact precipitated ^{125}I-insulin was counted. Insulin degradation was measured as soluble radioactivity and expressed as the percentage of total radioactivity in the buffer.

The degradation of insulin by both erythrocytes and adipose tissue (adipocytes and fat segments) was determined by examining the ability of the radioactive insulin remaining in the incubation medium to precipitate with TCA (% intact insulin). The percentage of the ^{125}I-insulin which remained intact was then determined by the following formula:

$$\frac{\%\text{ of intact incubated insulin}}{\%\text{ of intact unexposed insulin}} \times 100$$

Unexposed ^{125}I-insulin has been reported to be at least 98% precipitable by TCA (302).

The radioimmunoassay of insulin.

Radioimmunoassay is dependent upon the competition between

labelled and unlabelled antigen (Ag) for binding sites on specific antibodies (Ab) (316). Increasing amounts of unlabelled Ag in the sample produce a proportional decrease in the binding of labelled Ag to the Ab (218). Thus the level of radioactivity associated with the Ab-Ag complex is related to the concentration of unlabelled Ag in the original sample.

In the present work the insulin concentration was determined in both serum and plasma samples by the double antibody technique, of Hales and Randle (317) where separation of the bound from free labelled Ag was carried out by centrifugation.

The essential principle of the test was the reaction of a limited, fixed quantity of anti-insulin serum with a mixture of the sample or standard insulin to be assayed together with a constant amount of ^{125}I-insulin. After the reaction had been allowed to approach completion the antibody bound insulin was separated from free insulin by centrifugation and the distribution of the radioactivity determined. The binding of labelled insulin to antibody was progressively inhibited by increasing amounts of unlabelled insulin owing to competition for specific binding sites on the antibody and the concentration of insulin in the sample under test was determined by reference to a standard curve prepared at the same time. In this technique the first Ab (an anti-insulin Ab) raised against porcine insulin in guinea-pigs and the second Ab (induced in rabbits against the γ -globulin of the guinea-pig) were used to precipitate the primary Ab-Ag complex.

Preparation of radioimmunoassay reagents.

Diluent buffer: The following reagents were added to 1 litre

of double distilled water; 6.2 g sodium dihydrogen orthophosphate ($Na H_2 PO_4 2H_2O$), 0.25 g thiomersal (thiomersalate), 5.1 g bovine serum albumin fraction V, and the pH was adjusted to 7.4.

Insulin binding reagent: The lyophilised reagent consisted of a mixture of guinea-pig anti-insulin serum and rabbit anti-guinea pig-globulin serum (Wellcome Laboratories, Dartford, Kent) and was reconstituted in 8 ml of distilled water on the day of the assay. This antibody dilution bound approximately 40% of a selected concentration of labelled insulin at the lower end of physiolocial range (0.25 ng).

^{125}Iodinated-insulin: ^{125}I-insulin was prepared to an average specific activity of between 230-300 µCi/µg by the chloramine-T iodination method and purified by gel filtration as described previously (see page 68). The specific activity was reduced to 50 µCi/µg by the addition of an appropriate amount of unlabelled insulin. It was important not to add an excessively large amount of labelled insulin as the resulting saturation of insulin binding sites is known to severely reduce the assay sensitivity (284).

Preparation of insulin standards: The contents of one bottle of Wellcome human insulin standard (2 mill-units) was dissolved in 2 ml of diluent buffer. 0.2 ml aliquots of this solution were dispersed into stopped LP3 tubes and stored frozen at -20°C. When required on the day of assay, one of these tubes was diluted with 0.8 ml of diluent buffer to provide a top standard insulin concentration of 200 µU/ml. Four further serial dilutions were made of this first stock standard tube to provide a range of standard insulin concentrations from 12.5 to 200 µU/ml.

Preparation of samples for assay: Serum and plasma samples from patients and lean mice were assayed directly. Serum and plasma from hyperinsulinaemic obese mice was diluted 1 in 10 with diluent buffer (this dilution factor was taken into account when computing results).

Procedure

Reagents were added to LP3 tubes in the order indicated in the reaction protocol shown in Appendix 12, page 304. Insulin standards, zeros, blanks and total counts were usually set up in triplicate although the insulin content of samples was generally determined in duplicate. 50 µl aliquots of insulin standards (12.5-200 µU/ml) or samples were pipetted into plastic LP3 tubes. A 50 µl aliquot of insulin binding reagent was added to all tubes (except the total counts and blank tubes). The contents of each tube was vortex mixed without frothing (Hoak and Tucker Ltd, London) and incubated in a refrigerator at 4^{0}C for six hours. A 50 µl aliquot of ^{125}I-insulin ($\simeq$ 10,000 cpm of specific activity 50 µCi/µg)was then added to all tubes. Tubes containing only ^{125}I-insulin were used for the determination of the total ^{125}I-insulin activity (cpm). After gentle vortex mixing, all tubes were incubated for a further 16-18 hours at 4^{0}C. After the second incubation 0.5 ml of diluent buffer was added to all tubes except the total counts and the contents vortex mixed. Free and Ab-bound insulin was separated by centrifugation (1,500 g, Mistral Coolspin Centrifuge, MSE Scientific Instruments, Surrey) for 30 minutes at room temperature. The supernatant containing free labelled insulin was carefully decanted off and the remaining drops of buffer at the top of the tubes aspirated with the aid of a pasteur

pipette connected to a vacuum pump. The last drop of supernatant was then removed from the mouth of each tube with absorbant paper. The tubes were then left at an angle of 30° for approximately 4 hours to dry at room temperature. The ^{125}I activity associated with the precipitate was counted for 1 minute on a gamma counter (ICN, Tracerlab, with 57% counting efficiency) and counts were corrected for background.

Construction of standard curve and computation of results.

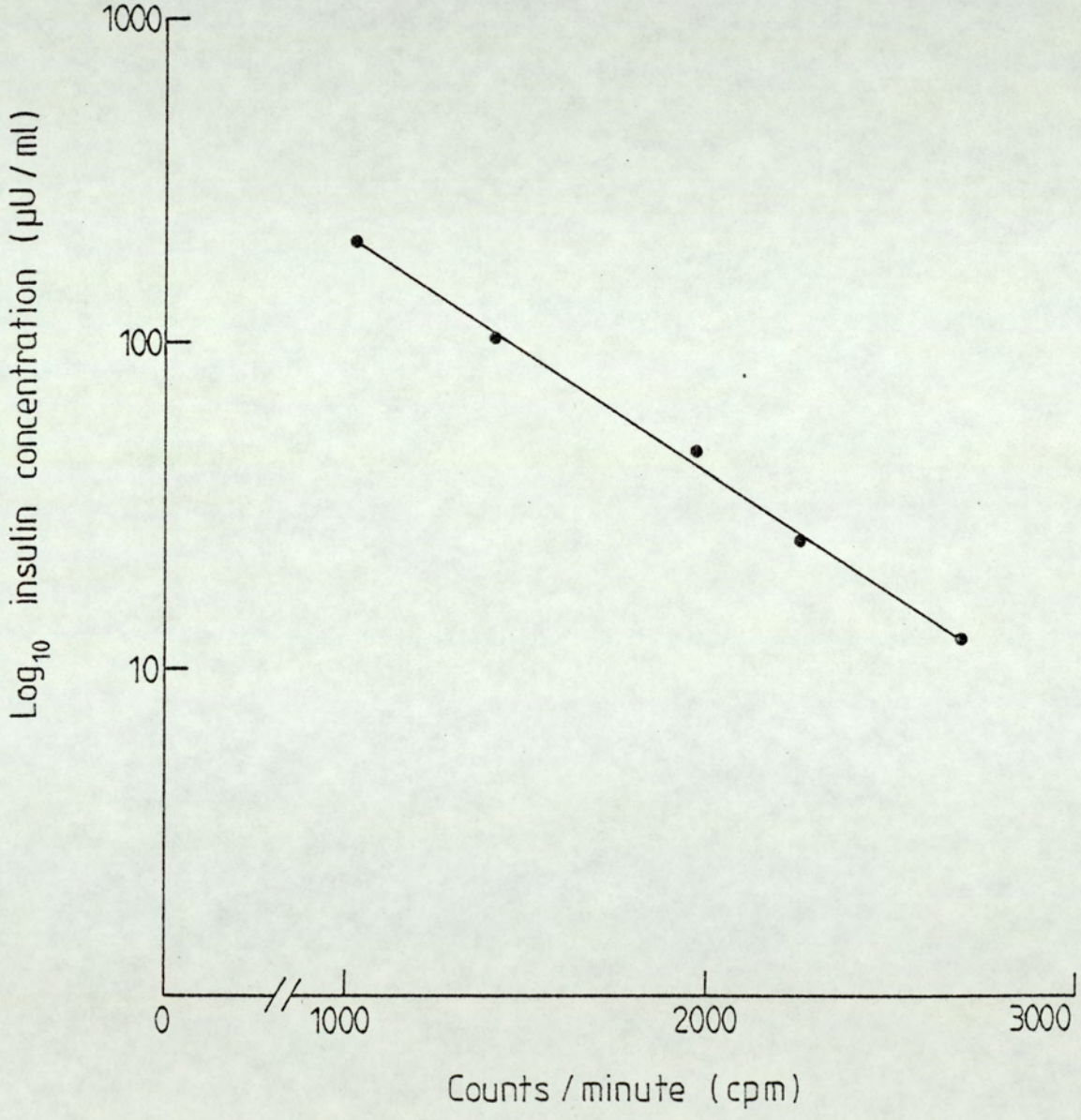
Initially standard curves were constructed using the $\log_{10}$ of the standard insulin concentration plotted against the corrected bound radioactivity in counts per minute (cpm). A line of best fit was constructed by the method of least squares to allow the determination of the sample insulin concentration by direct extrapolation from the graph. Figure 18 shows a typical standard curve for insulin radioimmunoassay. Computation of results for subsequent insulin assays was carried out using a computer program (INS) which allowed reference to both human and mouse insulin standards (Appendix 13, page 305).

Criteria for a satisfactory assay.

The following are indications of a satisfactory assay:

1. There must be good agreement between replicate values for zeros, standards, blanks and total counts. Replicate count rates were kept within 3-5 percent of the mean values in most instances. However, replicates differing by more than 200 counts/minute were discarded.

Figure 18. A typical standard curve for the double antibody radioimmunoassay of insulin. Correlation coefficient, r = 0·994.



The most common causes of poor precision were inaccurate pipetting, inadequate centrifugation and a bad decanting technique.

2. The count rate for the zeros should be approximately 30% of the total count rate. The day-to-day variation in this value did not normally exceed five percent. The zero count indicated the amount of radioactivity associated with the insulin Ab complex in the absence of unlabelled insulin.

3. The blank count rate should be less than 5% of total counts.

4. In the present studies insulin assays had an intra-assay coefficient of variation of 4% and an interassay coefficient of variation of 5.7%.

Blood sampling.

In man, fasted blood samples were either taken from the median basilic vein by venipuncture or obtained via an indwelling catheter as part of a glucose tolerance test. Serum or plasma was separated, stored at -20°C and subsequently used for the determination of glucose, insulin calcium, routine haematology and a biochemical profile.

In mice about 150 µl of whole blood was obtained from the cut tip of the tail by milking. Serum or plasma was separated and stored in 200 µl microfuge tubes at -20°C until required for assay.

The esimation of glucose in plasma and serum.

Glucose concentrations were estimated in both plasma and serum (10 µl) using an automated glucose oxidase procedure (Beckman Glucose

Analyser, Beckman Riic Ltd., High Wycombe) (318). Values were obtained in mmol/l glucose.

The estimation of calcium in plasma and serum.

Calcium levels (mmol/l) were determined in both plasma and serum (10ul) using a Corning Calcium Analyser 940 (367).

The estimation of glucose tolerance.

Glucose tolerance tests were carried out on overnight fasted patients using a 75 g oral glucose tolerance test (OGTT). Venous blood samples were withdrawn at 0, 15, 30, 45, 60, 90 and 120 minutes. The 75 g dose of glucose recommended by the National Institutes of Health (NIH) Working Group was chosen because the 50 g dose previously recommended by the British Diabetic Association was insufficiently provocative to demonstrate glucose intolerance, whereas the 100 gm dose recommended by the American Diabetic Association has been shown to be associated with symptoms of nausea during the test (428).

Biochemical profile and routine haematology of patients.

These parameters were determined at Dudley Road Hospital.

The estimation of sodium and potassium:

Sodium and potassium were determined by routine flame photometric analysis using a Lithium internal standard. Samples were diluted 1 in 200 and aspirated into a propane-air flame. The 589 nm

line of Na^+ and the 766 nm line of K^+ were isolated by suitable interference filters and transmitted to individual photoreceptors. The resulting photocurrent was amplified and read out on a digital display (319).

The estimation of uric acid (uricase or urate oxidase):

In this method uricase was used to catalyze the oxidation of uric acid to give allantoin and H_2O_2. The latter was measured by oxidative coupling of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) and anthanilic acid (O-aminobenzoic acid) in the presence of peroxidase. The coloured complex formed was measured at 570 nm (320).

The estimation of urea:

Serum urea was made to react with acidic diacetyl monoxime for a short period in the presence of thiosemicarbazide, which intensified the resulting colour and decreased the photosensitivity (321). Rather than measuring the colour formed by the reaction between diacetyl and urea photometrically the fluorescence was mesasured instead at 415 nm.

The estimation of total cholesterol:

The CHOD-PAP method was used (kinetic colorimetric test) specifically designed for an automatic analyser (322). The principle of the test is shown below:

Cholesterol ester + H_2O $\xrightarrow[\text{esterase}]{\text{Cholesterol}}$ Cholesterol + RCOOH

Cholesterol + O_2 $\xrightarrow[\text{oxidase}]{\text{Cholesterol}}$ Δ^4 - Cholesterone + H_2O_2

$2H_2O_2$ + 4-aminophenazone + phenol $\xrightarrow{\text{POD}}$ 4 - (benzoquinone - monoimino)-phenazone + 4 H_2O

The resultant colour was measured photometrically at 560 nm.

The estimation of serum triglycerides:

The enzymatic determination of triglyceride was based on the following series of reactions (323):

Triglyceride $\xrightarrow{\text{Lipase}}$ Glycerol + fatty acids

Glycerol + ATP $\xrightarrow{\text{Glycerol kinase}}$ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 $\xrightarrow{\text{Glycerol-3-phosphate oxidase}}$ Dihydroxyacetone-phosphate + H_2O_2

H_2O_2 + Acid-3-hydroxy 2-4-6 triiodobenzoic + Amino-4-antipyrine $\xrightarrow{\text{Peroxidase}}$ coloured quinonemine + H_2O

The colour formed was measured at 564 nm.

The estimation of glycosylated haemoglobin:

Was measured by an affinity chromatographic method (Glyco-gel; Pierce Chemical Company, Illinois, U.S.A.) (324).

Routine haematology:

Haemoglobin, white cell count, platelet count and mean corpuscular volume (MCV) were measured by standard laboratory methods (325).

Normal values for haematology and biochemistry are given in Appendix 11, page 303.

General statistical analysis.

Data in this thesis are presented as the mean ± SEM (standard error of the mean). Straight lines were fitted by the method of least squares and calculated using an Olivetti Programma 101. Linear correlation was assessed by Pearson's correlation coefficient (Pearson's r). Estimation of the significance of difference between mean values was carried out using Student's paired and unpaired t-tests.

Experimental protocol.

The following protocol summarises the experimental work that has been described in detail in the following chapters.

a) The effect of overnight fasting on erythrocyte insulin receptor binding in normal male volunteers.

b) The effect of the antihypertensive drug bendrofluazide and subsequent potassium supplementation on erythrocyte insulin receptor binding, glucose tolerance, serum glucose and insulin levels and general biochemistry of mildly hypertensive patients with and without non-insulin dependent diabetes mellitus.

c) The effect of long term nifedipine treatment, subsequent withdrawal and restoration of nifedipine treatment on oral glucose tolerance and serum glucose and insulin levels of mildly hypertensive patients with and without non-insulin dependent diabetes mellitus.

d) The effect of dietary fibre (guarem) on erythrocyte insulin receptor binding in poorly controlled non-insulin dependent diabetics.

e) The effect of dietary fibre (guarem) on adipocyte insulin receptor binding in genetically obese diabetic (ob/ob) mice.

f) Adipocyte insulin receptor binding in lean and genetically obese diabetic (ob/ob) mice.

g) Insulin receptor binding and glucose oxidation in subcutaneous and abdominal fat of genetically obese diabetic (ob/ob) mice.

CHAPTER THREE

The Effect of Overnight Fasting on Erythrocyte Insulin Receptor Binding in Normal Male Volunteers

Introduction

The quantity and composition of the diet is of importance for glucose tolerance and insulin sensitivity (326). Conflicting data have been reported for the response of monocyte insulin receptors to feeding (239,327) and similarly conflicting data are now begining to appear for erythrocytes. Bhathena and colleagues (328) have reported an increase in insulin binding to both erythrocytes and monocytes following a glucose load. In contrast, a more recent study has showed that insulin binding did not change after the glucose loading (329).

Long periods of fasting appear to affect insulin receptor binding. Spanheimer and colleagues (331) demonstrated that a 14 day fast by obese patients increased insulin binding in both circulating monocytes and erythrocytes. The increase in erythrocyte insulin binding was largely due to a change in receptor affinity, while the increase in monocyte insulin binding was thought to be secondary to an increase in both affinity and the concentration of the insulin receptors. It would appear that different tissues respond differently with regard to insulin receptor concentration during fasting. An increased concentration of insulin receptors after 3 days fasting has been reported for human monocytes (332). In human monocytes and adipocytes, fasting for 3 days was suggested to lead to an increased insulin binding because of an increase in the affinity of the receptor for insulin (196,334). This increase could be primarily accounted for by a decrease in the rate at which insulin dissociated from its receptor with no change in other kinetic features of the insulin receptor interaction (334,335).

Insulin receptor binding to human erythrocytes has been shown to increase following an overnight fast. The increase in insulin binding appeared to be due to an increase in receptor affinity (329).

In animal studies the binding of ^{125}I-insulin to isolated hepatocytes from fed rats bound significantly more ^{125}I-insulin than hepatocytes from fasted rats and this increase could largely be accounted for by an increase in receptor concentration without alteration in receptor affinity. In contrast to hepactocytes, liver plasma membranes of fasted rats showed significantly increased insulin binding when compared to liver plasma membranes from fed rats. Analysis of the data indicated a significant increase in the affinity of liver plasma membrane receptors but no change in the receptor concentration (336). These observations demonstrate the variations in the responses of different tissues to insulin receptor binding during fasting. Increases in insulin receptor concentration have been found in different tissues of genetically obese (ob/ob) mice after fasting, e.g. liver plasma membranes (255), thymic lymphocytes (205) and heart plasma membranes (124), but not adipose tissue plasma membranes (337). In genetically obese (ob/ob) mice 24hr of fasting increased the number of insulin receptors on liver plasma membranes but did not affect receptor affinity (339). Almira and Reddy (336) reported that 72h fasting in rats produced an increased insulin binding to liver plasma membranes. Their data indicated a significant increase in the affinity of insulin receptors but no change in receptor concentration. 24h fasting in rats increased the ability of isolated adipocytes to bind insulin and this was largely due to an increase in receptor affinity. In these animals binding continued to increase between 24h and 48h, but there was no further increase between 48h and 72h (302,338).

The present study has investigated the effect of an overnight fast (12 hours) on insulin binding to human erythrocytes freshly isolated from male human volunteers. This study was initiated because subsequent clinical investigations involved patients that had been subjected to a 12h overnight fast prior to blood sampling and experimentation

Materials and Methods.

Subjects:

Whole blood samples were obtained by venipuncture from 7 freely fed normal male volunteers with a mean age of 37.6 ± 1.4 years and a mean body weight of 76.5 ± 2.5 kg (±SEM). All subjects had a normal body weight to age ratio. All had family and personal histories negative for diabetes mellitus, and no subject was allowed drug therapy before or during experimentation. For this study blood samples were taken at 09.00 hrs on the day preceeding the assay and then in the evening of that day volunteers were subjected to a 12hr overnight fast. Volunteers were recalled at 09.00 hrs on the following day (with 2 hours of morning ambulatory activity) for blood sampling and subsequent insulin receptor assay.

Erythrocyte insulin receptor binding:

10-15 ml of whole blood was obtained by venipuncture and transferred to a heparinized vacutainer tube. Purified erythrocytes were separated from plasma and resuspended in a volume of buffer G that resulted in a suspension containing 4.4 x 10^9 cells per millilitre. The total erythrocyte concentration was determined using

a coulter counter (see page 83). More than 95% of the erythrocytes were found to be viable , as determined by trypan blue exclusion (299). The binding of ^{125}I-insulin to purified erythrocytes was effected by the incubation of 400 µl of erythrocyte suspension (1.76 x 10^9 cells) in buffer G with ^{125}I-insulin (10,000 cpm of specific activity, 242 µCi/µg) in the presence of increasing concentrations of unlabelled insulin (0 - 10^5 ng/ml) as described previously (page 98). After reaction the cell bound and free insulin were separated by centrifugation through di-n-butyl phthalate (see page 98). The specific cell bound fraction at each concentration of unlabelled insulin was determined by subtracting the amount of ^{125}I-insulin bound in the presence of an excess of unlabelled insulin (10^5 ng/ml), the non-specific binding at each concentration of unlabelled insulin.

The binding data was analysed in four ways: firstly, the total insulin specifically bound was plotted as a function of the total insulin concentration. Then the percentage of ^{125}I-insulin specifically bound was plotted as a function of the total insulin concentration (competition curve) to provide values of receptor affinity. This was followed by plotting the specific cell bound/free insulin ratio as a function of the specific cell bound insulin (Scatchard analysis) (267) to provide values of receptor concentraiton and the various affinity constants. The binding capacity for the two classes of receptors (Ro) was obtained by extrapolation of each curve for each class to its intercept on the abscissa. Finally an average affinity profile, was constructed to demonstrate the relationship between the average affinity for insulin ($\bar{K}$) and the receptor occupancy ($\bar{Y}$) (see page 102). The profile allowed the computation of $\bar{K}_e$ and $\bar{K}_f$ the empty and full sites affinity respectively.

Results

Patient data and the effect of overnight fasting on erythrocyte insulin receptor binding has been summarised in Table 5. When ^{125}I-insulin was incubated with purified human erythrocytes a specific binding of the hormone was observed. Non-specific ^{125}I-insulin binding in the presence of 10^5 ng/ml insulin was not significantly affected by fasting. At low insulin concentrations (0-10 ng/ml) erythrocytes from fasted volunteers bound marginally more insulin than erythrocytes from non-fasted volunteers, but this difference was not statistically significant, Figure 19. The total ^{125}I-insulin bound to erythrocytes at the lowest insulin concetration used (0.08 ng/ml) was 9.14 $\pm$ 0.53% and 9.57 $\pm$ 0.47% before and after an overnight fast respectively, Table 5. Erythrocytes from both fasted and non-fasted volunteers showed a displacement of bound ^{125}I-insulin as the concentration of unlabelled insulin increased, Figure 20. The inhibition of ^{125}I-insulin binding by unlabelled insulin was almost linear over the range 1-15 ng/ml of insulin. The concentration of unlabelled cold insulin required to inhibit 50% of the ^{125}I-insulin binding to erythrocytes from both fast and unfasted volunteers was about 5 ng/ml. This percentage inhibition was observed with concentrations of insulin within the physiological range for normal individuals. The similarity in the profiles obtained for the competition-inhibition curves indicated that the ability of unlabelled insulin to inhibit ^{125}I-insulin binding was similar for erythrocytes from both fasted and non-fasted volunteers, Figure 20. Since the ability of unlabelled insulin to inhibit ^{125}I-insulin binding to erythrocytes is thought to reflect the binding

Table 5. Patient data and the effect of overnight fasting on erythrocyte insulin receptor binding (mean values ± SEM).

	n	Before Overnight Fasting	After Overnight Fasting
Weight (Kg)	7	76.5 ± 2.5	-
Age (Years)	7	37.6 ± 1.4	-
Red blood cell concentration (cells x 10^9/ml)	7	4.003 ± 0.14	3.9 ± 0.15
Non specific binding (%)	7	16.71 ± 1.5	14.71 ± 0.75
Specific binding (%)	7	9.14 ± 0.53	9.57 ± 0.47
High affinity receptor concentration per cell	7	14.14 ± 1.22	16.14 ± 1.63
Low affinity receptor concentration per cell	7	176.1 ± 14.4	184.7 ± 13.7
Total affinity receptor concentration per cell	7	190.3 ± 15.1	200.9 ± 14.5
Total Sites per square micrometre (μm^2)	7	1.36 ± 0.11	1.43 ± 0.10
$\overline{K}_e$, affinity constant of empty sites (nM^{-1} x 10^{-2})	7	9.15 ± 0.87	9.65 ± 0.55
$\overline{K}_f$, affinity constant of full sites (nM^{-1} x 10^{-2})	7	1.38 ± 0.23	1.3 ± 0.20
$\overline{K}_m$, apparent affinity constant (nM-1 x 10-2)	7	5.23 ± 0.36	5.39 ± 0.45

Figure 19. Total insulin specifically bound (ng/ml) to erythrocytes from normal volunteers.
(*— —*) before overnight fasting.
(o——o) after overnight fasting.
Each point represents the mean of 7 determinations ± SEM.

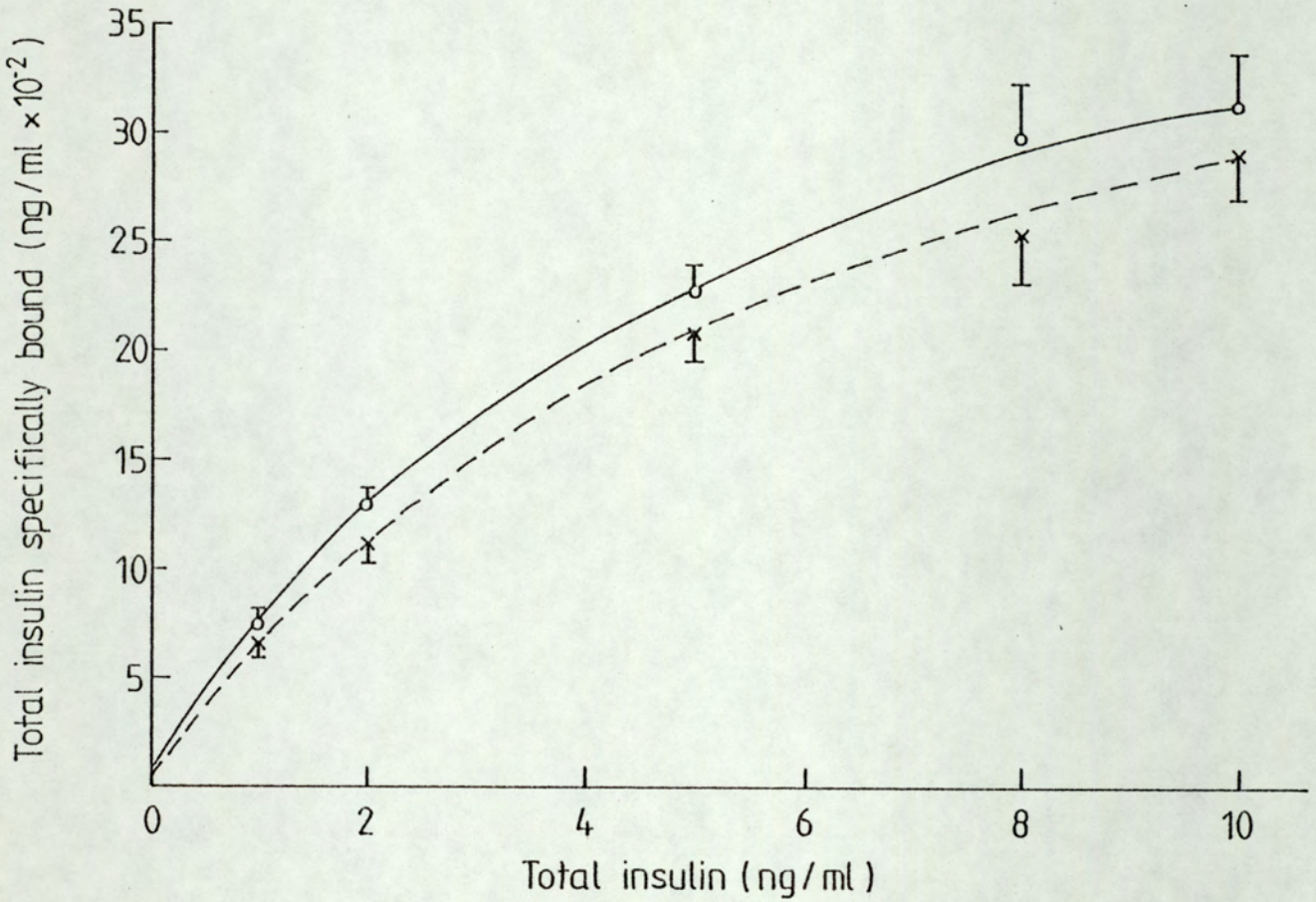
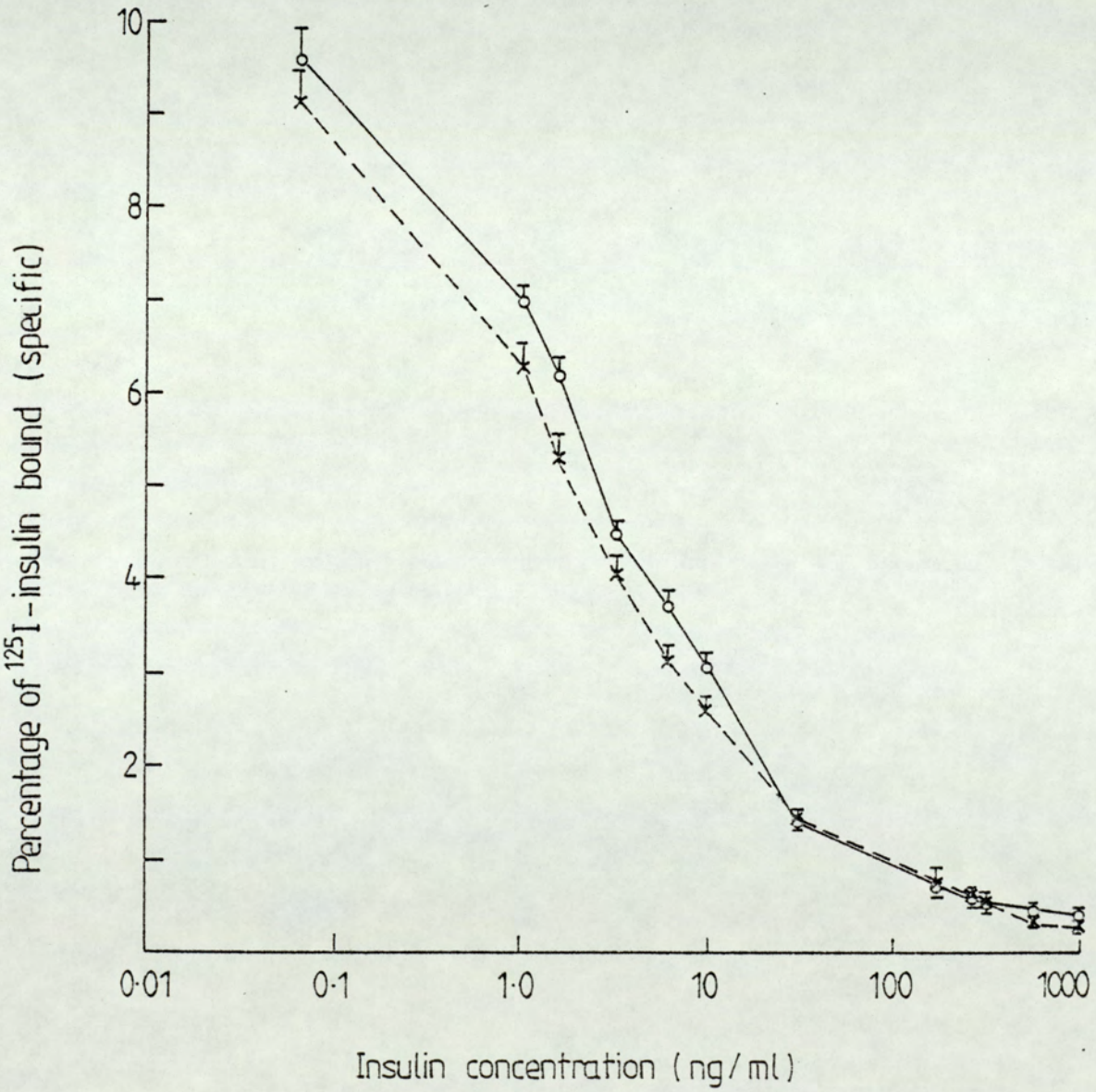


Figure 20. Competition - inhibition curves showing the percentage of ^{125}I - insulin specifically bound to human erythrocytes from normal volunteers before and after overnight fasting.
Each point represents the mean value of 7 determinations ± SEM.
(×– –×) before an overnight fast.
(o——o) after an overnight fast.



affinity, the slope of the competition-inhibition curves in Figure 20, suggested that the affinity of erythrocyte insulin receptors for insulin was the same in volunteers before and after fasting. This observation was supported by the characteristic curvilinear Scatchard plots of the binding data, Figure 21. Using Scatchard analysis 4 x 10^9 erythrocytes per ml of blood from free fed volunteers were calculated to bind 10 x 10^{-10} moles of insulin and each erythrocyte was estimated to have 190.3 $\pm$ 15.1 receptor sites. After fasting overnight 3.9 x 10^9 erythrocytes per ml were shown to bind 10.2 x 10^{-10} moles of insulin and each erythrocyte was estimated to have 200.9 $\pm$ 14.5 receptor sites, Table 5. In order to analyse any changes in affinity more precisely, the present data was plotted as an average affinity profile, Figure 22. The average affinity of the receptor was plotted against the $\log_{10}$ of the fractional receptor occupancy. The overall affinity of erythrocytes for insulin in volunteers both before and after fasting was marginally greater at low insulin concentrations, when the receptors were empty ($\bar{K}_e$, low occupancy), than at high insulin cocnentrations, when the receptors were "full" or "occupied" ($\bar{K}_f$, high occupancy). The empty sites affinity ($\bar{K}_e$) had values of 0.0915 nM^{-1} and 0.0965 nM^{-1} before and after fasting, respectively. These values began to decrease with increasing occupancy of the receptors by insulin. The apparent $\bar{K}$ progressively decreased until $\bar{K}_f$ values of 0.0138 nM^{-1} and 0.013nM^{-1} were obtained for erythrocytes taken from volunteers before and after fasting, respectively. These latter values were obtained with about 10% of the available receptor sites occupied. The differences between the values obtained for the various affinity constants were not statistically significant, Table 5.

Figure 21. Scatchard analysis of insulin binding data before and after overnight fasting.
Each value represents the mean of 7 determinations.
(×– –×) before an overnight fast.
(o——o) after an overnight fast.

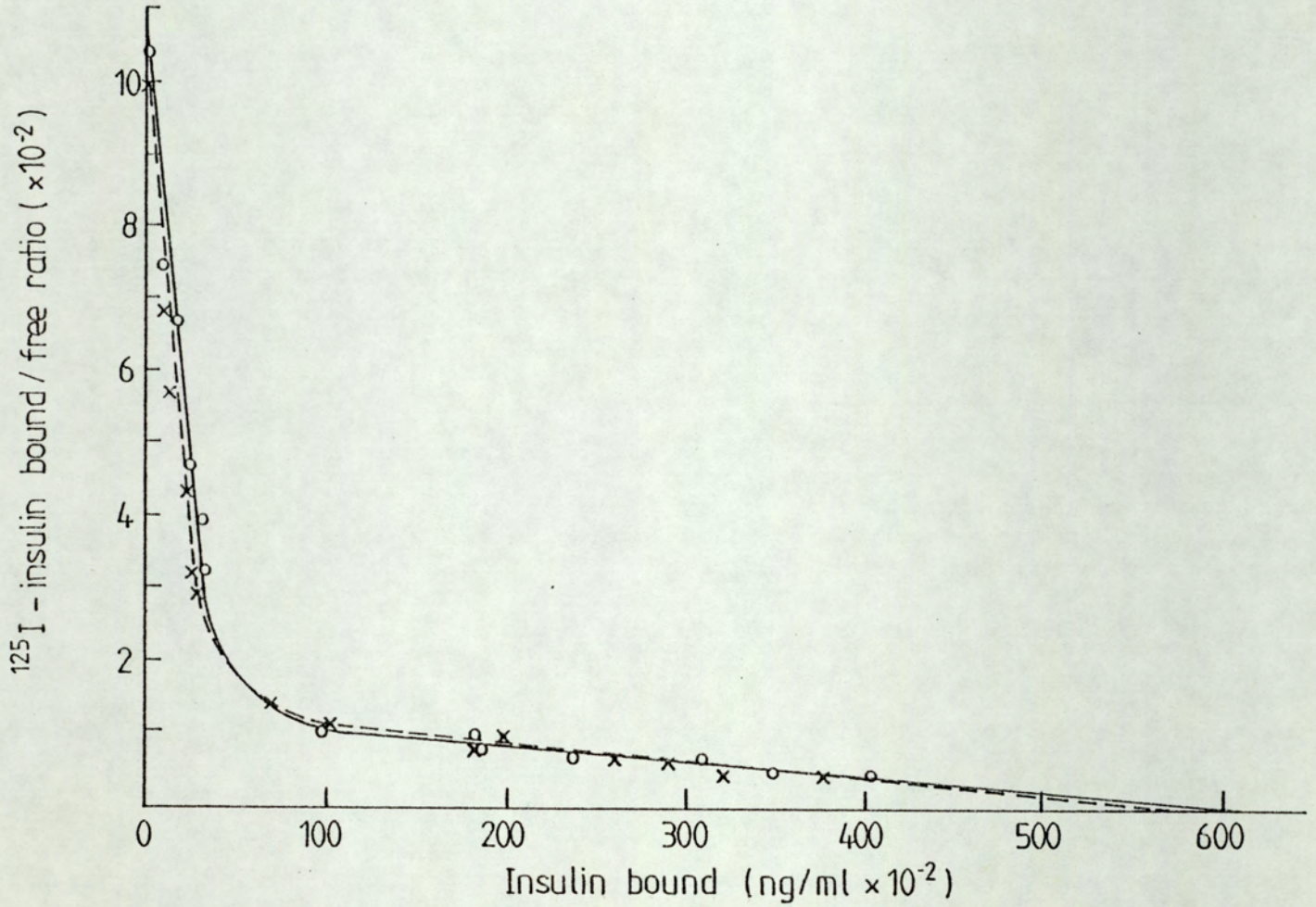
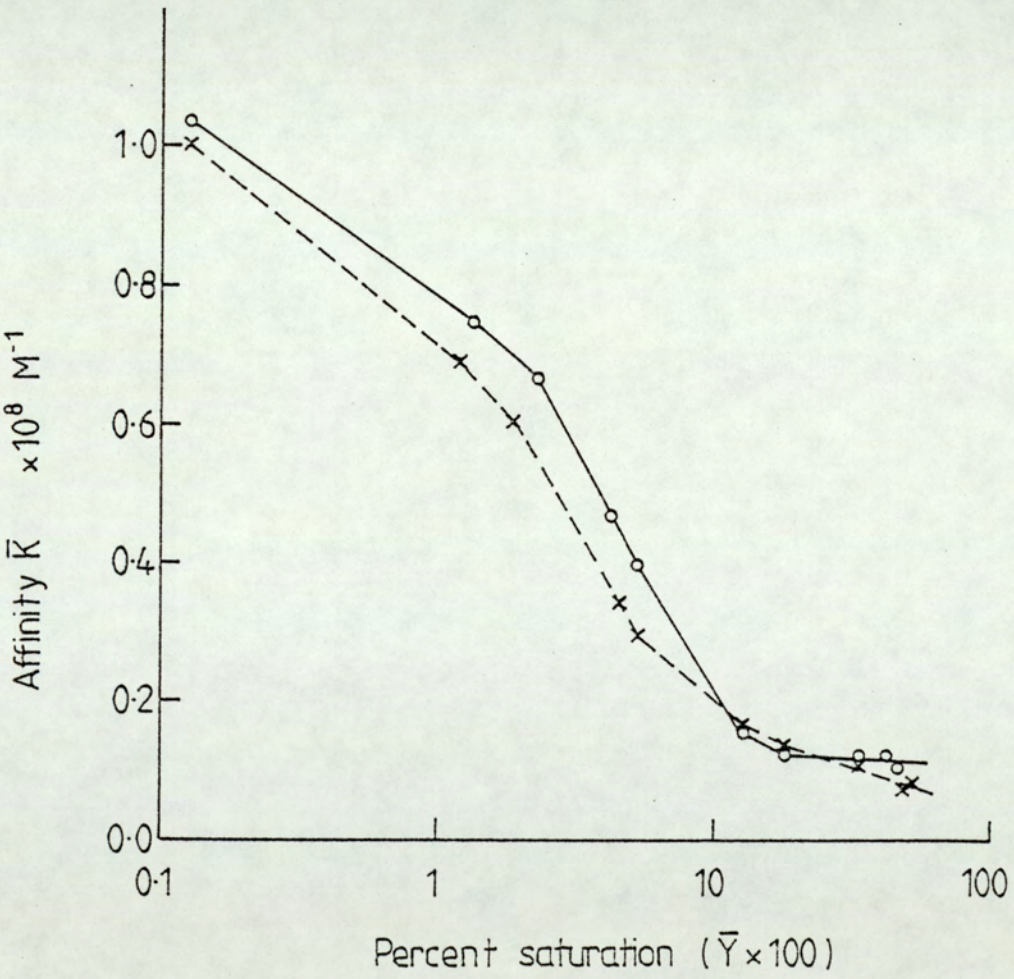


Figure 22. Average affinity profile of ^{125}I - labelled insulin binding data before and after fasting.
Each point represents the mean of 7 determinations.
(×—×) before an overnight fast.
(o—o) after an overnight fast.



Discussion

The present data confirmed that insulin binding to human erythrocytes is a physiological phenomenon. Human erythrocytes have been shown to have highly specific insulin receptors (121), and the characteristics of insulin binding to erythrocytes appear to be similar to those for insulin binding to human monocytes (209), granulocytes (119) and adipocytes (219). As with other cell types, the binding of a physiological concentration of unlabelled porcine insulin to human erythrocytes produced a greater than 50 percent inhibition of the total ^{125}I-insulin binding (121,340). Also ^{125}I-insulin binding to erythrocytes increased over the physiological range of insulin concentrations (0-10 ng/ml) suggesting that a similar interaction may take place *in vivo*. The binding of insulin to erythrocytes was represented by nonlinear Scatchard plots and characterised by decreased receptor affinity with increased receptor occupancy. Similar observations have been made for lymphocytes (312) and insulin sensitive target tissues such as adipocytes (229). In the present study the evaluation of insulin binding data, showed that fasting did not significantly affect the ability of erythrocytes to bind insulin over the whole range of unlabelled insulin concentrations used. The total amount of insulin bound was a function of both receptor affinity and concentration. Both the ability of unlabelled insulin to inhibit ^{125}I-insulin binding and the 50% inhibition of ^{125}I-insulin binding to erythrocytes by unlabelled insulin was not influenced by fasting, suggesting no change in receptor affinity. The mean values for erythrocyte insulin receptor concentration before and after fasting were 190.3 and 200.8 sites per

erythrocyte, respectively. These values were significantly lower than previously recorded values of 2000 sites per cell by Gambhir and co-workers (121) but higher than values computed by Kappy and Plotnick (340) of 20-140 receptor sites per erythrocyte. These seemingly large discrepencies are due to the different interpretations of the binding data. Gambhir and co-workers (121) used insulin concentrations of over 1000 ng/ml in order to calculate the maximum binding capacity. On the other hand Kappy and Plotnick (340) used a maximum insulin concentration of 100 ng/ml. In the present study the highest insulin concentration used for the Scatchard analysis was 1000 ng/ml.

One of the important assumptions for the interpretation of Scatchard plots is the absence of inter-site interactions. However, negative cooperativity has been postulated for insulin receptors (312). Therefore, the presence of negative cooperative interactions suggested that the receptor affinity constants obtained from the linear slope of the Scatchard plot probably do not have a great deal of precise physio-chemical meaning. Interpretation of the present data using the negatively cooperative model gave support to the previous analysis and suggested that overnight fasting had no significant effect on either the concentration or affinity of erythrocyte receptors. These observations are supported by the work of Bar and colleagues who have shown that fasting for 24 hours has no effect on insulin binding to circulating monocytes (196). On the other hand recent work by McElduff and Eastman has demonstrated an increased insulin receptor binding in human erythrocytes after an extended overnight fast (329).

CHAPTER FOUR

A - The Effect of the Antihypertensive Drug Bendrofluazide and Subsequent Potassium Supplementation on Erythrocyte Insulin Receptor Binding, Glucose Tolerance, Serum Glucose and Insulin Levels and General Biochemistry of Mildly Hypertensive Patients With and Without Non-Insulin Dependent Diabetes Mellitus.

B - The Effect of Long-term Nifedipine Treatment, Subsequent Withdrawal and Restoration of Nifedipine Treatment on the Oral Glucose Tolerance and Serum Insulin and Glucose Levels of Mildly Hypertensive Patients With and Without Non-insulin Dependent Diabetes Mellitus.

A. The effect of the antihypertensive drug bendrofluazide and subsequent potassium supplementation on erythrocyte insulin receptor binding, glucose tolerance, serum glucose and insulin levels and general biochemistry of mildly hypertensive patients with and without non-insulin dependent diabetes mellitus.

Introduction

Diuretic therapy has been used as a successful form of antihypertensive therapy for many years (65,77,85). However, the exact mechanism of action of the diuretic agents in reducing the blood pressure has still not been fully elucidated. Sodium and water excretion by the kidney undoubtedly play an important part, but whether it is purely the reduction in extracellular fluid volume or a more subtle alteration in the sodium content of various body compartments in particular the vascular smooth muscle wall, which leads to the reduction of blood pressure is still not known (341). Diuretic agents on their own are only mild antihypertensive agents, but they are very important adjuncts to other forms of antihypertensive therapy, most of which tend to cause a compensatory fluid retention and thus a loss of antihypertensive efficiency. It is important to administer low dose of diuretics because, with increasing doses, the number and degree of unwanted biochemical side effects increase, whereas the antihypertensive action does not increase correspondingly (88,342). Although this has been well known for a long time, diuretics are often still given in doses that exceed by far those necessary for the maximum effect on blood pressure. A major advantage in the use of thiazides is that in addition to

producing a modest fall in blood pressure, they potentiate the effect of all other drugs which lower the blood pressure (343). Continuous treatment with diuretics often leads to potassium depletion in man (345). Most patients taking these thiazides for any length of time are given potassium prophylactically (79). The body-potassium is, however, located mainly within the cells, less than 3% being in the extracellular fluid, so that potassium status is only indirectly related to the plasma-potassium concentration. The estimation of intracellular potassium is difficult practically (79). Many clinicians feel that potassium supplementation is not necessary with the routine use of diuretic agents in the treatment of otherwise uncomplicated mild hypertension (344). In the case of both potassium retaining diuretics and potassium supplementation, the risk associated with diuretic therapy is increased, since potassium-retaining drugs may induce hyperkalaemia, particularly in patients whose renal function is impaired. In addition potassium supplementation may cause nausea and local damage to the gastrointestinal mucosa, depending on the type of pharmaceutical preparation used (76). Thiazide diuretics,have been shown to lower the serum potassium levels of treated hypertensive patients (346,347). The lowering effect appeared after about 6 weeks administration (345) and within one year (348). Other studies have reported that the administration of bendrofluazide to mildly hypertensive men and women did not significantly affect the serum potassium after 12 months of therapy (349). However, the serum potassium of diabetics has been observed to fall after 5 years continuous therapy with bendrofluazide without a change in total body potassium (85). There is evidence to suggest that diuretics do have an hyperglycaemic effect (65,78,80).

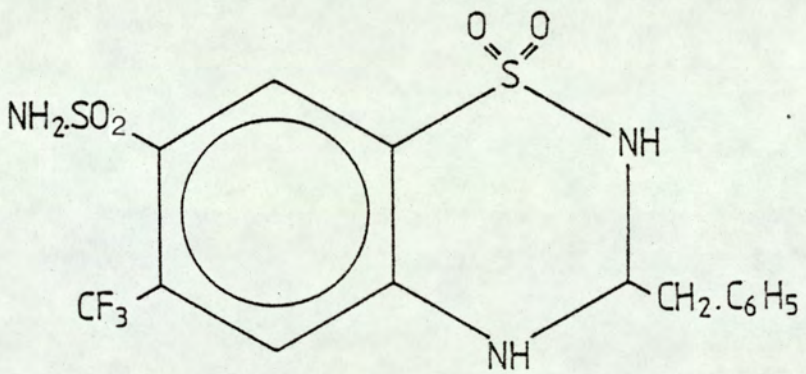
Breckenridge and colleagues (350) observed high blood glucose levels together with low levels of serum insulin in hypertensive patients after 3 years of thiazide therapy. In another study no change in glucose tolerance was observed in hypertensive patients treated with bendrofluazide for one year, but a significant deterioration was observed after 6 years. These workers suggested that prolonged oral treatment with thiazide was diabetogenic (80). Recently Pacy and colleagues (66) have demonstrated a deterioration in the glycaemic control of mild hypertensive diabetic patients after 3 months on bendrofluazide therapy. Other investigators have disputed that thiazide are causally related to glucose intolerance (85). Diabetes may be precipitated by the use of thiazide diuretics, particularly in the elderly (351). There have been a number of reports in the literature stating that thiazides and related diuretics can cause or exacerbate diabetes mellitus (350,352) via a deterioration in glucose tolerance (352), but a role for thiazides in provoking glucose intolerance in previously glucose-tolerant subjects is controversial. Decreased insulin secretion by the pancreas (353), a decrease in tissue sensitivity to insulin (354), increased insulin output, accelerated development of insulin depletion in the prediabetic state (355) and an effect on the enteropancreatic insulin axis (356) have all been offered as causal mechanisms. Certainly an impairment in glucose tolerance is most marked in those patients in whom the serum potassium has been shown to decrease over a 2 year period of therapy (78).

In addition to potassium depletion and glucose intolerance, hyperuricaemia and changes in the lipid pattern have also been observed during thiazide therapy for mild hypertensive patients

(83,357). Serum urea and uric acid levels have been shown to rise in men and women taking bendrofluazide for periods of less than 12 months (349). The increase in serum uric acid can be a practical problem, because of the association between hypertension and gout. In theory hyperuricaemia could contribute to the deterioration of renal function in hypertension and aggravate generalised arterial disease (358). However other workers have not been able to show a significant change in serum uric acid levels after 12 or even 24 months of treatment with bendrofluazide (84). There is evidence to suggest that thiazide diuretics elevate both serum cholesterol and triglyceride levels (81,82) but other studies have shown that bendrofluazide therapy does not significantly influence either mean serum chlosterol or triglyceride levels (66). Elevated serum lipid levels are known to be risk factors for the development of coronary heart disease (359) and in mild hypertension changes in the plasma lipid pattern may antagonize any possible benefits of blood pressure reduction and be responsible for the failure of diuretics to prevent myocardial infarction in hypertensive patients (360). Thiazide diuretics have been shown to cause hypercalcaemia and an association between bendrofluazide treatment and impotence has also been reported (347). With the exception of diabetes, gout and hypokalemia, the clinical significance of changes in biochemical parameters is uncertain (361). Any deterioration in either potassium, glucose, cholesterol or uric acid must be weighed against the benefits of treatment which may be modest in patients with mild hypertension (65). However, thiazides are not recommended for the treatment of non-insulin requiring diabetic patients with hypertension since thiazides can aggravate glucose tolerance (362). Despite this,

thiazide diuretic therapy is still widely used in the treatment of both insulin and non-insulin dependent diabetic patients with hypertension (66).

In the present study bendrofluazide was used for the treatment of mildly hypertensive patients with and without diabetes. Bendrofluazide is a thiazide diuretic that produces a prolonged diuresis (18 hrs). The thiazide retails as Aprinox(R); Bendroflumethiazide; Berkozide(R); and Neo-NaClex(R) and has the following structural formula (3-Benzyl-3,4-dihydro-6-trifluoromethyl-2H-benzo-1,2,4-thiadiazine-7-sulphonomide 1,1-dioxide.



$C_{15}H_{14}F_3N_3O_4S_2 = 421.4$

Structure of bendrofluazide

Bendrofluazide is readily absorbed after oral administration, and after an oral dose of 10mg peak plasma concentrations of 50 to 100 ng/ml are attained in 2 to 2.5 hours. The plasma half-life, during the period 4 to 10 hours after oral administration is about 2.7 hours. About 95% of the dose is bound to plasma proteins and 45 to 77% is excreted unchanged in the urine within 24 hrs (363). The frequency of metabolic side effects during bendrofluazide treatment of mild to moderately severe essential hypertension is low and has

been grossly exaggerated (85). It is well established that the deterioration in glucose tolerance following the administration of thiazides occurs more rapidly in diabetic patients (364). Many mechanisms have been proposed to account for thiazide induced glucose intolerance including effects on insulin secretion (353), insulin sensitivity and receptor and postreceptor actions (354,256,370). The actions upon insulin secretion may be a consequence of thiazide induced hypokalaemia (87,365). Potassium has been shown to be an important ion for the release of insulin *in vitro* (365). Insulin. *per se* may play an important role in the regulation of serum potassium levels (366). Some reports have suggested that thiazide induced glucose intolerance may be prevented or even reversed by the administration of potassium supplementation (87). This may provide an important way of reducing not only the potential adverse effects of hypokalaemia but also glucose intolerance.

The purpose of this study was to examine the effects of the thiazide, bendrofluazide and subsequent potassium supplementation on gluocse tolerance, serum glucose and insulin levels, insulin receptor binding and general biochemistry and haemotology in mild hypertensive patients with and without non-insulin dependent diabetes mellitus over a period of 12 months with placebo control.

Materials and Methods

Newly diagnosed mild untreated hypertensive patients with and without non-insulin dependent diabetes mellitus were recruited for the study from the Diabetic Clinic at Dudley Road Hospital, Birmingham. All diabetic patients were controlled by dietary

carbohydrate restriction prior to study, no patients were taking any other medication in particular oral antidiabetic therapy. Only male and postmenopausal females were recruited because there is evidence to suggest that insulin binding may change during the stages of the menstrual cycle and may exhibit a diurnal variation (108). All patients had seated blood pressures greater than 160/95 mm Hg measured on two occasions by trained observers (Hawksley random zero sphygmomanometer) using phase 5 diastolic blood pressure. Secondary hypertension was excluded. The study was conducted in a single-blind manner. Patients with non-insulin dependent diabetes were diagnosed on the basis of a 75 g oral glucose test showing a 60 minute value of greater than 11 mmol/l glucose. Twenty patients were originaly recruited into the study. However, two non-diabetic patients did not complete the study. One patient had a myocardial infarction and subsequently died. The other repeatedly vomitted during the oral glucose tolerance tests. The results of these two patients were subsequently excluded from analysis when all the visits were compared. Of the eighteen patients that completed the study nine were non-diabetic (four male and five female) and nine were diabetic patients (eight male and one female) with mean ages of 56.8 $\pm$ 2.3 and 63.8 $\pm$ 2.1 years, respectively and mean body weights of 79.5 $\pm$ 4.2 and 70.6 $\pm$ 2.3 Kgs (mean $\pm$ SEM) respectively. The ages and body weights of diabetic and non-diabetic patients were not significantly different. Each patients served as his or her own control. 75 g oral glucose tolerance tests (OGTT - page 119) were performed at recruitment, after one month of bendrofluazide placebo treatment (methylcellulose), after one month treatment with bendrofluazide 5 mg daily, after a further 3 months of bendrofluazide and after one month

of bendrofluazide with potassium supplementation. Potassium was administered as the chloride salt (20 mg three times daily as Kloref S).

Patients presented at 09.00 hrs in the clinic after a 12 hour overnight fast. Body weight, pulse and blood pressure measurements were taken seated and then approximately 30 ml of venous blood was taken by venipuncture for haematology (full blood count and platelets), biochemistry (urea, electrolytes, liver function tests, serum glucose and insulin and lipid profile). Fasting blood was taken for erythrocyte insulin receptor binding and results were analysed by computer-assisted Scatchard analysis (page 102) using two classes and negatively cooperative models. During the OGTT venous blood samples were taken for glucose and insulin at 0,15,30,45,60,90 and 120 minute intervals. Urine samples were analysed for total protein, glucose and haemoglobin. Glucose was determined using an automated glucose oxidase procedure (318) and insulin concentration determined using a double-antibody radioimmunoassay (317) (page 112). The area under the curve for glucose and insulin during the oral glucose tolerance test were calculated using parallogram approximation between each consecutive pair of time intervals (Appendix 9, page 299 and Appendix 14, page 308). A complete protocol of the study has been included in Appendix 10, page 300. In this study analysis of variance was used to examine group effects (between diabetics and non-diabetics), and treatment effects between groups. Parameters in which treatment effects were observed at the $P < 0.05$ level were further analysed by a priori contrasts comparing each bendrofluazide treatment period with placebo and the effect of potassium supplementation with the level achieved after 4 months bendrofluazide treatment. The study was approved by the hospital

ethical committee. Patient compliance was assessed by medication counting and subjects were allowed to continue the study if more than 80 percent of medication had been taken.

Results

Diabetic and non-diabetic patients were studied at recruitment and after 1 month on placebo, prior to treatment with bendrofluazide as indicated in the protocol (Appendix 10, page 300). Analysis of the data indicated that the placebo had no significant effect upon either the insulin receptor binding status, glucose and insulin levels or biochemical and haemotological parameters of non-diabetic and diabetic patients. Hence in this study, placebo values were used as controls and compared with data obtained after treatment with bendrofluazide and bendrofluazide supplemented with potassium.

Mildly hypertensive non-diabetic patients:

In non-diabetic mild hypertensive patients, bendrofluazide and subsequent potassium supplementation significantly reduced systolic and diastolic blood pressures, Table 6. In this group bendrofluazide therapy and subsequent potassium supplementation had no significant effect upon body weight, fasting blood glucose and fasting insulin levels, Table 6.

Glucose tolerance appeared to deteriorate in these patients during treatment but did not achieve statistical significance, Figure 23. Bendrofluazide and subsequent potassium supplementation had no significant effect upon either the area under the OGTT curve or the gluocse/insulin ratio, Table 6, Figures 24 and 25. Fasting serum

Table 6. The effect of bendrofluazide treatment on the body weight, blood pressure, fasting glucose and insulin of mildly hypertensive non-diabetic patients (means values ± SEM).

Treatment	n	Body Weight (Kg)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Fasting blood glucose level (mmol/l)	Fasting insulin level (µU/ml)	Area under the OGTT curve (mmol/l/ 120 minutes)	Area under the OGTT insulin curve (µU/ml/ 120 minutes)
1 month placebo (control) (P)	9	80.02 ± 4.1	161.8 ± 5.08	110.2 ± 10.9	4.6 ± 0.31	37.8 ± 8.6	781.5 ± 62	25975 ± 5987.6
1 month bendrofluazide (B1)	9	78.6 ± 3.8	146 * ± 1.53	94.0 * ± 1.3	4.44 ± 0.13	46.6 ± 12.2	779.8 ± 47.4	28619.2 ± 5709.3
3 months bendrofluazide (B3)	9	79.1 ± 3.5	143.3 * ± 4.2	90.9 * ± 2.3	4.7 ± 0.23	51.3 ± 13.5	847.9 ± 63.9	24271.7 ± 4655.9
1 month potassium supplementation with bendrofluazide (K)	9	79.3 ± 3.8	144.4 * ± 4.37	94.0 * ± 2.16	4.57 ± 0.13	27.2 ± 4.52	809 ± 48	18290.8 * ± 3453.8

* $p < 0.05$ compared with values obtained after one month on placebo.

Figure 23. The effect of bendrofluazide and subsequent potassium supplementation on the oral glucose tolerance of mildly hypertensive non-diabetic patients. (mean values, SEM omitted for clarity).

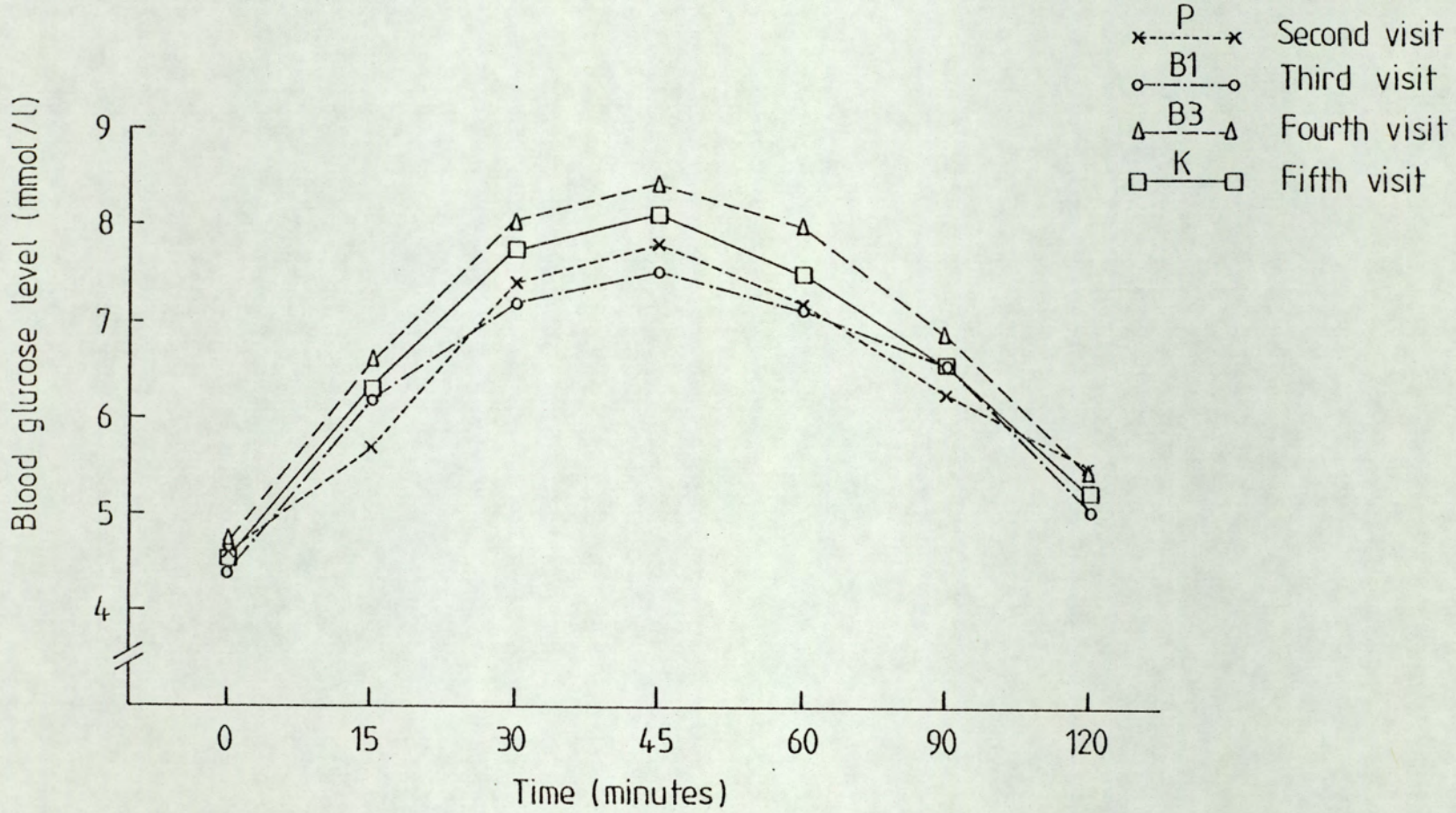


Figure 24 . The effect of bendrofluazide and subsequent potassium supplementation on the area under the curve of the OGTT (mean values ± SEM) .

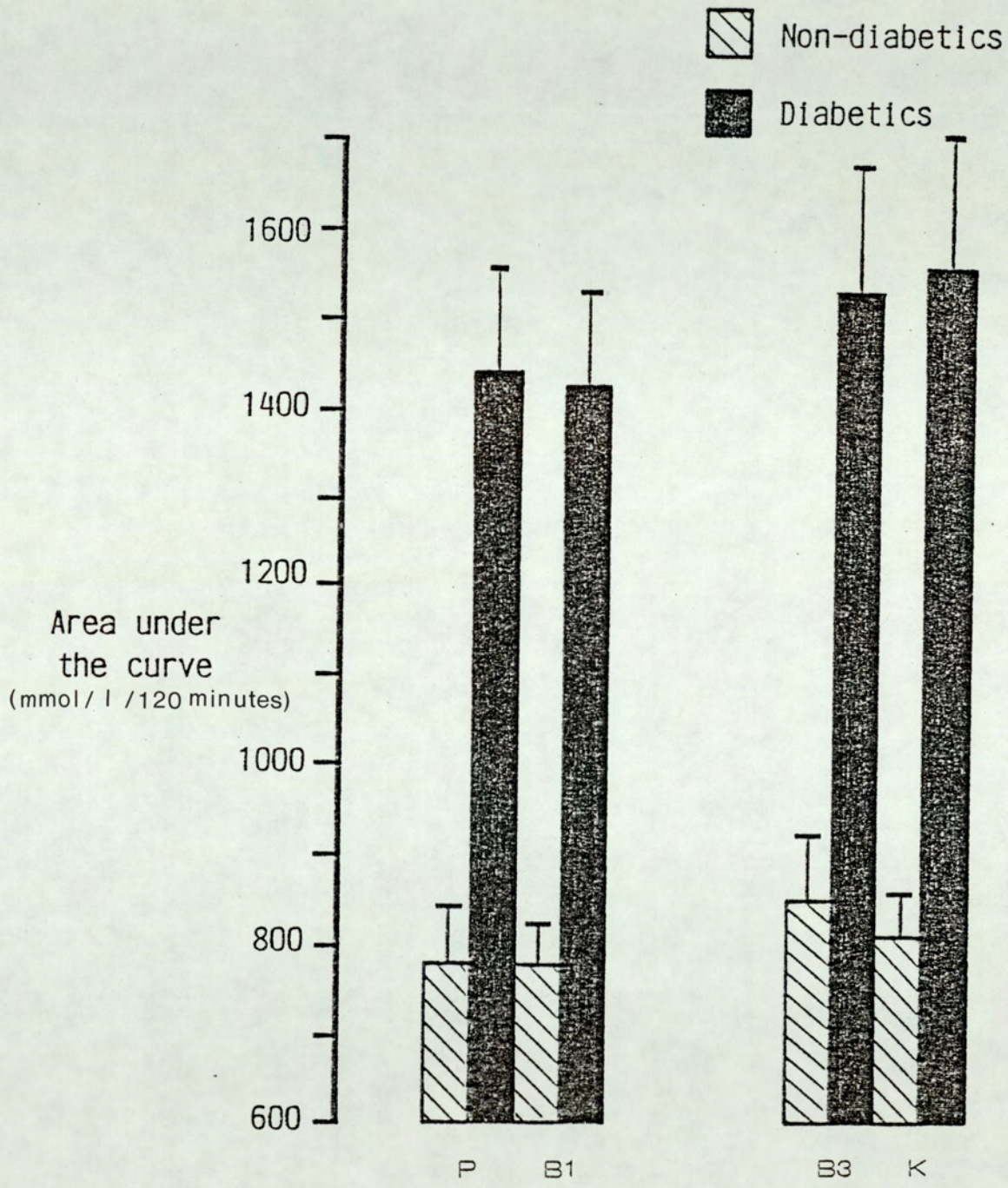
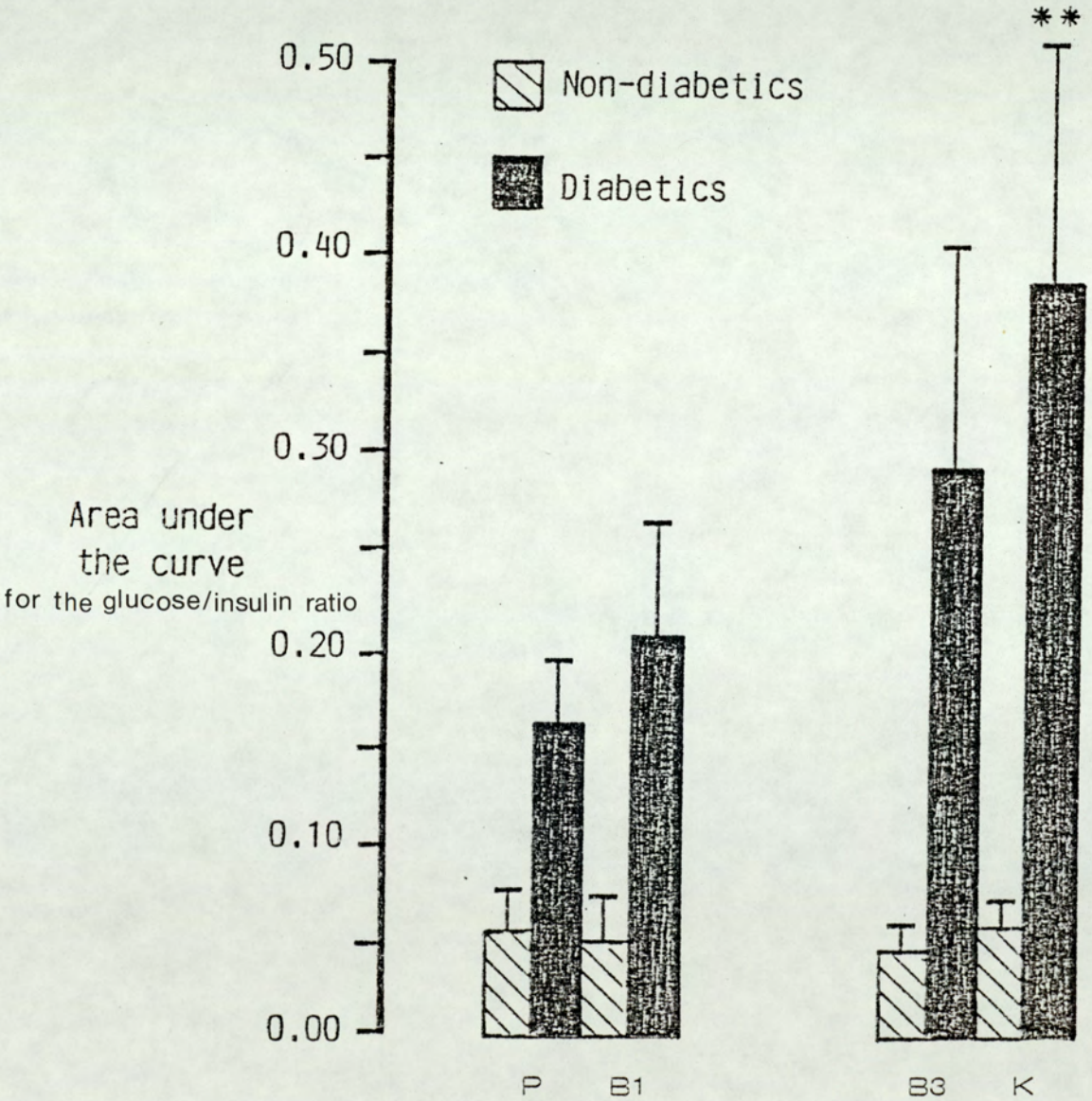


Figure 25 . The effect of bendrofluazide and subsequent potassium supplementation on the glucose/insulin ratio (mean values ± SEM) .



** $p < 0.01$ compared with placebo value .

insulin levels, Table 6, and serum insulin levels estimated during OGTT, Figure 26 were marginally elevated after bendrofluazide treatment but decreased when the bendrofluazide was supplemented with potassium. Potassium supplementation significantly reduced the area under the curve of the OGTT insulin levels in mildly hypertensive non-diabetic subjects, Figure 27.

Bendrofluazide treatment and subsequent potassium supplementation had no significant effect on the biochemical profile of non-diabetic mild hypertensive patients, Table 7. Following potassium supplementation, the serum potassium increased by 0.06 mmol/l but just failed to achieve the serum potassium level obtained with placebo.

Bendrofluazide significantly increased the total insulin receptor concentration of erythrocytes from mildly hypertensive non-diabetic patients, Table 8, and this increase was largely accounted for by a significant increase in the concentration of low affinity receptors, Figure 28. The total insulin receptor concentration (sites per cell) was increased significantly after 1 month of bendrofluazide therapy and this increase was maintained at 3 months and after potassium supplementation, Figure 29. There were no significant changes in the various affinity constants ($\bar{K}_e$, $\bar{K}_f$ or $\bar{K}_m$) with bendrofluazide treatment, Table 8.

Mildly hypertensive diabetic patients:

Bendrofluazide treatment and subsequent potassium supplementation had no significant effect on the body weight of mildly hypertensive diabetic patients but significantly reduced both

Figure 26. Serum insulin levels during oral glucose tolerance tests on mild hypertensive non-diabetic patients treated with bendrofluazide and subsequent potassium supplementation. (mean values, SEM omitted for clarity).

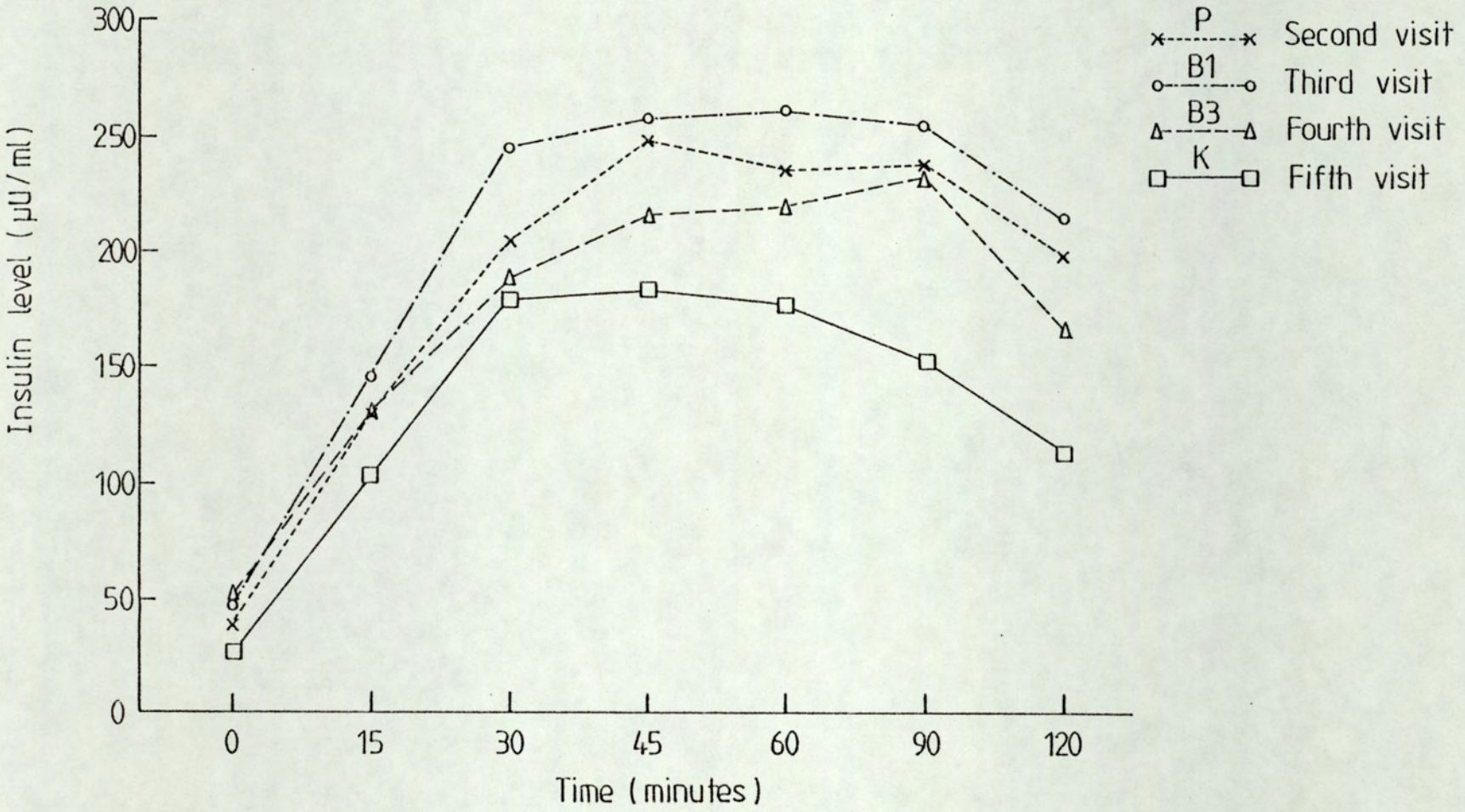
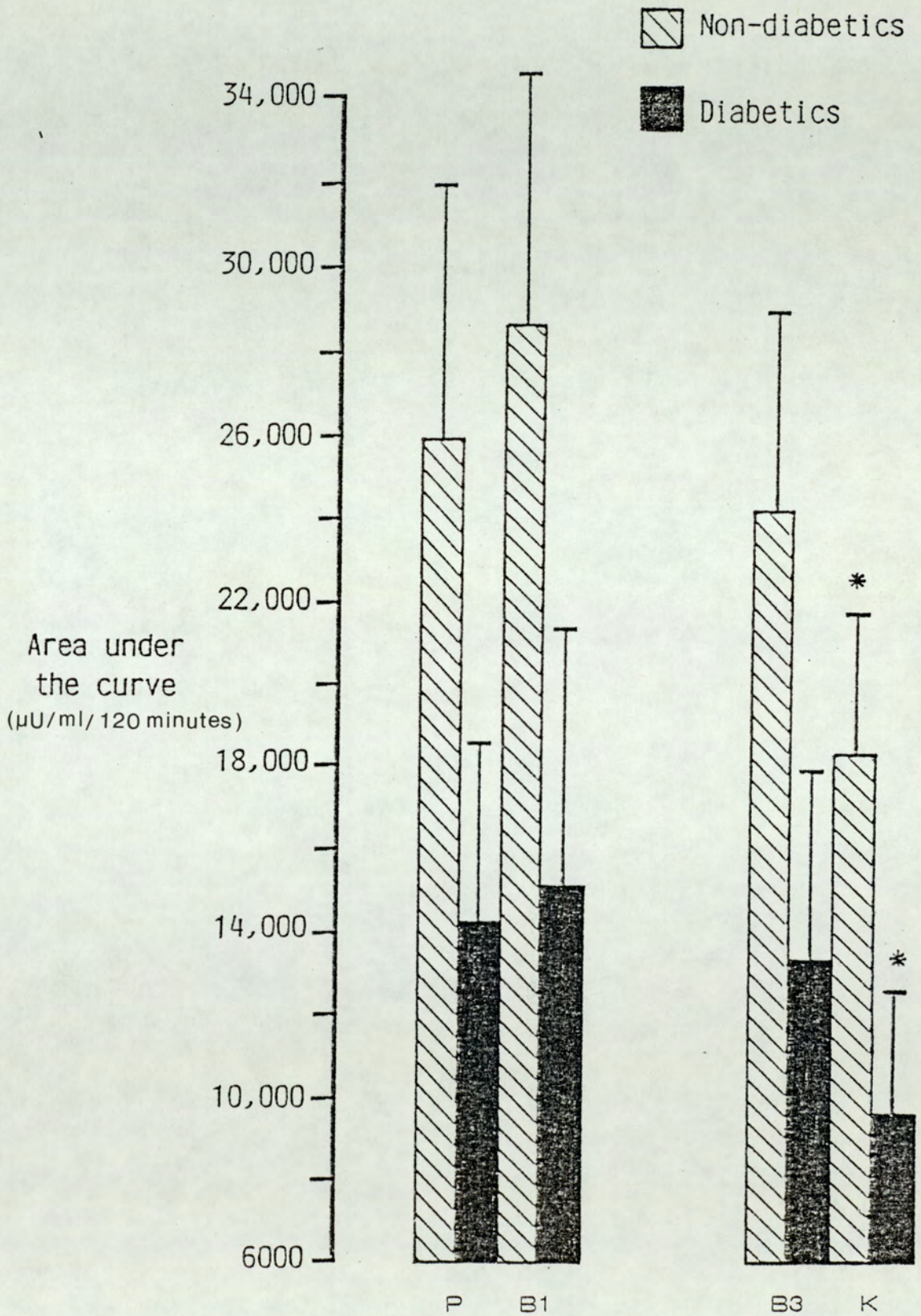


Figure 27 . The effect of bendrofluazide treatement and subsequent potassium supplementation on the area under the curve of the OGTT insulin levels (mean values ± SEM) .



* $p < 0.05$ compared with placebo value .

Table 7. Biochemical profile for mildly hypertensive non-diabetic patients treated with bendrofluazide and subsequent potassium supplementation (Mean values ± SEM).

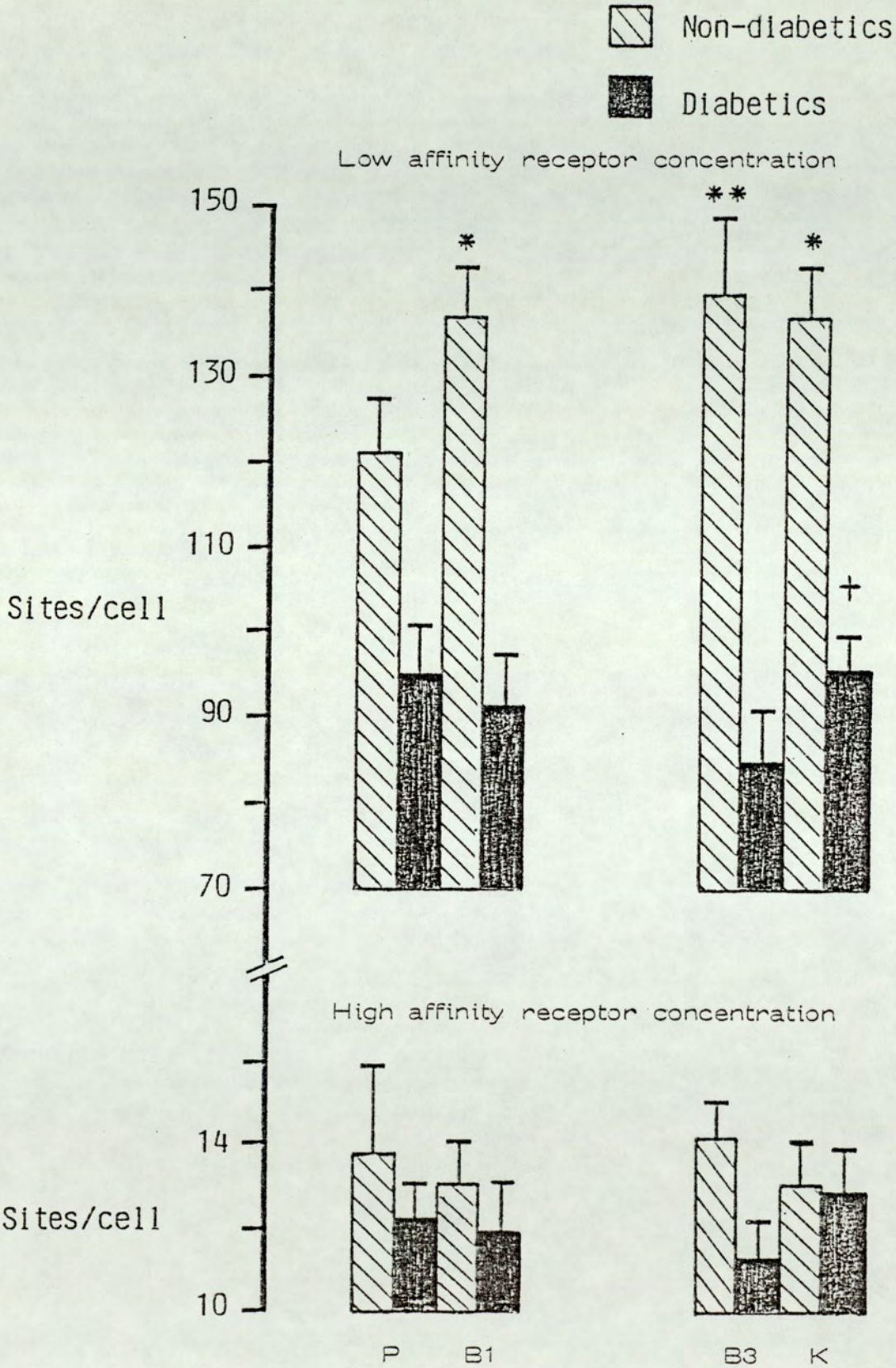
Treatment	n	Cholesterol (mmol/l)	Urea (mmol/l)	Uric Acid (mmol/l)	Serum potassium (mmol/l)	Platelets ($x10^9$/l)	MCV (fl)	Triglycerides (mmol/l)
1 month placebo (control) (P)	9	5.53 ± 0.25	4.74 ± 0.29	0.384 ± 0.04	4.5 ± 0.12	247.7 ± 22.6	85.6 ± 2.1	1.38 ± 0.21
1 month bendrofluazide (B1)	9	6.03 ± 0.21	5.73 ± 0.63	0.434 ± 0.05	4.34 ± 0.13	274.4 ± 27.3	85.0 ± 2.2	1.45 ± 0.28
3 months bendrofluazide (B3)	9	5.87 ± 0.26	5.72 ± 0.62	0.395 ± 0.04	4.27 ± 0.12	273.1 ± 11.1	83.6 ± 2.1	1.47 ± 0.25
1 month potassium supplementation with bendrofluazide (K)	9	6.02 ± 0.33	5.48 ± 0.51	0.42 ± 0.04	4.33 ± 0.1	299.4 ± 14.4	83.6 ± 2.1	1.64 ± 0.29

Table 8. Erythrocyte insulin receptor status of mildly hypertensive non-diabetic patients treated with bendrofluazide and subsequent potassium supplementation (mean values ± SEM).

Treatment	n	Specific binding (%)	High Affinity receptor concentration per cell	Low Affinity receptor concentration per cell	Total Affinity receptor concentration per cell	$\bar{K}_e$, affinity constant of empty sites (nM^{-1})	$\bar{K}_f$, affinity constant of full sites (nM^{-1})	$\bar{K}_m$, apparent affinity constant (nM^{-1})
1 month placebo (control) (P)	9	6.68 ± 0.24	13.84 ± 2.1	121.1 ± 6.3	134.93 ± 6.5	0.10416 ± 0.005	0.01709 ± 0.002	0.05508 ± 0.003
1 month bendrofluazide (B1)	9	8.08* ± 0.52	13.06 ± 1.1	136.9* ± 5.6	150* ± 5.68	0.11868 ± 0.012	0.01514 ± 0.001	0.06204 ± 0.006
3 months bendrofluazide (B3)	9	7.95* ± 0.37	14.2 ± 0.85	139.54** ± 8.4	153.7** ± 8.4	0.11434 ± 0.008	0.01657 ± 0.002	0.06007 ± 0.004
1 month potassium supplementation with bendrofluazide (K)	9	7.46* ± 0.43	13.02 ± 0.6	136.7* ± 5.9	149.7* ± 5.64	0.1047 ± 0.008	0.01562 ± 0.001	0.05486 ± 0.004

* $p < 0.05$, ** $p < 0.01$ when compared with corresponding placbo value.

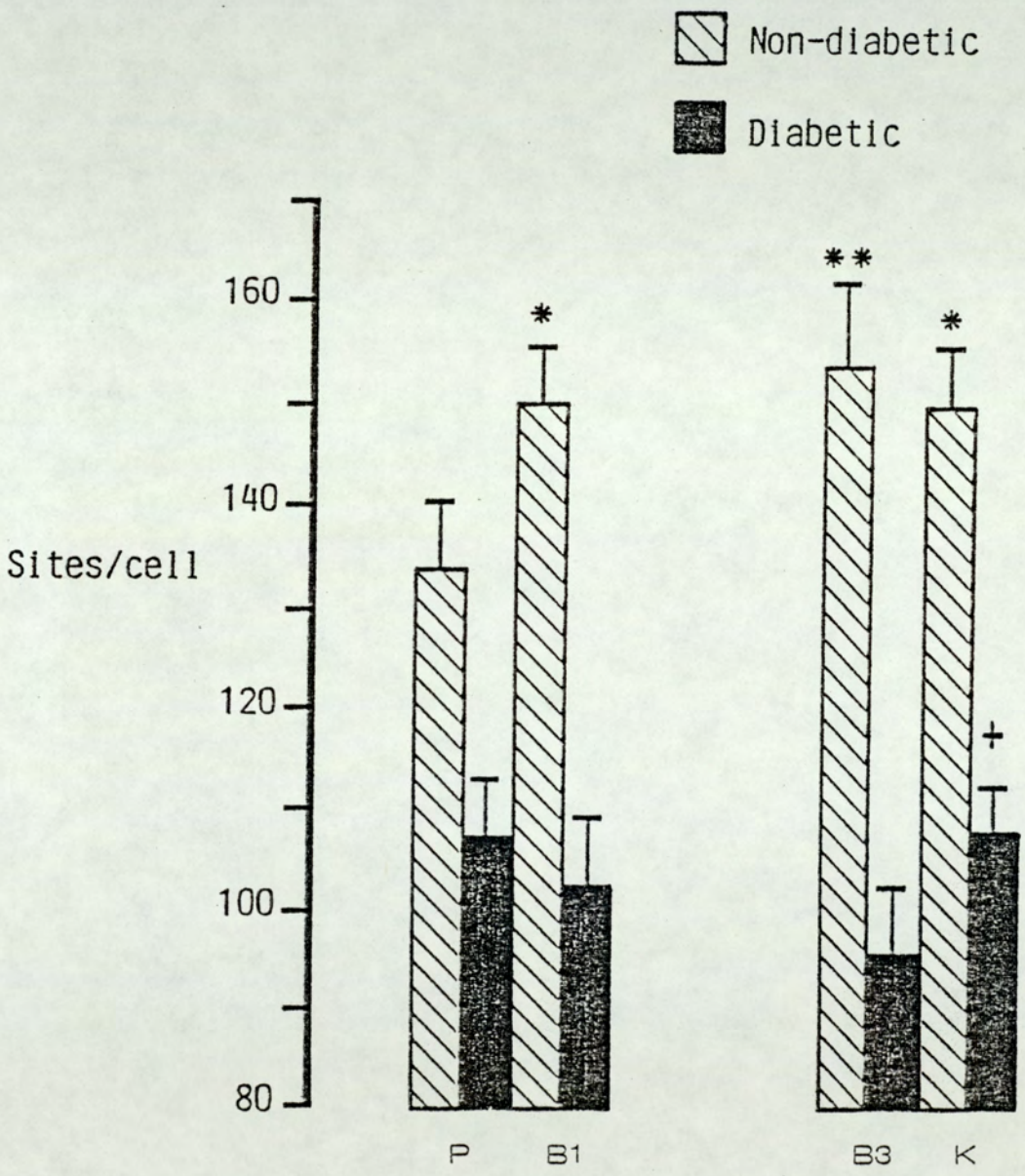
Figure 28 . The effect of bendrofluazide therapy on the low and the high affinity concentrations of mildly hypertensive non - diabetic and diabetic patients (mean values ± SEM) .



* p < 0.05 ,** p < 0.01 compared with placebo value.

+ p < 0.05 , compared with 3 months bendrofluazide value .

Figure 29 . The effect of bendrofluazide and subsequent potassium supplementation on the total insulin receptor concentration of mildly hypertensive non - diabetic and diabetic patients (mean values ± SEM) .



* $p < 0.05$,** $p < 0.01$ compared with placbo value .

+ $p < 0.05$ compared with 3 months bendrofluazide value .

systolic and diastolic blood pressures, Table 9. The fasting blood glucose rose significantly in diabetics after 4 months of bendrofluazide treatment but was subsequently reduced by potasssium supplementation, Table 9. Potassium supplementation did not lower the fasting blood glucose of diabetic patients back to placebo levels. Gluocse tolerance, Figure 30, and the area under the OGTT, Figure 24, deteriorated in diabetic patients treated with bendrofluazide and subsequent potassium supplementation but these changes did not achieve statistical significance. The glucose/insulin ratio tended to increase with bendrofluazide treatment reaching statistical significance when the bendrofluazide was supplemented with potassium, Figure 25. The fasting serum insulin levels of diabetic patients, though slightly elevated after the first month of bendrofluazide treatment, declined after three further months of treatment, Table 9. In the diabetic group insulin levels continued to diminish despite potassium supplementation although statistical significance was never achieved. Insulin levels of diabetic patients during OGTT, Figure 31, and the areas under the insulin curves obtained during the OGTT, Figure 27, were reduced as a result of bendrofluazide treatment, but only reached statistical significance after subsequent potassium supplementation.

Bendrofluazide treatment and subsequent potassium supplementation had no significant effect on the biochemical and haematological profiles of mildly hypertensive diabetic patients, Table 10.

After one month on placebo the total specific insulin binding to erythrocytes and the total and low affinity receptor concentrations were all significantly lower in mildly hypertensive diabetic patients

Table 9. The effect of bendrofluazide and subsequent potassium supplementation on the body weight, blood pressure, glucose and insulin levels of mildly hypertensive diabetic patients (mean values ± SEM).

Treatment	n	Body Weight (Kg)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Fasting blood glucose level (mmol/l)	Fasting insulin level (μU/ml)	Area under the OGTT curve (mmol/l/ 120 minutes)	Area under the OGTT insulin curve(μU/ml/ 120 minutes)
1 month placebo (control) (P)	9	70.4 ± 2.3	164.9 ± 8.1	88.9 ± 3.1	6.66 ± 0.71	34.1 ± 6.41	1439.5 ± 118.1	14315.8 ± 4316.5
1 month bendrofluazide (B1)	9	69.2 ± 2.2	147.6* ± 7.8	82.9* ± 2.6	7.14 ± 0.9	46.1 ± 8.9	1427.6 ± 109.6	15189.2 ± 6169.5
3 months bendrofluazide (B3)	9	69.2 ± 1.8	154.7* ± 8.9	82.9* ± 2.7	8.2* ± 0.9	30 ± 8.24	1521.3 ± 143.3	13336.7 ± 4656.1
1 month potassium supplementation with bendrofluazide (K)	9	68.8 ± 2.1	145.1* ± 8.8	80.8* ± 4.4	7.33 ± 0.98	25.4 ± 9.0	1554.4 ± 153.9	9534.2* ± 3667.1

* $p < 0.05$ when compared with corresponding placebo value.

Figure 30. 75 gm oral glucose tolerance tests on mildly hypertensive diabetic patients treated with bendrofluazide and subsequent potassium supplementation. (mean values, SEM omitted for clarity).

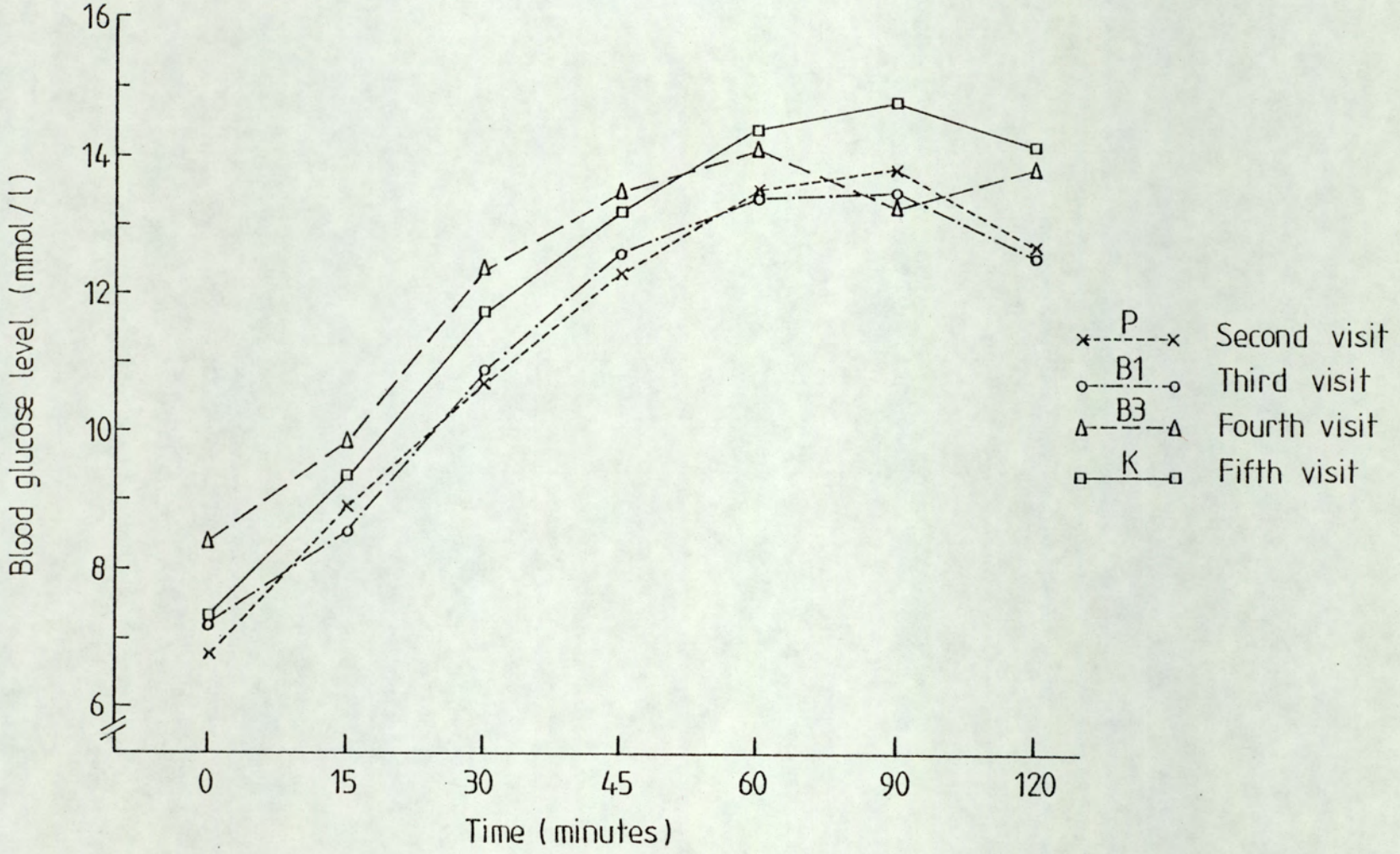


Figure 31. Insulin levels during oral glucose tolerance tests in mild hypertensive diabetic patients treated with bendrofluazide and subsequent potassium supplementation. (mean values, SEM omitted for clarity).

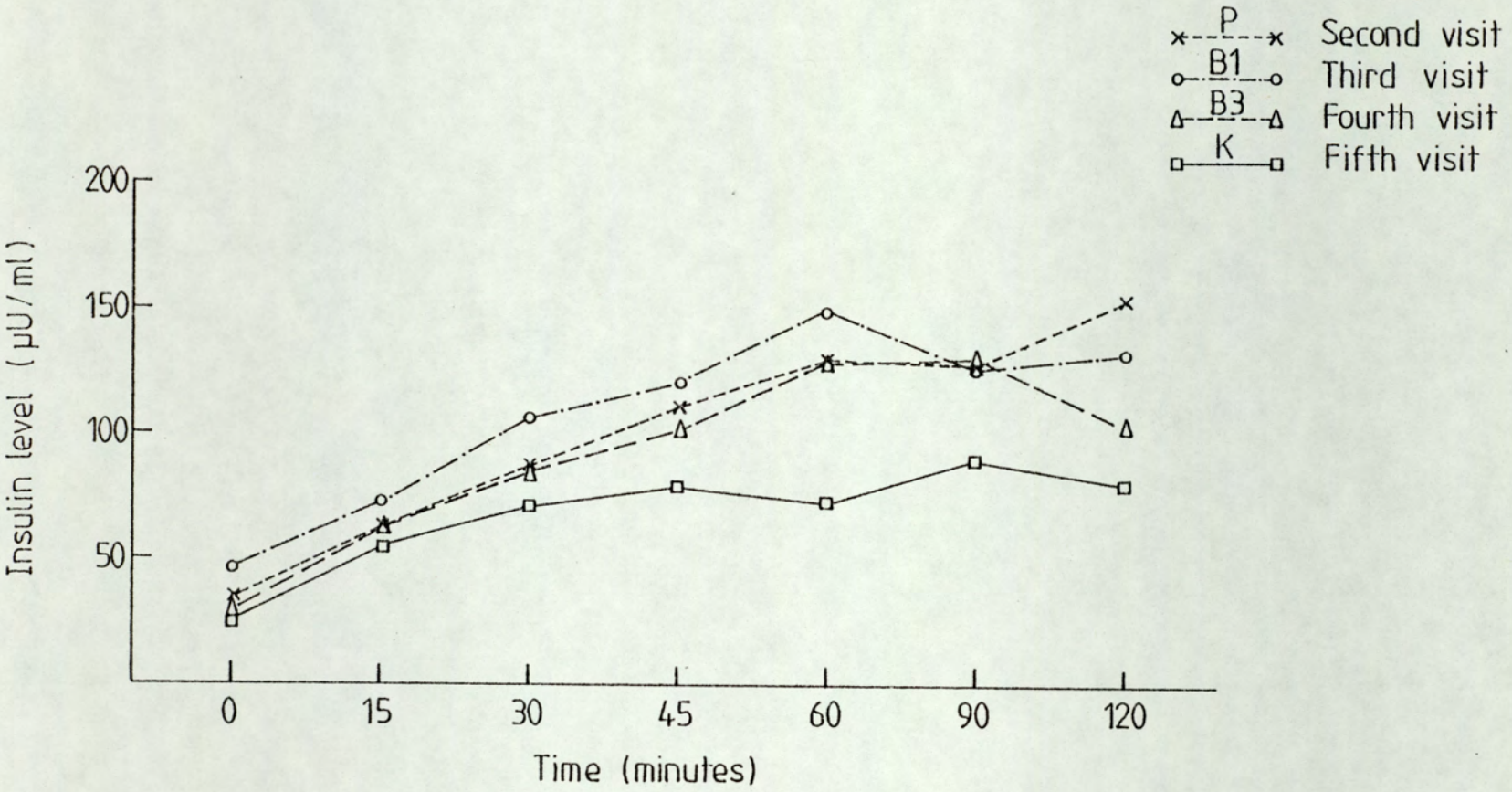


Table 10. Biochemical profile of mildly hypertensive diabetic patients treated with bendrofluazide and subsequent potassium supplementation (mean values ± SEM).

Treatment	n	Cholesterol (mmol/l)	Urea (mmol/l)	Uric Acid (mmol/l)	Serum potassium (mmol/l)	Platelets ($x10^9$/l)	MCV (fl)	Triglycerides (mmol/l)
1 month placebo (control) (P)	9	5.27 ± 0.34	4.91 ± 0.36	0.342 ± 0.02	4.46 ± 0.13	234.4 ± 22.7	87.0 ± 1.03	1.24 ± 0.13
1 month bendrofluazide (B1)	9	5.49 ± 0.32	5.53 ± 0.43	0.386 ± 0.03	4.39 ± 0.09	252.2 ± 22.4	85.7 ± 0.9	1.63 ± 0.23
3 months bendrofluazide (B3)	9	5.6 ± 0.33	5.4 ± 0.42	0.343 ± 0.04	4.23 ± 0.15	227.4 ± 19.9	83.9 ± 1.06	1.25 ± 0.18
1 month potassium supplementation with bendrofluazide (K)	9	6.06 ± 0.39	6.32 ± 0.40	0.373 ± 0.03	4.18 ± 0.11	250.4 ± 13.1	84.4 ± 1.08	2.32 ± 0.72

than in mildly hypertensive non-diabetic patients, Tables 8 and 11. Bendrofluazide treatment for either 1 or 3 months had no significant effect upon insulin receptor binding in diabetic patients, Table 11. However, subsequent potassium supplementation significantly increased both the total specific binding and the total insulin receptor concentration in these patients. The increase in total receptor concentration could be largely attributable to an increase in low affinity receptor concentration, Table 11. However, the values obtained were always significantly lower than the corresponding values for mildly hypertensive non-diabetic patients after potassium supplementation, Table 8, and not significantly different from placebo values obtained for mildly hypertensive diabetic patients, Table 11.

Pooling of non-diabetic and diabetic patients.

Treatment with bendrofluazide alone or supplemented with potassium did not significantly influence the body weight, Table 12. Both systolic and diastolic blood pressures were significantly reduced following 1 and 3 months treatment with bendrofluazide and bendrofluazide with subsequent potassium supplementation, Table 12.

The pooling fasting serum glucose level was significantly increased after 3 months treatment with bendrofluazide. Potassium supplementation marginally lowered this value but the decrease was not statistically significant when compared with values for placebo and 3 months of bendrofluazide treatment, Table 12. Examination of the pooled oral glucose tolerance curves for non-diabetic and diabetic patients did not reveal a significant deterioration, Figure

Table 11. Erythrocyte insulin receptor status of mildly hypertensive diabetic patients treated with bendrofluozide and subsequent potassim supplementation (mean values ± SEM).

Treatment	n	Specific binding (%)	High Affinity receptor concentration per cell	Low Affinity receptor concentration per cell	Total Affinity receptor concentration per cell	$\bar{K}_e$, affinity constant of empty sites (nM^{-1})	$\bar{K}_f$, affinity constant of full sites (nM^{-1})	$\bar{K}_m$, apparent affinity constant (nM^{-1})
1 month placebo (control) (P)	9	5.46 ǂ ± 0.32	12.28 ± 0.93	95.04 ǂ ± 5.8	107.32 ǂ ± 5.98	0.10223 ± 0.008	0.01695 ± 0.003	0.05394 ± 0.004
1 month bendrofluazide (B1)	9	5.42 ± 0.54	12.02 ± 1.36	91.36 ± 6.18	103.4 ± 7.21	0.10640 ± 0.01	0.01493 ± 0.001	0.05657 ± 0.006
3 months bendrofluazide (B3)	9	5.43 ± 0.28	11.3 ± 0.9	84.6 ± 6.1	95.9 ± 6.83	0.11067 ± 0.007	0.01595 ± 0.001	0.05884 ± 0.004
1 month potassium supplementation with bendrofluazide (K)	9	6.1 † ± 0.14	12.93 ± 0.91	95.1 † ± 4.0	108.03 † ± 4.42	0.11225 ± 0.009	0.01664 ± 0.001	0.05959 ± 0.005

† $p < 0.05$ when compared with the corresponding values for 3 months treatment with bendrofluazide.

ǂ Significantly reduced ($p < 0.05$) when compared with the corresponding placebo values for mildly hypertensive non-diabetic patients (Table 8, page 156).

Table 12. The effect of pooling body weights, blood pressures, serum glucose and insulin levels of non-diabetic and diabetic mildly hypertensive patients (mean values ± SEM).

Treatment	n	Body Weight (Kg)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Fasting blood glucose level (mmol/l)	Fasting insulin level (μU/ml)	Area under the OGTT curve (mmol/l/ 120 minutes)	Area under the OGTT insulin curve(μU/ml/ 120 minutes)
1 month placebo (control) (P)	18	75.21 ± 2.56	163.4 ± 4.64	99.6 ± 2.21	5.63 ± 0.5	35.94 ± 5.22	1110.5 ± 62.8	20145.4 ± 3606.1
1 month bendrofluazide (B1)	18	73.92 ± 2.41	146.8* ± 3.9	88.45* ± 1.98	5.794 ± 0.55	46.35 ± 7.33	1103.7 ± 54.6	21904.2 ± 4557.2
3 months bendrofluazide (B3)	18	74.15 ± 2.24	149.0* ± 4.98	86.9* ± 2.25	6.45* ± 0.62	40.7 ± 8.09	1184.6 ± 72.1	18804.2 ± 3768.9
1 month potassium supplementation with bendrofluazide (K)	18	74.05 ± 2.56	144.8* ± 4.75	87.4* ± 2.87	5.95 ± 0.59	26.3 ± 4.89	1181.7 ± 70.3	13912.5* ± 2692.7

* $p < 0.05$ when compared with the corresponding placebo value.

32 and there were no significant changes in the areas under the curves, Table 12. The fasting insulin level was slightly elevated after one month on bendrofluazide but subsequently fell after 3 months with a more pronounced reduction after potassium supplementation. However, none of these changes achieved statistical significance, Table 12. Potassium supplementation marginally reduced the serum insulin levels and significantly reduced the area under the insulin curves estimated during the oral glucose tolerance tests, Figure 33.

Pooled values for serum cholesterol, uric acid and urea were significantly elevated after bendrofluazide treatment and subsequent potassium supplementation compared with placebo values, Table 13 and Figures 34,35,36. Platelet levels were significantly elevated after potassium supplementation, Figure 37, while triglycerides remained unchanged throughout the treatments, Table 13. Serum potassium levels did not change significantly after 1 month on bendrofluazide, but were reduced significantly after 3 months treatments and subsequent potassium supplementation, Table 13 and Figure 38. The mean erythrocyte volume (MCV) decreased significantly after treatment with bendrofluazide and subsequent potassium supplementation, Figure 39.

As might be expected pooling the insulin binding data effectively cancelled out the alterations observed in receptor concentration for the two groups, Table 14, and there were no changes in receptor affinity. For clarity the results of the bendrofluazide section have been summarised in Table 15.

Figure 32. Pooled OGTT curves for non-diabetic and diabetic mildly hypertensive patients treated with bendrofluazide and subsequent potassium supplementation. (mean values, SEM omitted for clarity).

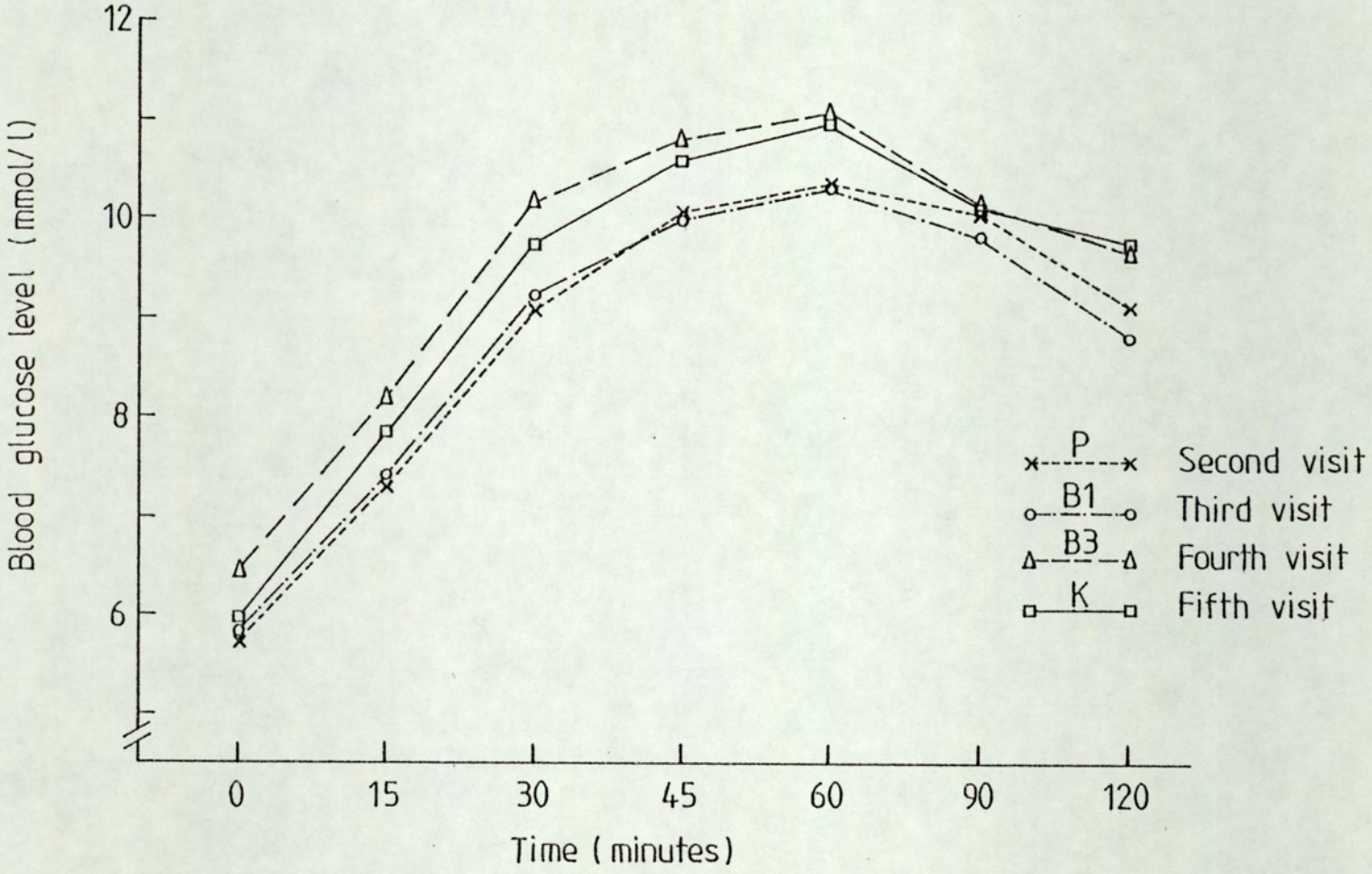


Figure 33. Serum insulin levels during oral glucose tolerance tests for both non-diabetic and diabetic mildly hypertensive patients treated with bendrofluazide and subsequent potassium supplementation.
(mean values, SEM omitted for clarity).

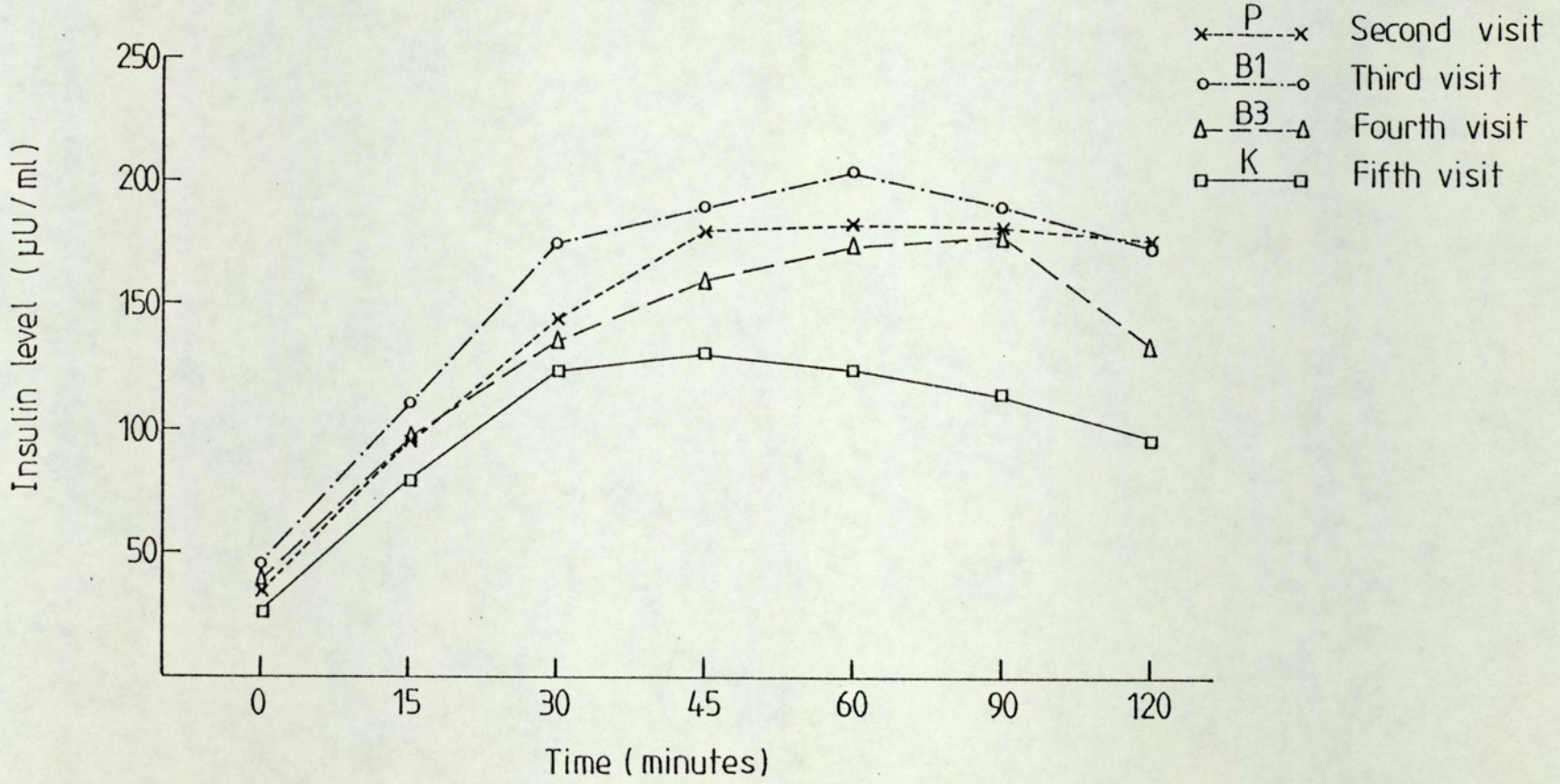
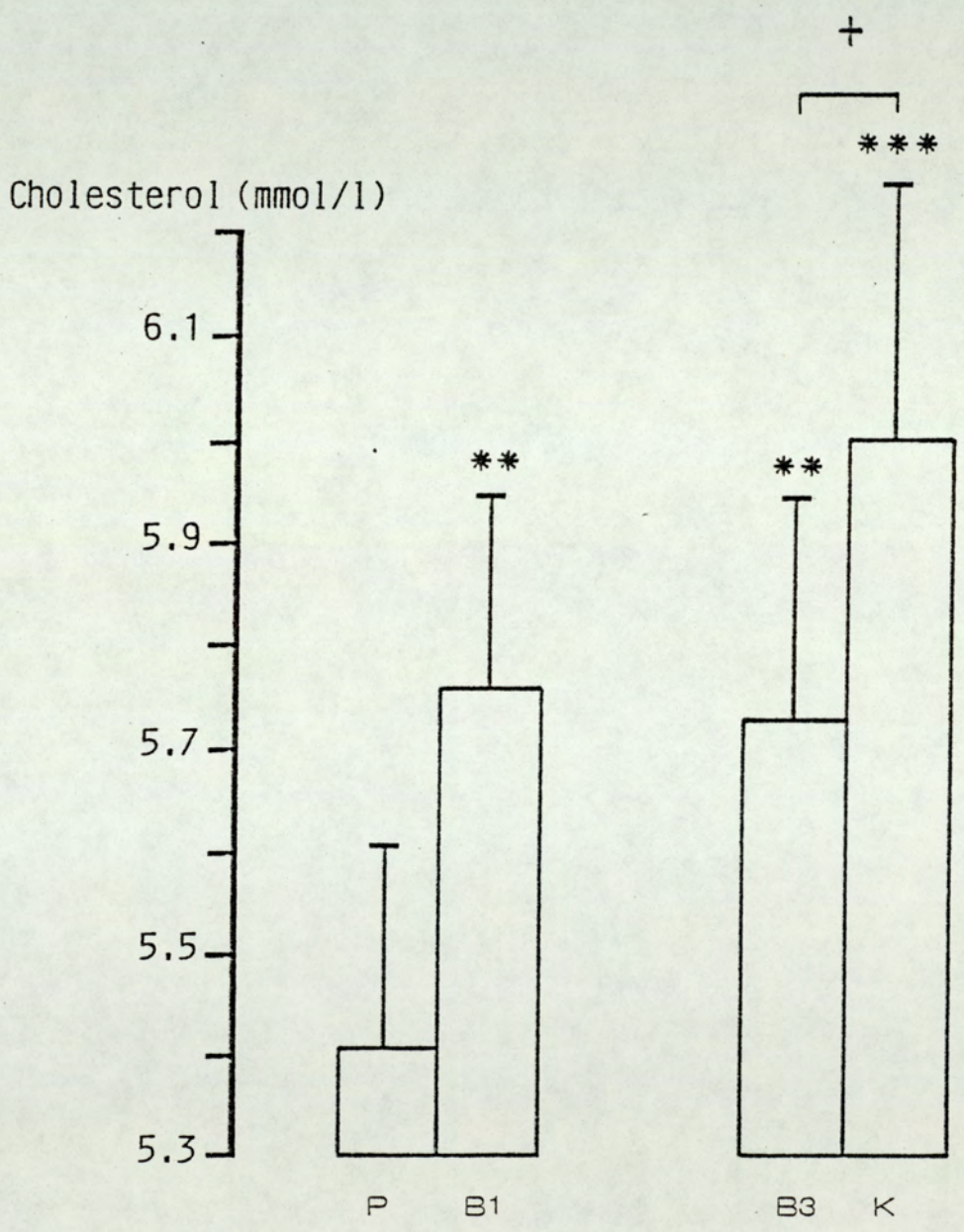


Table 13. Pooled biochemistry of diabetic and non-diabetic mildly hypertensive patients treated with bendrofluazide and subsequent potassium supplementation (mean values ± SEM).

Treatment	n	Cholesterol (mmol/l)	Urea (mmol/l)	Uric Acid (mmol/l)	Serum potassium (mmol/l)	Platelets ($x10^9$/l)	MCV (fl)	Triglycerides (mmol/l)
1 month placebo (control) (P)	18	5.40 ± 0.21	4.83 ± 0.22	0.363 ± 0.023	4.48 ± 0.09	241.1 ± 15.6	86.3 ± 1.16	1.309 ± 0.122
1 month bendrofluazide (B1)	18	5.76** ± 0.2	5.63** ± 0.37	0.41*** ± 0.03	4.37 ± 0.08	263.3 ± 17.3	85.4* ± 1.14	1.542 ± 0.175
3 months bendrofluazide (B3)	18	5.735** ± 0.21	5.56* ± 0.362	0.368 ± 0.03	4.25** ± 0.09	250.3 ± 12.4	83.8*** ± 1.12	1.362 ± 0.15
1 month potassium supplementation with bendrofluazide (K)	18	† 6.04*** ± 0.25	5.9*** ± 0.33	† 0.40** ± 0.03	4.26** ± 0.07	274.9* ± 11.1	84.0*** ± 1.16	1.98 ± 0.39

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared with the corresponding placebo value.
† $p<0.05$ when compared with the corresponding values for 3 months treatment with bendrofluazide.

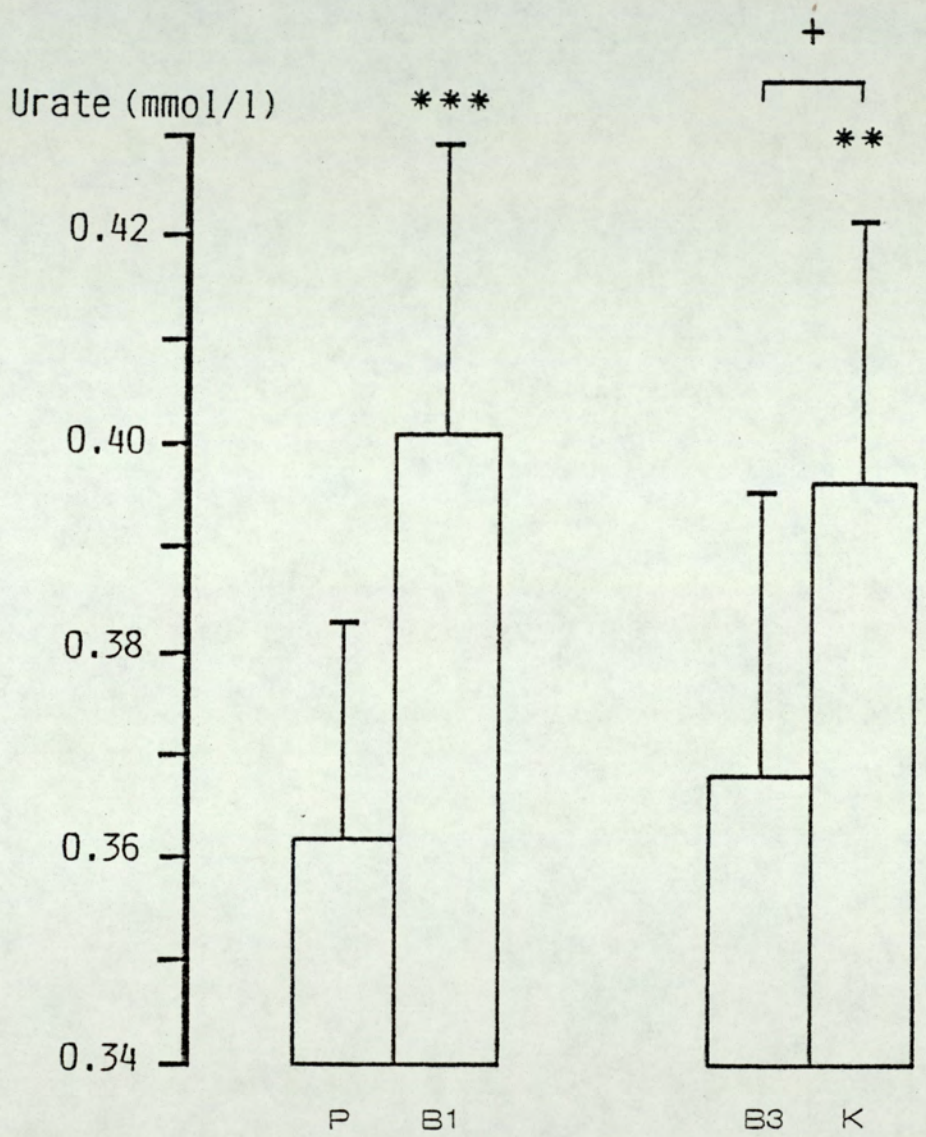
Figure 34 . The effect of bendrofluazide and subsequent potassium supplementation on the pooled serum cholesterol levels of non - diabetic and dibetic mildly hypertensive patients (mean values ± SEM).



** p < 0.01 , *** p < 0.001 compared with placebo value .

\+ p < 0.05 compared with 3 months bendrofluazide value .

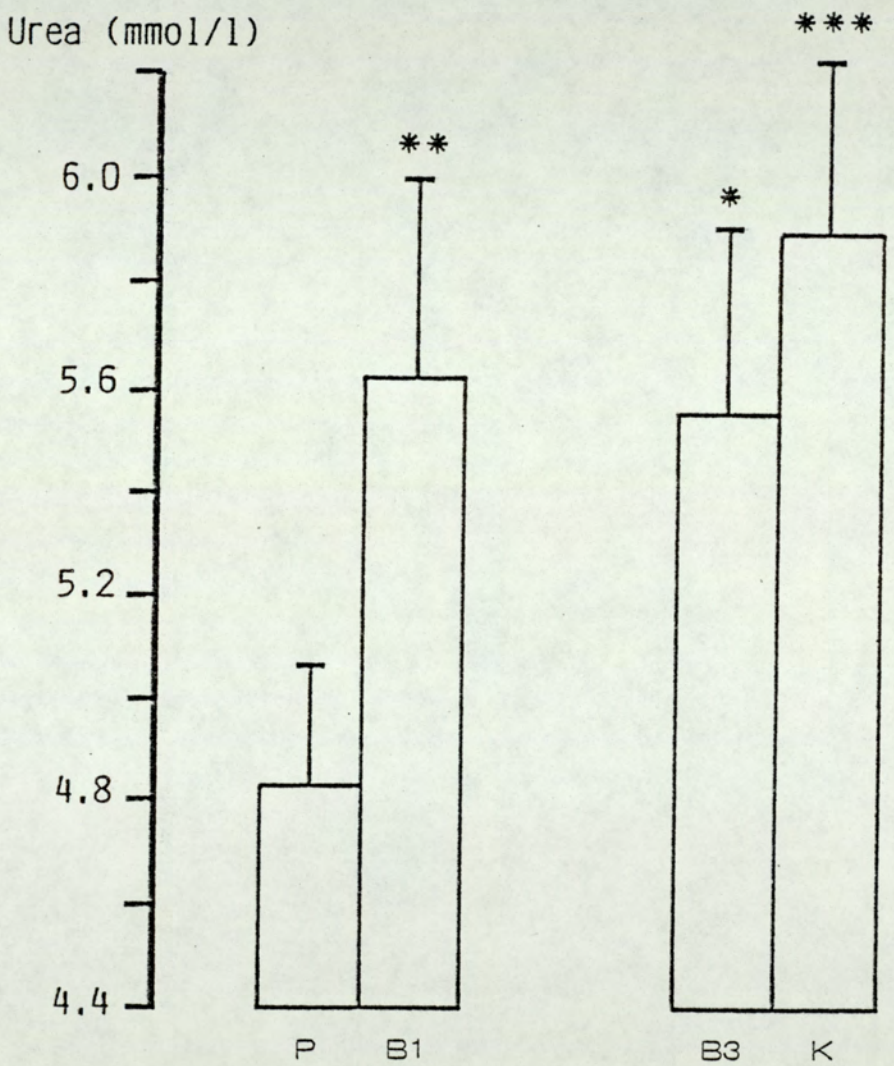
Figure 35 . The effect of bendrofluazide and subsequent potassium supplementation on the pooled serum urate (uric acid) levels of non - diabetic and diabetic mildly hypertensive patients (mean values ± SEM) .



** p <0.01 , *** p <0.001 compared with placebo value .

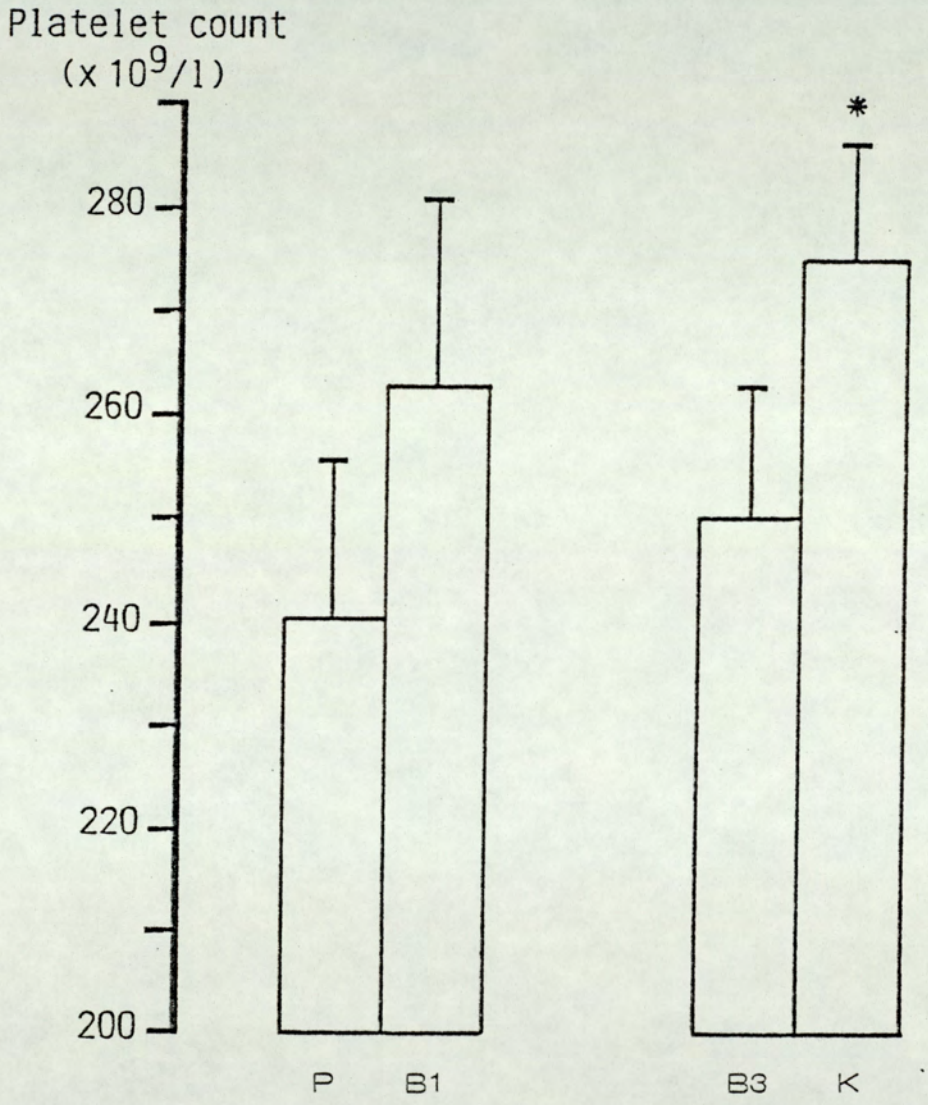
+ p <0.05 compared with 3 months bendrofluazide value .

Figure 36 . The effect of bendrofluazide and subsequent potassium supplementation on the pooled serum urea levels of non - diabetic and diabetic mildly hypertensive patients (mean values ± SEM) .



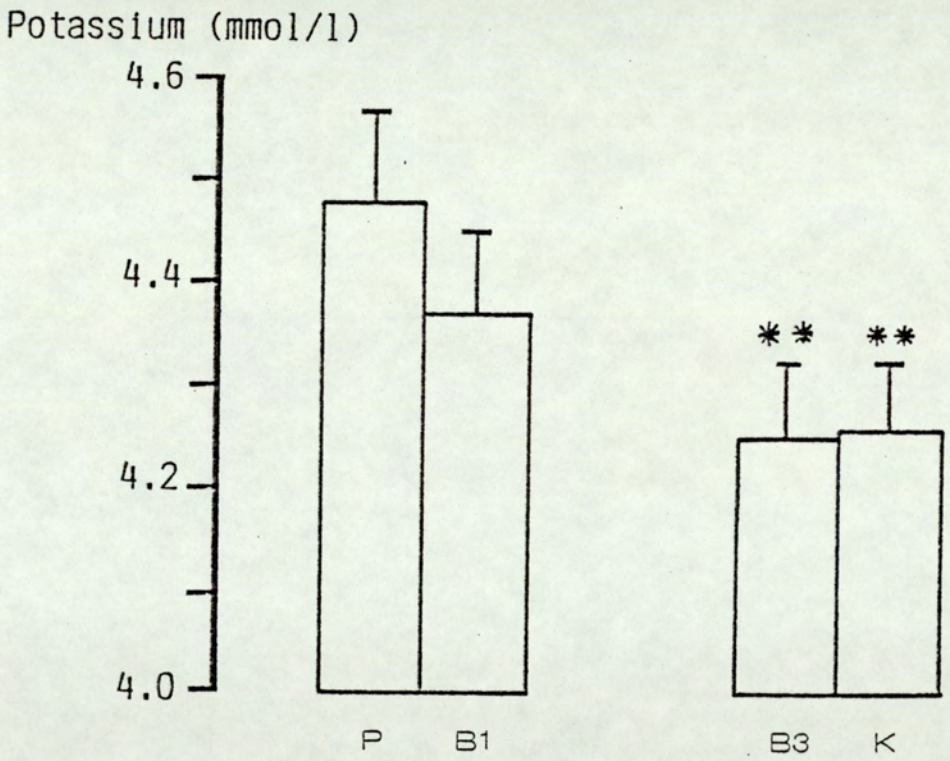
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with placebo value .

Figure 37 . The effect of bendrofluazide and subsequent potassium supplementation on the pooled platelet levels of non - diabetic and diabetic mildly hypertensive patients (mean values ± SEM) .



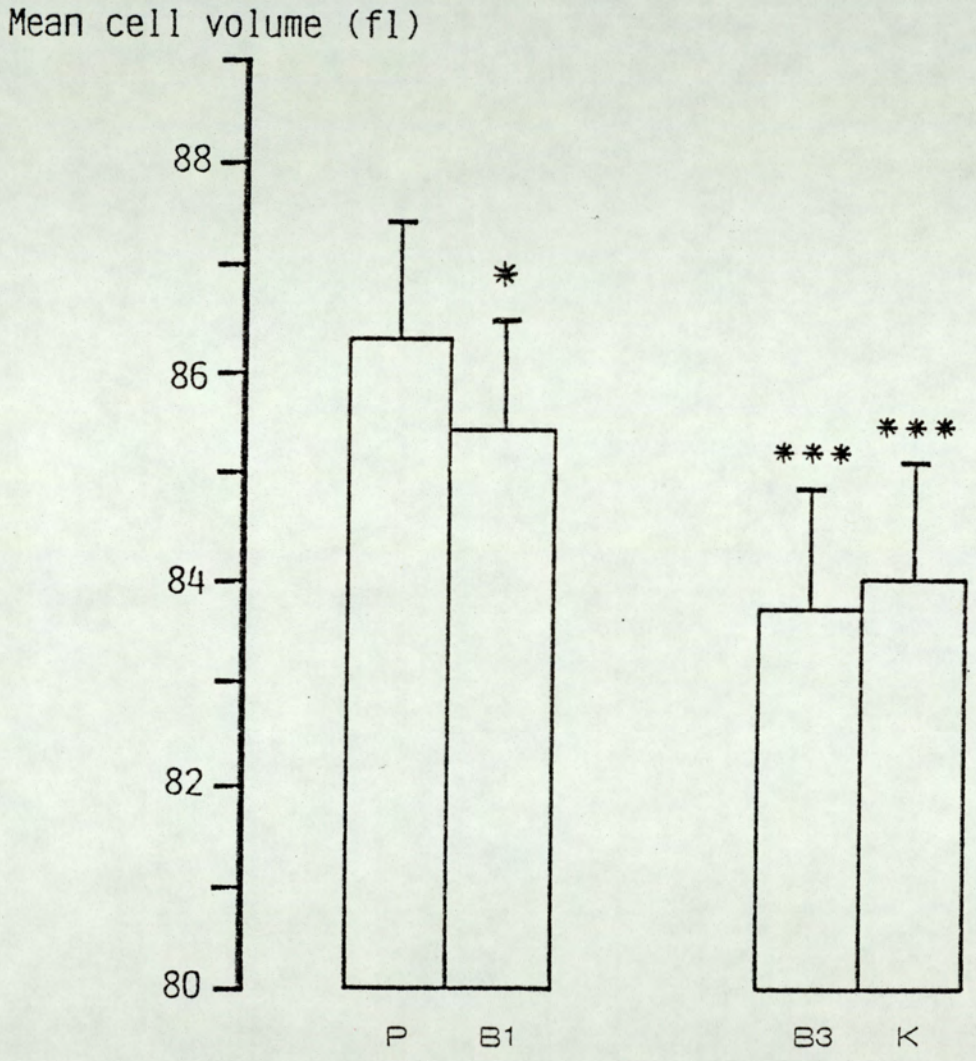
* $p < 0.05$ compared with placebo value .

Figure 38 . The effect of bendroflazide and subsequent potassium supplementation on the pooled potassium levels of non - diabetic and diabetic mildly hypertensive patients (mean values ± SEM).



** $p < 0.01$ compared with placebo value .

Figure 39 . The effect of bendrofluazide and subsequent potassium supplementation on the pooled values for MCV in non - diabetic and diabetic mildly hypertensive patients (mean values ± SEM) .



* $p < 0.05$, *** $p < 0.001$ compared with placebo value .

Table 14. Pooled erythrocyte insulin receptor binding data for non-diabetic and diabetic mildly hypertensive patients treated with bendrofluazide and subsequent potassium supplementation (mean values ± SEM).

Treatment	n	Specific binding (%)	High Affinity receptor concentration per cell	Low Affinity receptor concentration per cell	Total Affinity receptor concentration per cell	$\bar{K}_e$, affinity constant of empty sites (nM^{-1})	$\bar{K}_f$, affinity constant of full sites (nM^{-1})	$\bar{K}_m$, apparent affinity constant (nM^{-1})
1 month placebo (control) (P)	18	6.07 ± 0.24	13.06 ± 1.11	108.1 ± 5.21	121.1 ± 5.45	0.1032 ± 0.004	0.01702 ± 0.002	0.05451 ± 0.002
1 month bendrofluazide (B1)	18	6.75 ± 0.49	12.54 ± 0.8	114.2 ± 6.85	126.7 ± 7.2	0.11254 ± 0.008	0.01504 ± 0.001	0.05931 ± 0.004
3 months bendrofluazide (B3)	18	6.69 ± 0.38	12.75 ± 0.7	112.1 ± 8.4	124.8 ± 8.75	0.11251 ± 0.005	0.01625 ± 0.001	0.05946 ± 0.003
1 month potassium supplementation with bendrofluazide (K)	18	6.78 ± 0.28	12.98 ± 0.52	115.9 ± 6.12	128.9 ± 6.13	0.10848 ± 0.006	0.01613 ± 0.001	0.05723 ± 0.003

Table 15. Summary of the changes observed in mildly hypertensive non-diabetic and diabetic patients after bendrofluazide treatment (B) and subsequent potassium supplementation (K) (-) no change, (↓) decreased and (↑) increased.

Treatment	Parameters	Hypertension without diabetes (n = 9)	Hypertension with diabetes (n = 9)	Pooled Observations (n = 18)
B,K	Body Weight (kg)	-	-	-
B,K	Systolic blood pressure (mm Hg)	↓	↓	↓
B,K	Diastolic blood pressure (mm Hg)	↓	↓	↓
B	Fasting blood glucose level (mmol/l)	-	↑	↑
K	Fasting blood glucose level (mmol/l)	-	-	-
B,K	Fasting insulin level (µU/ml)	-	-	-
B,K	Area under OGTT curve	-	-	-
B	Area under OGTT insulin curve	-	-	-
K	Area under OGTT insulin curve	↓	↓	↓
B,K	Cholesterol (mmol/l)	-	-	↑
B,K	Urea (mmol/l)	-	-	↑
B,K	Uric acid (mmol/l)	-	-	↑
B,K	Serum potassium (mmol/l)	-	-	↓
B	Platelets (x 10^9/l)	-	-	-
K	Platelets (x 10^9/l)	-	-	↑
B,K	MCV (fl)	-	-	↓
B,K	Triglycerides (mmol/l)	-	-	-
	Insulin receptor status:			
B,K	Specific binding (%)	↑	-	-
B,K	Insulin receptor concentration	↑	-	-
B,K	Insulin receptor affinity	-	-	-

Discussion

In the present study the mean body weights of the two groups were unchanged and this observation supported the earlier work of Murphy and colleagues (65) who demonstrated no change in the body weight of hypertensive patients treated with bendrofluazide. On the other hand Pacy and colleagues (66) were unable to detect an increase in body weight in hypertensive diabetic patients treated with bendrofluazide. Berglund and colleagues demonstrated that patients with arterial hypertension had impaired glucose tolerance compared with normotensive controls even when their body weight and body fat was similar (371).

Systolic and diastolic blood pressures were significantly decreased in the present study in both non-diabetic and diabetic groups after 1 month and 3 months of bendrofluazide therapy. In addition bendrofluazide treatment had a hyperglycaemic effect in diabetic patients but had no effect on the fasting blood glucose of non-diabetic patients. In the diabetic patients fasting blood glucose levels were significantly elevated after 3 months. Glucose tolerance continued to deteriorate with bendrofluazide treatment but the area under the curve for the oral glucose tolerance was not significantly increased even after 3 months of bendrofluazide therapy. These observations suggest that diabetes *per se* may influence the action of bendrofluazide in hypertensive patients. Three months bendrofluazide therapy has recently been reported to be associated with a deterioration in glycaemic control in diabetic hypertensive patients (66). The reported deterioration in glucose tolerance was small and control of blood pressure in patients with

moderate hypertension was considered to more than offset by the theoretical risk of a small increase in blood glucose (372). There was some evidence to suggest a decreased tissue senstivity to insulin since, the glucose/insulin ratio tended to deteriorate. All the effective diuretics in common use seem capable of producing potassium depletion. The most probable explanation is that the increased amount of sodium delivered to the sodium/potassium exchange of the distal tubule and collecting ducts, results in the reduction of sodium reabsorption in more proximal parts of the nephron (373). Many investigations have shown that prolonged treatment of hypertension with various diuretics is associated with a reduction in the plasma potassium concentration, but measurements of total body potassium have been less conclusive. A fall in exchangeable potassium has been observed by some workers (344) but others (374) have found no significant fall in body potassium. Hypertensive patients treated with diuretics over a long period have been shown to have a reduced serum potassium concentration and a reduced total body potassium (345). The plasma potassium has been shown to be more labile than the total body potassium so that considerable variations in the former are not surprising. Potassium depletion may be involved in the mechanism whereby thiazides impair carbohydrate metabolism (356). In the present study pooled serum potassium levels did not change after 1 month of bendrofluazide but were significantly reduced after 3 months although none of the patients achieved clinical hypokalaemia. Potassium supplementation halted the fall in serum potassium but did not restore it to pretreatment placebo values. Potassium supplementation also led to a significant decrease in the fasting blood glucose levels in diabetic patients. This

action did not appear to be due to a restoration of insulin levels which continued to decrease during this phase. This observation was in contrast to previous studies (87) where potassium supplementations has corrected the deficiency in insulin secretion. Murphy and colleagues (65) were unable to find a positive correlation between changes in plasma potassium and changes in glucose tolerance, although in patients who had persistent hypokalaemia (plasma potassium below 3.6 mmol/l) glucose tolerance deteriorated significantly more than in those with plasma potassium consistently above 3.6 mmol/l.

In the present study insulin levels did not change significantly in either non-diabetic or diabetic patients after bendrofluazide therapy. After potassium supplementation insulin levels continued to decrease and a significant reduction in the area under the OGTT curve for insulin was observed for both non-diabetic and diabetic groups. It may be that the reduced insulin levels were the result of a deficiency in insulin secretion resulting from thiazide induced hypokalaemia. Potassium is an essential cation for insulin release from pancreatic B-cells (365). Thiazide may increase the permeability of the B-cell membrane to potassium (223). This then would inhibit the influx of calcium ions upon which insulin secretion is thought to be dependent (377). An excellent correlation has been shown to exist between the decrease in total body potassium and the defect in insulin secretion in normal patients (369). The study demonstrated that potassium depletion of the level encountered in clinical practice could cause glucose intolerance associated with impaired insulin secretion. In addition to the decreased secretion of insulin, patients with severe chronic hypokalaemia may also

secrete a higher ratio of proinsulin (which has less biological activity) to insulin (365). It has also been reported that insulin itself may play an imprtant role in the regulation of serum potassium levels (366). Some previous studies on glucose intolerance during thiazide administration have demonstrated a decrease in insulin secretion (87) while others have suggested that thiazides may increase insulin secretion (368). In the present study there is great doubt that the administration of bendrofluazide decreased serum potassium, however, though this correlated with the glucose level, it did not relate to insulin. This may merely reflect the poor correlation of serum potassium with total body levels of this ion. Alternatively potassium may not be as important in the regulation of insulin release as previously supposed. Thiazide diuretics are known to affect other cations involved in insulin release and these may be more important determinants of deficient insulin secretion. It is well known that thiazides also effect serum magnesium and calcium levels and these ions may be more directly involved in the maintainence of insulin release (375). In addition it has been reported that a magnesium deficiency may aggravate the symptoms of hypokalaemia (376).

Many of the other characteristic metabolic and biochemical actions of thiazides have been observed in this study. Bendrofluazide therapy resulted in an elevation in urea, uric acid and cholesterol in both diabetic and non-diabetic patients. Potassium supplementation failed to correct these changes. Berglund and colleagues (84) were unable to demonstrate a significant increase in uric acid after 12 and 24 months of bendrofluazide therapy. On the other hand a recent multicentre study (349) has demonstrated a

significant rise in serum uric acid after 6 months and 12 months of treatment with bendrofluazide. Hyperuricaemia, hypokalaemia and hyperglycaemia are now recognised biochemical side effects of thiazide treatment (351). Hyperuricaemia may occur spontaneously in hypertensive patients who are generally overweight (378) and treatment with thiazide diuretics has been shown to increase serum urate (uric acid) by competitively inhibiting the tubular secretion of urate (379). In the present study serum cholesterol was significantly elevated after bendrofluazide therapy. The effects of thiazides on serum lipids have been extensively studied (380). The actual rise in cholesterol in the treated group, may be evidence of a thiazide-induced increase in cholesterol such as that reported by Grimm and colleagues (81), Ames and Hill (380), and Helgeland et al (361), on the other hand Pacy and colleagues (66) reported that treatment of hypertensive diabetic patients for 3 months with bendrofluazide did not show significant changes in the serum cholesterol level. Triglyceride levels were unchanged in the present study after bendrofluazide treatment. Previous workers had reported an increase in the serum levels of triglyceride (81,361) but recent work by Pacy and colleagues (66) confirmed that bendrofluazide had no significant effect on serum triglyceride levels.

Reduction in MCV and increased platelet concentrations after bendrofluazide and potassium supplementation remain difficult to account for.

The present study is the first to examine the effect of bendrofluazide treatment on insulin receptor binding in mildly hypertensive diabetic and non-diabetic patients. The total insulin receptor concentration increased in non-diabetic patients within one

month of bendrofluazide treatment and remained significantly high after 3 months treatment. The increase in binding could be largely accounted for by an increase in low affinity insulin receptor concentration. In non-diabetic patients the increase in insulin receptor concentration would help to maintain normal glucose tolerance. Alterations in insulin receptor number in response to changes in insulin levels have been reported previously (108,257) although the relationship was not entirely clear. Bar and colleagues reported that the total receptor concentration was inversely related to the circulating levels of insulin (196). While in the foetus, hyperinsulinaemia does not appear to lead to receptor downregulation (126). In the present study the serum insulin concentration did not significantly change after bendrofluazide treatment so insulin release does not appear to be the real cause of the increase in insulin receptor number. Since the receptor number remained significantly elevated after potassium supplementation this would suggest that potassium supplementation had no additional influence on insulin receptor number. In the diabetic group the insulin receptor number was not influenced by bendrofluazide treatment and the receptor/insulin ratio deteriorated. These effects might explain why glucose tolerance in diabetic patients administered thiazide diuretics deteriorates rapidly, whereas in non-diabetic patients, glucose tolerance might take many years to develop. Potassium supplementation increased the insulin receptor number significantly in diabetic patients when compared with values obtained after 3 months treatment with bendrofluazide alone. The increase in receptor number in the diabetic group after potassium supplementation may have allowed the diabetic group to maintain glucose tolerance and further reduce the level of glycaemia. The effect of potassium may well have

been mediated through an action upon intracellular glucose metabolism. Another possibility might be that potassium may affect other mechanisms involved in the maintenance of carbohydrate tolerance (421,476).

The insulin receptor data obtained in the present study might have been more reliable if it had been obtained from studies using target tissues for insulin such as hepatocytes or fat cells. However, it would be impossible to obtain repeated samples of these tissues. Erythrocyte insulin receptor number correlates well with receptor numbers on monocytes, though these do not necessarily correlate well with receptor numbers in other target tissues. Previously the effect of thiazides upon insulin receptor binding has only been studied for diazoxide a particularly diabetogenic thiazide vasodilator, but so far results have been conflicting (256,370).

It is difficult to envisage the mechanisms by which insulin receptor number might change in erythrocytes which have only a limited capacity for protein biosynthesis. Three mechanisms have been proposed for the change in insulin receptor number. Firstly, new cells including reticulocytes may be recruited into the circulating pool and these may differ in their insulin receptor number from those already in the circulation. Reticulocytes have a higher binding capacity than erythrocytes (213). The above mechanism alone would be unlikely to account for the degree of change in insulin receptor status observed in the present study. Secondly, bendrofluazide might affect the endocytotic internalisation of receptors and their recycling to the external plasma membrane via the lysosome (134). Thirdly, thiazides may cause a conformational change in the insulin receptor. This effect may be mediated by the thiazide diuretic itself or through changes in insulin concentration.

B. The effect of long-term nifedipine treatment, subsequent withdrawal and restoration of nifedipine treatment on the oral glucose tolerance and serum insulin and glucose levels of mildly hypertensive patients with and without non-insulin dependent diabetes mellitus.

Introduction

Calcium ions play a vital role in many cellular processes and ionic calcium is particularly important for the function of the myocardium (457). Calcium fluxes through the slow inward channels are responsible for the plateau of the transmembrane action potential and play an essential role in myocardial excitation-contraction coupling probably by triggering the release of intracellular calcium ions. Calcium also tightens cell membranes thereby decreasing their permeability to various substances, including glucose (458). In addition, calcium has been shown to mediate the cellular response to glucagon stimulation (459) and thus affects hepatic glucose output via s stimulation of glycogenolysis. Calcium might influence glucose metabolism via several pathways and different overall effects on the blood glucose concentration may be forthcoming depending on which of these pathways is the dominating one. *In vitro* experiments have shown that calcium affects factors of importance for the regulation of glucose metabolism. Accordingly, calcium has been shown to trigger the stimulus-secretion coupling process which leads to insulin release from the pancreatic B-cell (375) and any drug which modified cellular calcium metabolism/mobilisation could also affect insulin release. Calcium antagonists, have been shown to interfere with calcium at the cell membrane and intracellularly (460) and the

term may be used to denote agents which specifically inhibit calcium dependent processes or regulatory mechanisms. Such drugs may be subdivided into those that decrease the availability of calcium to the myoplasm and those that decrease the cellular effects of calcium without lowering the intracellular calcium concentration. Accordingly, calcium channel blockers, such as nifedipine and verapamil (461) form a subgroup of calcium availability inhibitors, since they have been shown to block the influx of extracellular calcium through ion selective channels in the membrane of both cardiac and smooth muscle (460). However, some of these drugs have been shown to have additional sites of action, particularly in smooth muscle (460) and this fact must be taken into consideration when they are to be used as investigational tools. Calcium antagonists have been found to be active in lowering high blood pressure and are increasingly prescribed for hypertension (94). In practically all cases of essential hypertension the increase in blood pressure has been shown to be due to an elevation in vascular resistance the product of smooth muscular tension in arterioles. The final determinant of vascular smooth muscle tension has been shown to be the concentration of free calcium in the sarcoplasm (462) and it would appear that the antihypersensitive effect produced by the inhibition of calcium influx increases in proportion with the pre-treatment blood pressure. The acute administration of nifedipine results in a distinct fall in blood pressure in patients with essential hypertension but not in normotensive subjects (94).

Nifedipine is a dihydropyridine derivative widely used in the treatment of angina pectoris. Information on the pharmacokinetics of nifedipine has been hard to assemble due to analytical difficulties. Nifedipine is rapidly absorbed after sublingual

application with a peak concentration after 1 hour. It is metabolised to a number of inactive metabolites and 70-80% of the given dose is excreted through the kidneys as water soluble metabolities (457). The incidence of side effects has been shown to be low with nifedipine but do include flushing, local oedema and headache.

In spite of the well documented in vitro effects of calcium on some of the metabolic processes which determine the glucose tolerance (463), relatively few clinical studies have been carried out to investigate whether calcium antagonists influence glucose tolerance in man. However, a few studies, dealing with the effect of nifedipine on human glucose tolerance have been performed recently (97,461,464). The results obtained in those investigations have been highly conflicting. Nifedipine has been shown to increase fasting plasma glucose and induce apparent glucose intolerance in subjects with normal glucose tolerance (461,98) and reduce further both insulin secretion and glucose tolerance in patients with glucose intolerance (98). Other studies have been unable to demonstrate changes in either glucose concentration or peak insulin responses after nifedipine treatment (97) and the effects of nifedipine on glucose tolerance were minimal when the drug was given in low doses (98,465). These studies in normal subjects did not exclude the possibility of a significant effect of conventional doses of the drug in diabetic patients, who have a limited capacity to secrete insulin. A further impairment of glucose tolerance in non-insulin dependent diabetic subjects has been reported after nifedipine treatment (98) but this has not been confirmed in other studies involving diabetic patients (464,465). The fact that nifedipine might be capable of inducing glucose intolerance in normal subjects

and delaying the insulin response to an oral glucose load suggested that nifedipine might be hazardous if given to diabetic subjects (97). Furthermore, it had previously been observed that a patient with non-insulin dependent diabetes had appreciably decreased mean blood glucose concentrations after the withdrawal of nifedipine treatment (461). This begged the question as to whether nifedipine might possess some diabetogenic properties.

The present study was designed to investigate the effect of long-term nifedipine treatment, a short period of withdrawal and subsequent restoration of nifedipine treatment on serum glucose and insulin and oral glucose tolerance in mildly hypertensive patients with and without non-insulin dependent diabetes mellitus.

Materials and Methods

Twelve mildly hypertensive volunteers (6 non-diabetic and 6 non-insulin dependent diabetics) who had been treated with nifedipine (40 mg/day) for longer than six months were recruited from the Diabetic and Hypertensive Clinics at Dudley Road Hospital, Birmingham. The volunteers were either male or postmenopausal females and diabetic patients were controlled by dietary restriction alone. Patients with cardiac conduction disturbances and cardiac insufficiency were excluded from the study. All volunteers were asked to fast for 12 hours prior to attending the hypertensive clinic. At presentation volunteers were given a 75g oral glucose tolerance test and fasting blood samples were taken for haematology (Hb, WBC, platelets and MCV), a biochemical profile (urea, uric acid and electrolytes), serum glucose and insulin. Blood pressure was measured using a Hawksley random zero sphygmomanometer using phase 5 diastolic in the sitting

position. Pulse and body weight were also recorded. Patients were then asked to stop taking nifedipine for one month. At the end of the month (second clinic visit) the blood tests, parameters of the biochemical profile, oral glucose tolerance tests and serum glucose and insulin estimations were all repeated. Patients were then instructed to resume nifedipine treatment (40 mg/day) and after one month (third clinic visit) all the measurements were repeated. Informed consent was obtained from all the patients and all patients completed the study. A complete protocol of the study has been included in Appendix 10, page 300.

Results

The effect of long-term nifedipine treatment, subsequnet withdrawal and restoration of nifedipine treatment in pooled mildly hypertensive patients with and without non-insulin dependent diabetes have been summarised in Table 16. Body weights and diastolic blood pressures did not change significantly throughout the study. However, the pooled systolic blood pressure was significantly elevated after nifedipine withdrawal but decreased again after the restoration of nifedipine treatment. When patients were separated into diabetic and non-diabetic groups, Tables 17 and 18, the elevation in systolic blood pressure was no longer statistically significant.

Serum glucose, serum insulin, haemotological and biochemical parameters were not significantly changed by manipulation of nifedipine treatment, either when values were pooled or separated into diabetic and non-diabetic groups. The withdrawal of nifedipine treatment increased the level of glycosylated haemoglobin in diabetic

Table 16. The effects of long term nifedipine treatment, subsequent withdrawal, and restoration of nifedipine treatment in pooled mildly hypertensive patients with and without non-insulin dependent diabetes mellitus. (mean values ± SEM).

	n	After 6 months of nifedipine treatment (1st visit)	After 1 month withdrawal from nifedipine (2nd visit)	1 month after restoration of nifedipine (3rd visit)
Age (Years)	12	60.3 ± 1.7	-	-
Body Weight (Kg)	12	77.4 ± 3.4	77.5 + 3.3	77.2 ± 3.3
Systolic blood pressure (mmHg)	12	156.7 + 5.11	165.6 ± 4.5 *	157.2 ± 5.3
Diastolic blood pressure (mmHg)	12	95.9 + 2.9	96.4 ± 2.9	95 ± 2.6
Serum glucose level (mmol/l)	12	5.77 ± 0.77	5.67 ± 0.76	5.62 ± 0.67
Serum insulin level (µU/ml)	12	18.16 ± 1.98	18.8 ± 1.9	19.8 ± 2.1
Glycosylated haemoglobin (%)	11	7.77 ± 0.55	8.39 ± 0.73	7.41 ± 0.52
Platelets ($x10^9$/l)	12	245.4 ± 23.4	253.7 ± 29	237.4 ± 27.3
MCV (fl)	12	86.1 ± 2.15	87.4 ± 1.8	89.5 ± 1.6
Urea (mmol/l)	12	5.49 ± 0.46	5.19 ± 0.37	5.08 ± 0.34
Uric acid (mmol/l)	12	0.376 ± 0.02	0.364 ± 0.02	0.389 ± 0.03
Sodium (mmol/l)	12	138.7 ± 1.0	139.5 ± 0.7	139.8 ± 0.6
Potassium (mmol/l)	12	4.39 ± 0.11	4.21 ± 0.12	4.26 ± 0.11
Calcium (mmol/l)	12	2.31 ± 0.02	2.25 ± 0.02	2.26 ± 0.03

* $p < 0.05$ compared with first visit.

Table 17. The effects of long term nifedipine treatment, subsequent withdrawal and restoration of nifedipine treatment in mildly hypertensive non-insulin dependent diabetic patients (mean values ± SEM)

	n	After 6 months of nifedipine treatment (1st visit)	After 1 month withdrawal from nifedipine (2nd visit)	1 month after restoration of nifedipine (3rd visit)
Age (Years)	6	58.2 ± 2.7	-	-
Body Weight (Kg)	6	78.8 ± 2.4	79.4 + 2.4	78.8 ± 2.5
Systolic blood pressure (mmHg)	6	150.4 + 8.9	164.8 ± 7.5	158.3 ± 4.9
Diastolic blood pressure (mmHg)	6	92.8 + 3.4	95.8 ± 5.2	95.3 ± 2.9
Serum glucose level (mmol/l)	6	7.28 ± 1.28	7.23 ± 1.28	6.88 ± 1.12
Serum insulin level (µU/ml)	6	20.5 ± 3.4	19.7 ± 3.7	23.7 ± 3.5
Glycosylated haemoglobin (%)	5	7.88 ± 1.37	9.82 ± 1.3 *	7.82 ± 1.11
Platelets ($x10^9/l$)	6	224.6 ± 43	224.2 ± 46.4	250.0 ± 48.8
MCV (fl)	6	83.7 ± 3	88.0 ± 1.2	89.8 ± 1.9
Urea (mmol/l)	6	5.1 ± 0.59	4.85 ± 0.4	4.78 ± 0.44
Uric acid (mmol/l)	6	0.377 ± 0.04	0.38 ± 0.04	0.387 ± 0.04
Sodium (mmol/l)	6	137.0 ± 1.2	137.8 ± 1.1	139.5 ± 0.7
Potassium (mmol/l)	6	4.15 ± 0.08	4.07 ± 0.14	4.13 ± 0.08
Calcium (mmol/l)	6	2.34 ± 0.03	2.27 ± 0.03	2.3 ± 0.03

* $p < 0.05$ compared with first visit.

Table 18. The effects of long term nifedipine treatment, subsequent withdrawal and restoration of nifedipine treatment in mildly hypertensive non-diabetic patients (mean values ± SEM).

	n	After 6 months of nifedipine treatment (1st visit)	After 1 month withdrawal from nifedipine (2nd visit)	1 month after restoration of nifedipine (3rd visit)
Age (Years)	6	62.5 ± 1.4	-	-
Body Weight (Kg)	6	76.0 ± 6.6	75.6 + 6.4	75.6 ± 6.3
Systolic blood pressure (mmHg)	6	163 + 5.3	166.3 ± 4.7	156.0 ± 9.9
Diastolic blood pressure (mmHg)	6	99.0 + 4.02	97.0 ± 3.5	94.7 ± 4.6
Serum glucose level (mmol/l)	6	4.25 ± 0.25	4.1 ± 0.19	4.35 ± 0.24
Serum insulin level (uU/ml)	6	15.8 ± 1.85	18.0 ± 1.29	16.0 ± 1.1
Glycosylated haemoglobin (%)	6	7.67 ± 0.41	7.2 ± 0.45	7.07 ± 0.36
Platelets ($x10^9$/l)	6	266.2 ± 24.9	283.2 ± 36.2	224.8 ± 21.1
MCV (fl)	6	88.5 ± 2.7	86.8 ± 3.2	89.2 ± 2.5
Urea (mmol/l)	6	5.82± 0.73	5.53 ± 0.85	5.37 ± 0.61
Uric acid (mmol/l)	6	0.375 ± 0.03	0.348 ± 0.03	0.392 ± 0.04
Sodium (mmol/l)	6	140.3 ± 1.2	141.2 ± 0.3	140.0 ± 0.9
Potassium (mmol/l)	6	4.64 ± 0.18	4.35 ± 0.19	4.38 ± 0.19
Calcium (mmol/l)	6	2.28 ± 0.03	2.24 ± 0.04	2.22 ± 0.04

patients, Table 17, but marginally improved the oral glucose tolerance in pooled patients, Figure 40, as reflected by the area under the OGTT curve, Figure 42. When patients were separated into diabetic and non-diabetic groups only the diabetics showed a marginal improvement in glucose tolerance after nifedipine withdrawal, Figure 43. The restoration of nifedipine treatment for one month was associated with a marginal deterioration in glucose tolerance, Figure 43, which was reflected by an increase in the area under the OGTT curve for both diabetic and non-diabetic patients, Figure 45. The deterioration in glucose tolerance seen in non-diabetic patients after the restoratiion of nifedipine treatment was not associated with any significant reduction in insulin levels, Figures 44 and 45. Serum insulin levels of pooled patients, Figure 41, and diabetic patients, Figure 44, recorded during the oral glucose tolerance tests, tended to increase with each visit. On the other hand manipulation of nifedipine treatment in non-diabetic patients did not significantly affect insulin levels recorded during the oral glucose tolerance tests, Figure 44, or the areas under the OGTT insulin curves, Figure 45.

Discussion

As expected nifedipine withdrawal caused a marginal increase in systolic blood pressure (457) which reached statistical significance only when patient data was pooled together. Nifedipine treated patients recruited into the study were generally mildy hypertensive and it may be that the withdrawal of nifedipine for one month was not sufficiently long enough to show a significant deterioration in the blood pressure when analysed by group.

Figure 40. The effect of long term nifedipine treatment (1st visit), subsequent withdrawal (2nd visit) and restoration of nifedipine treatment (3rd visit) on the oral glucose tolerance of pooled mildly hyperhypertensive patients with and without non-insulin dependent diabetes mellitus. (each point represents the mean of 12 determinations. SEM omitted for clarity).

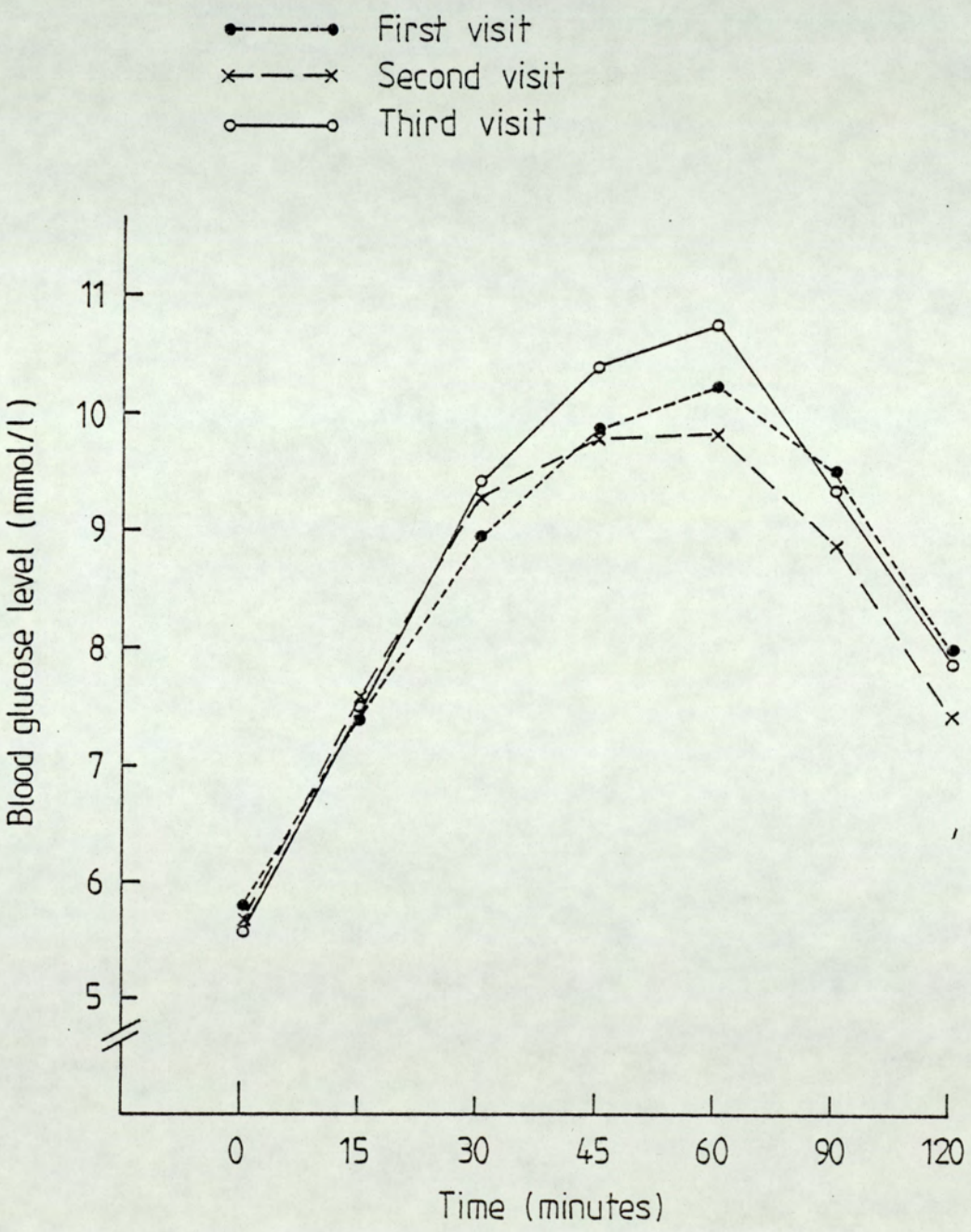


Figure 41. The effect of long term nifedipine treatment (1st visit), subsequent withdrawal (2nd visit) and restoration of nifedipine treatment (3rd visit) on serum insulin levels recorded during oral glucose tolerance testing of pooled mildly hypertensive patients with and without non-insulin dependent diabetes. (each point represents the mean of 12 determinations. SEM omitted for clarity).

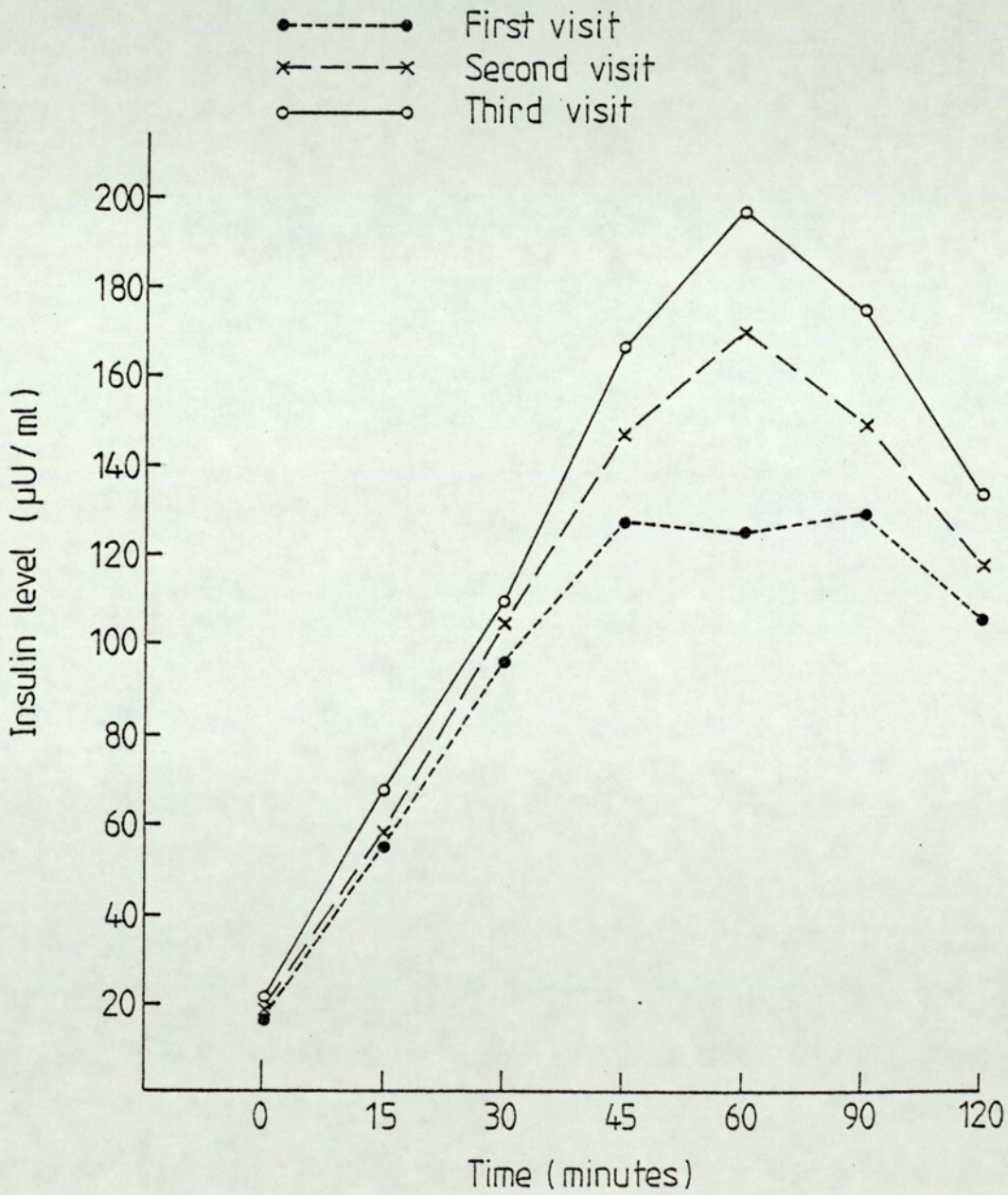


Figure 42. The effect of long term nifedipine treatment (1st visit), subsequent withdrawal (2nd visit) and the restoration of nifedipine treatment (3rd visit) on the area under the OGTT and OGTT insulin curves of pooled mildly hypertensive patients with and without non-insulin dependent diabetes mellitus. (each block represents the mean value of 12 determinations ± SEM).

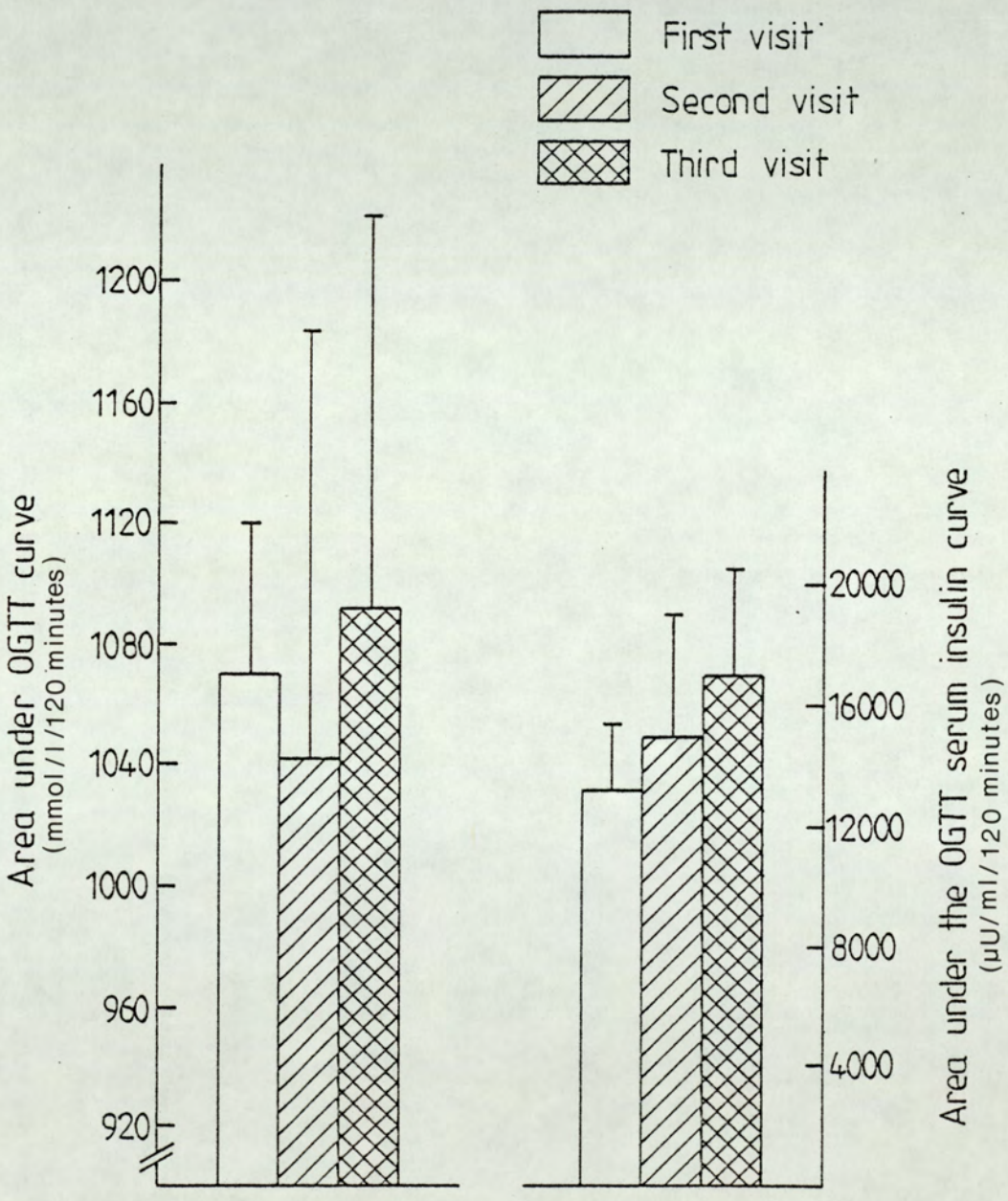


Figure 43. The effect of long term nifedipine treatment (1st visit), subsequent withdrawal (2nd visit) and restoration of nifedipine treatment (3rd visit) on the oral glucose tolerance of mildly hypertensive patients with and without non-insulin dependent diabetes mellitus (each point represents the mean of 6 determinations. SEM omitted for clarity).

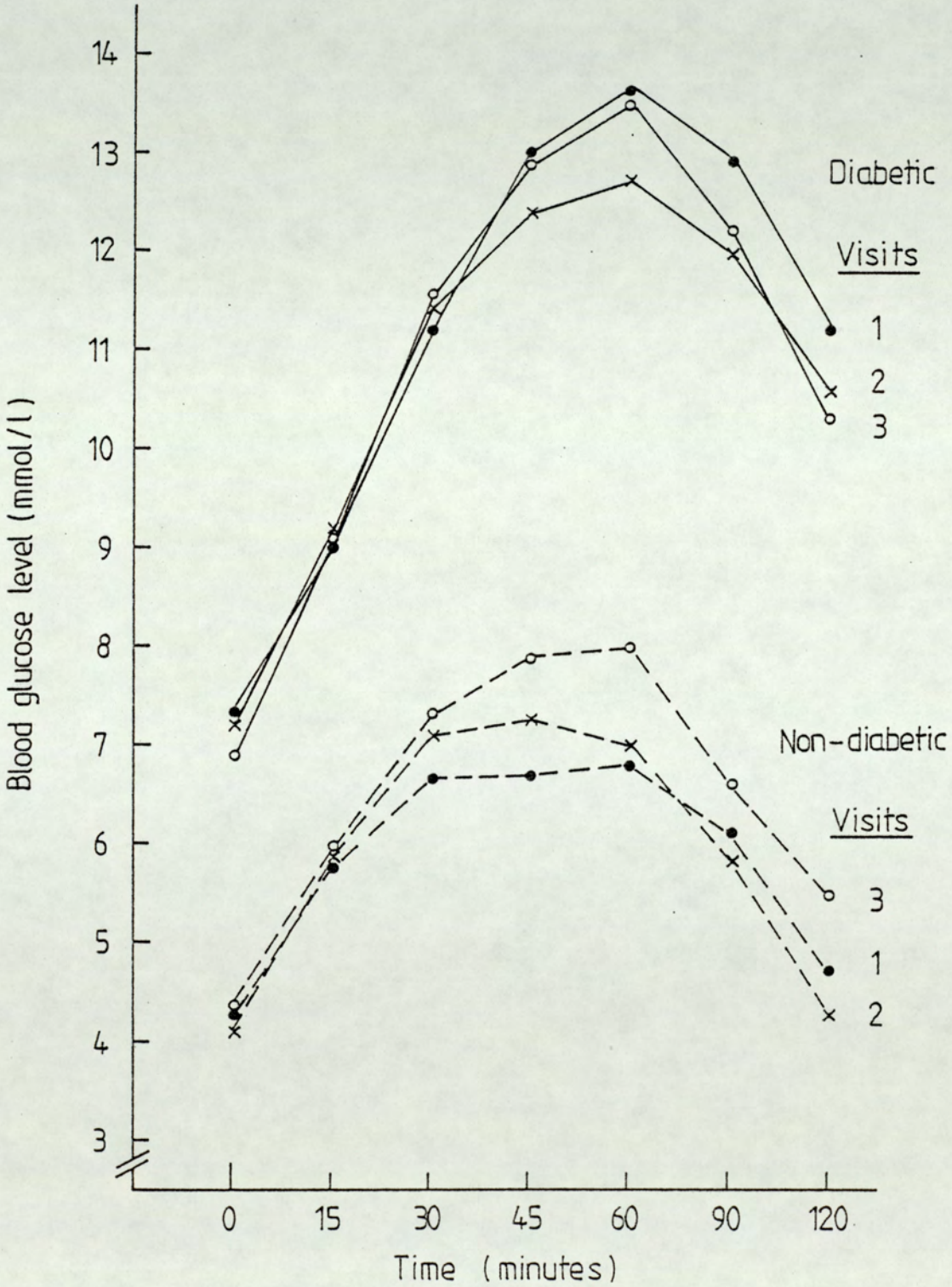


Figure 44. The effect of long term nifedipine treatment (1st visit), subsequent withdrawal (2nd visit) and restoration of nifedipine treatment (3rd visit) on the serum insulin levels recorded during oral glucose tolerance testing of mildly hypertensive patients with and without non-insulin dependent diabetes (each point represents the mean of 6 determinations. SEM omitted for clarity).

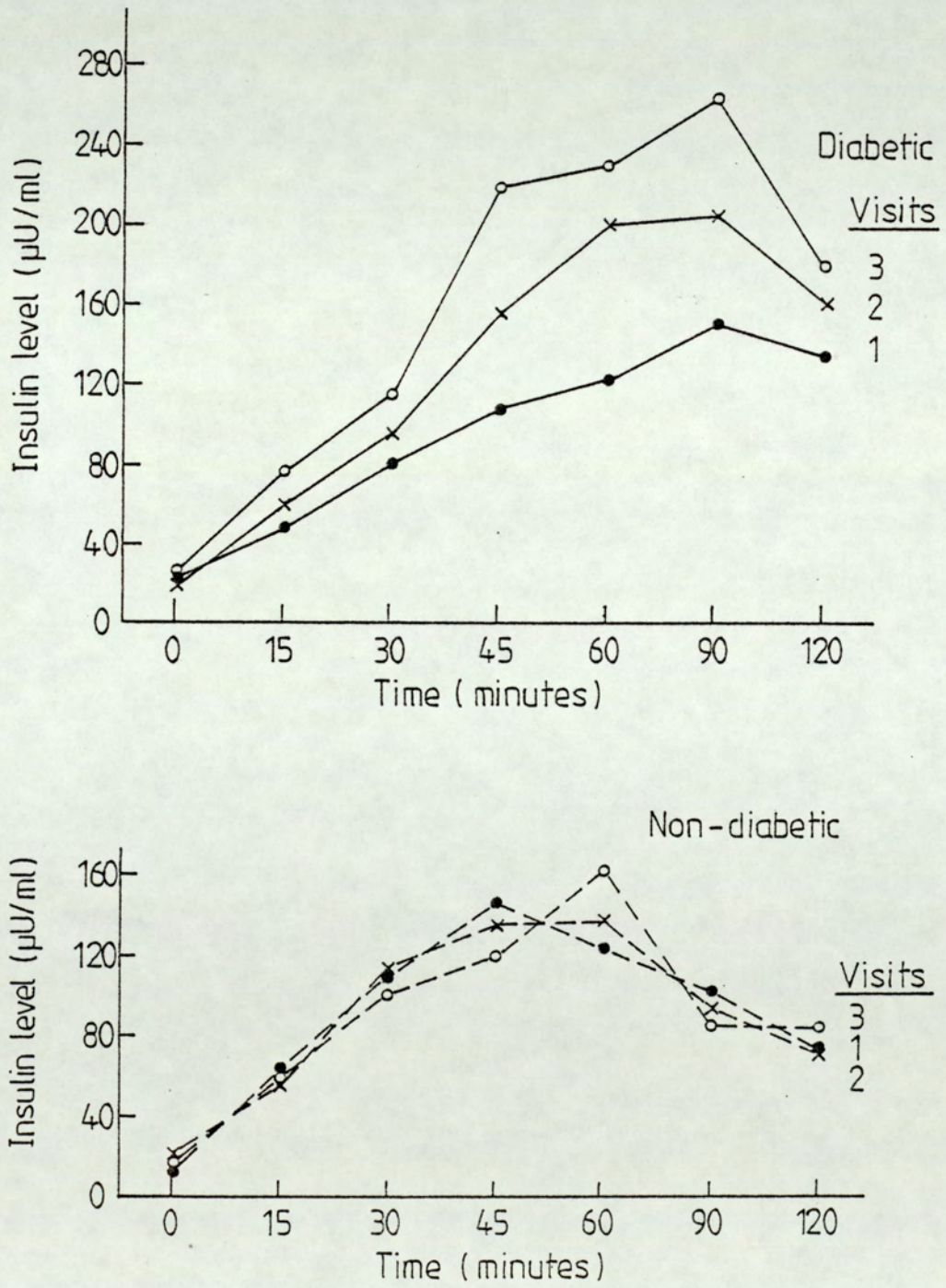
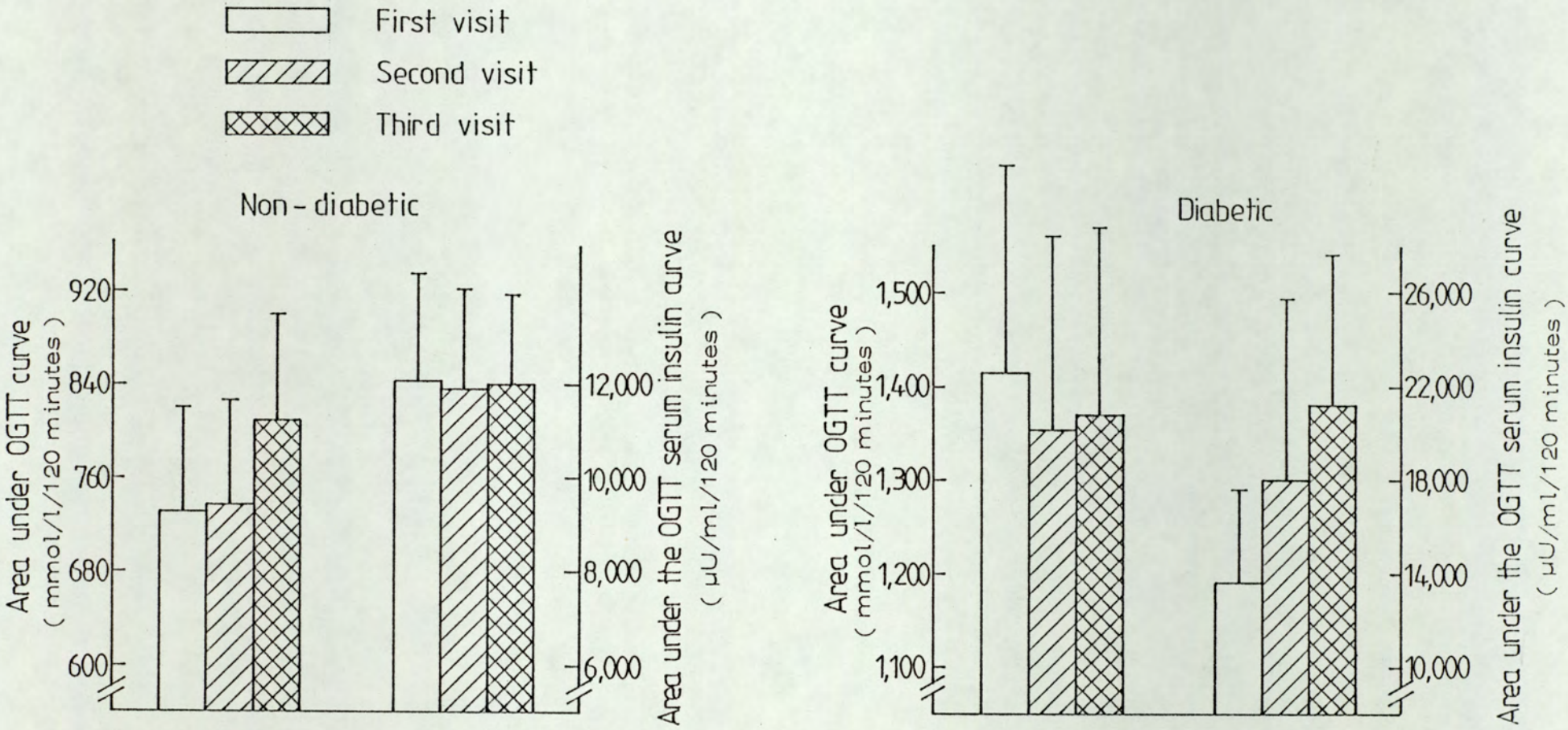


Figure 45. The effect of long term nifedipine treatment (1st visit), subsequent withdrawal (2nd visit) and the restoration of nifedipine treatment (3rd visit) on the area under the OGTT and OGTT insulin curves of mildly hypertensive patients with and without non-insulin dependent diabetes mellitus. (each block represents the mean value of 6 determinations ± SEM).



Nifedipine withdrawal did not significantly influence fasting levels of either serum glucose or serum insulin. Despite an inexplicable increase in glycosylated haemoglobin, there was some indication of a marginal improvement in the already impaired glucose tolerance of diabetic patients but no real change in the glucose tolerance of non-diabetic patients.

Previous studies have been unable to demonstrate an impaired glucose tolerance in normal individuals treated for three days with nifedipine (20 mg/day) (461) and to some extent the present work in consistent with the work of Giugliano and colleagues (98) who were unable to demonstrate any deterioration in glucose tolerance after 10 days nifedipine (30 mg/day) treatment of patients with normal glucose tolerance. Restoration of nifedipine treatment was not accompanied by changes in either fasting glucose or insulin levels but there was some evidence for a deterioration in glucose tolerance in both the diabetic and non-diabetic groups. An aggravation of the impaired glucose tolerance of non-insulin dependent diabetic patients has been postulated after 10 days treatment with nifedipine (30 mg/day) (98) but this has not been confirmed by other studies involving the treatment of diabetic patients for significantly longer periods of time (464,465,470). Any nifedipine induced impairment of glucose tolerance could well be the product of a reduction in insulin release, since nifedipine is a calcium antagonist and this cation is known to play a major role in B-cell glucose stimulus insulin secretion coupling (480). Nifedipine might equally act by affecting either intra B-cell stores of calcium, the metabolism of glucose in islets or the transport of other cations across the B-cell membrane (481).

However, in the present work fasting serum insulin levels were not significantly influenced by manipulation of the nifedipine treatment. Indeed the serum insulin levels recorded during the oral glucose tolerance tests of diabetic patients were significantly increased, even during the period of nifedipine withdrawal and could have accounted for the marginal improvement in glucose tolerance observed at that time. The aggravation of glucose intolerance observed in diabetic patients, after the restoration of nifedipine treatment, in the face of increased plasma insulin levels might suggest an overall reduction in insulin sensitivity.

It is conceivable that any changes in the circulating insulin level observed after nifedipine treatment might be the consequence of vasodilatation-induced activation of the sympathetic nervous system (98).

There is evidence to suggest that factors other than impaired insulin release may play a part in any nifedipine induced glucose intolerance. Particularly relevant would be raised plasma glucagon concentrations and the possibility of transient increases in plasma noradrenaline concentrations (482).

It is clear that the effect of nifedipine on oral glucose tolerance and insulin release should be taken into account when the drug is used for the treatment of non-insulin dependent diabetic patients with coronary heart disease and a limited capacity to secrete insulin, since any further deterioration in glucose tolerance produced by the drug may be harmful.

CHAPTER FIVE

Effect of Dietary Fibre (Guarem) on Insulin Receptor Binding to: A) Erythrocytes from Poorly Controlled Non-insulin Dependent Diabetics and B) Subcutaneous Adipocytes from Genetically Obese Diabetic (ob/ob) Mice.

Introduction

The term dietary fibre was first coined by Hipsley in 1953 to describe the components derived principally from plant cell walls in foods (382). Trowell subsequently used the term to describe those components of the cell walls of plants which were not digested in the small intestine of man (383). Trowell concluded that these components were protective against ischaemic heart disease and might have some use in the treatment of diabetes mellitus (384). Dietary fibre is a complex mixture and its composition will vary according to the type of food or diet in question. Polysaccharides and lignin of plant cell walls do not contain α-glucosidic bonds and this principal chemical feature distinguishes them from the other major polysaccharide in food, starch. This distinction is crucial because the endogenous secretions of the mammalian gastrointestinal tract only contain enzymes that hydrolyse α -glucosidic bonds. The polysaccharides of the plant cell wall are therefore not hydrolysed and are equivalent to the carbohydrates which McCance and Lawrence (385) described as unavailable. The non-structural polysaccharides include pectin and cellulose deriviatives and a range of gums which may be either exudate gums (e.g. gum arabic) or storage polysaccharide gums (e.g. guar) (386).

Guar gum is a gel-forming galactomannan, a storage polysaccharide obtained from the cluster bean (Cyamopsis tetragonaloba) grown in India. The mature bean is used as cattle food in India (387), while in the United Kingdom, it is used as a food additive, a stabiliser in ice cream and a thickener in sauces.

It has been reported that guar acts by slowing up the rate at

which carbohydrates are digested and absorbed in the intestine, although the way it carries this out is not fully understood. Most dietary carbohydrate is eventually turned into glucose by the body and guar supplementation of the diet can slow up the rate at which glucose arrives in the blood stream (388). The mechanisms by which guar is believed to act remain controversial. One hypothesis has suggested that guar acts by delaying gastric emptying, hence slowing the delivery of carbohydrates to the small intestine (313,389). In support of this hypothesis was the observation that viscous polysacchardies such as guar slow gastric emptying in man (390) and rats (391). In the pig, guar has been shown to have little effect on gastric emptying when given at levels known to reduce blood glucose levels in man (392). Recently Blackburn and coworkers (388) have demonstrated that the incorporation of guar into a hypertonic glucose drink significantly reduced postprandial blood glucose and insulin levels and in most subjects delayed the rate of gastric emptying. However, blood glucose levels were even reduced in patients whose gastric emptying was accelerated by guar (388). In addition no correlation was found between the change in the rate of gastric emptying induced by guar and the change in the peak increase in blood glucose concentration, suggesting that delayed gastric emptying was not the most predominant mechanism by which guar improved glucose tolerance. An alternative hypothesis based on the results of studies carried out in rats suggested that guar improved glucose tolerance by reducing the rate at which glucose was absorbed from the small intestine (292,393). This could be effected either by forming an intraluminal gel and delaying glucose absorption (394) or by increasing the resistance to diffusion of the intestinal unstirred

water layer (395) or even by inhibiting the effects of intestinal motility on fluid convection (388). The observation that plasma insulin levels were lowered by guar indicated that improved glucose tolerance was not due to an enhanced insulin response and instead may be related to a reduction in glucose absorption. It has been confirmed that guar exerts its effects on postprandial insulin levels by forming intra-luminal gels in the small intestine causing a delay in glucose absorption (396). However, several gastrintestinal hormones, notably gastric inhibitory polypeptide (GIP) and gut glucagon-like immunoreactivity (GLI) have been shown to stimulate insulin release (397). Several workers have suggested that the decrease in insulin levels after guar may be due to a reduction in the release of gastric inhibitory polypeptide from the duodenum and jejunum (398). Such a reduction in serum gastric inhibitory polypeptide concentration might enhance the hepatic disposal of glucose since gut hormones belonging to this family have been suggested to share some of the anti-insulin actions of glucagon on hepatic glucose metabolism (398).

Several approaches have been used to study the effects of dietary fibre on glucose metabolism in non-insulin dependent diabetic patients. Jenkins and colleagues (399) have investigated the effect of adding highly purified guar to the usual diets of non-insulin dependent diabetic patients and have also examined the effect of substituting commonly available foods rich in dietary fibre for low fibre items in the diet (400).

The possibility that dietary fibre could play a role in the management of diabetes was first suggested by Trowell (384). He indicated that primitive societies, such as those found in Uganda and

other developing countries consumed diets containing more "dietary fibre" and less refined carbohydrate (as sucrose) than Western communities (401). Members of these primitive societies rarely suffered from diabetes. As these countries became more developed and westernised, the prevalence of diabetes began to increase, and Trowell suggested that this trend was related to parallel changes in diet (401). The link between fibre and non-insulin dependent diabetes has been strengthened by the many well documented studies (399,400) demonstrating that fibre intake has therapeutic benefits for diabetic patients. Jenkins and coworkers (402) showed that gel-forming fibres like guar gum were more potent in preventing a rise in the blood glucose level following an oral glucose load, than the non-gelling fibres like bran. Studies have confirmed that, while the addition of guar gum to test meals improves glucose tolerance in both diabetic and normal subjects, non-viscous forms of dietary fibre such as bran are without effect (399,402). Preliminary results using the euglycaemic glucose clamp techniques support the concept of an effect of dietary fibre on the insulin response (403). An increased intake of high carbohydrate fibre with the diet has been found to improve glucose control in insulin dependent diabetics and/or lower the insulin requirement (404). Jenkins and colleagues have shown that meals rich in guar and pectin will reduce hyperglycaemia and improve glucose tolerance in normal and diabetic subjects (61,399,424). The addition of dietary fibre to the diet has been shown to improve non-insulin dependent diabetes by decreasing the fasting level of plasma glucose (405,406), improving the intravenous insulin tolerance (406) and decreasing the urinary excretion of glucose (406,407). The effect of a variety of dietary fibre materials including guar, pectin

and gum tragacanth have been investigated on oral glucose tolerance in normal subjects. The additiion of each substance significantly reduced blood glucose levels at one or more time points and generally reduced the serum insulin concentration (402). Jenkins and colleagues (408) also developed a crispbread preparation containing guar gum. When insulin-dependent diabetic patients consumed 14 and 26g of guar per day in this form they required less insulin and showed a lower incidence of glycosuria with no adverse effects on mineral metabolism (409). The addition of guar to test meals consumed by non-insulin dependent diabetic patients significantly reduced the rise in blood glucose normally observed during the first 2 hours of an OGTT and depressed serum insulin levels over the same time period (399). In experiments with normal subjects, the results were not so clear cut. The addition of guar to a test meal of bread and marmalade did decrease the postprandial blood glucose and insulin levels of normal patients for a short time (61). Similar observations have been made by Goulder and colleagues (394). In addition guar gum supplementation led to a significant reduction in both basal and postprandial hyperglycaemia and insulin levels in non-insulin dependent diabetics receiving no oral hypoglycaemic therapy (407). Three months treatment of mildly hypertensive diabetic patients with a high fibre diet, guar bread or granulated guar produced a significant reduction in glycosylated haemoglobin (HbA1) indicating an overal improvement in diabetic control (66,416).

It is well established that obese non-insulin dependent diabetic patients have considerably higher insulin levels than normal weight, normoglycaemic individuals (410). Weight reduction is therefore one of the cornerstones in the treatment of the often overweight non-

insulin dependent diabetic patient. However, there is evidence to suggest that fibre treatment can improve glucose control in diabetics even in the absence of any weight reduction (411). Dietary fibre might contribute directly to weight reduction by creating a state of satiety. There are several important aspects involving hunger and satiety regulation which may be influenced by the fibre content of food (412). Increased dietary fibre content leads to a calorie dilution of the food, as the availability of calories in fibre is poor. Dietary fibre supplements have been shown to reduce hunger and increase satiety (403). There appears to be a difference in this regard between the gelling and the non-gelling fibres. Krotkiewski has recently shown that guar taken before main meals leads to a more significant reduction in hunger than wheat bran taken in the same way (412). The average weight reduction tended to be greater with guar supplement than with wheat bran. Furthermore, Tuomilehto and colleagues have shown that the daily ingestion of 15g of guar gum results in a permanent weight loss (413). Other workers have shown that guar gum supplementation does not reduce the body weight of either obese healthy subjects (414) or non-insulin dependent diabetics (407). In rats, guar supplementation does not affect the normal rate of body weight gain (415).

Vascular disease and coronary artery disease are the major causes of the reduced life expectancy of non-insulin dependent diabetics, Hypercholesterolaemia, high concentrations of low density lipoproteins (LDL) and hypertriglyceridaemia have been linked with vascular disease, particularly, coronary artery disease (381). The risk of disease increases with increased blood cholesterol levels. The concentration of high density lipoproteins (HDL) appears to be

inversely associated with the risk of coronary artery disease (333). The changes seen in plasma lipids and lipoproteins in normal subjects and in non-diabetic hyperlipidaemic patients in response to various dietary manipulations may be difficult to interpret, owing to alterations in other variables such as calorie intake, weight and compliance with therapy. In non-insulin dependent diabetic patients such studies have the added problems of variations in blood glucose, insulin, metabolites and the possibility of oral hypoglycaemic therapy. The hypolipidaemic effects of high fibre diets in normal non-diabetic subjects has been examined by several groups (66,406). In these studies the diet was supplemented with various types of fibre at doses of between 5 and 100g per day and included wheat bran, carrot, cabbage, citrus pectin and guar gum (417). 15-40 g of guar gum per day for periods of 2-8 weeks significantly reduced the serum cholesterol levels of both normal subjects and hyperlipidaemic non-insulin dependent diabetic subjects (418). Guar gum supplementation in non-insulin dependent diabetics caused a significant decline in the serum cholesterol level (407,414) without a change in serum triglyceride (414). Peterson (416) has reported that fasting triglycerides fall significantly in patients on guar gum and the most striking improvement was a reduction in total cholesterol. Furthermore, the reduction in the cholesterol level was almost exclusively due to a fall in low density lipoprotein cholesterol, as the high density lipoprotein cholesterol fraction did not change throughout (416). On the other hand Tuomilehto and coworkers (413) have been unable to demonstrate consistent changes in either serum cholesterol, triglycerides or HDL cholesterol in hyper-cholesterolaemic female patients taking guar gum. A change in body

weight has been suggested to cause changes in serum lipid levels (419). Guar gum incorporated into hypercholesterolaemic rat diets significantly lowered serum cholesterol (415), liver cholesterol and total liver lipid (420) but fasting plasma triglycerides were not affected (415).

The available data on the use of dietary fibre in obesity and non-insulin dependent diabetes mellitus have strongly suggested that the reported improvement in glucose tolerance is mainly brought about through a reduction in insulin resistance. A high fibre diet has been shown to be associated with an increased tissue sensitivity to insulin. Increased insulin binding to circulating monocytes and a 25% increase in the number of insulin receptors on monocytes has been demonstrated in adult insulin dependent diabetic patients on high fibre diets (422). Feeding insulin-dependent diabetics with a low-fat/high-starch/high-fibre diet for 4 weeks was accompanied by a significant rise in monocyte insulin bidning with no change in insulin binding to erythrocytes (423). Increased insulin receptor binding to adipocytes and monocytes but not erythrocytes has also been observed in non-insulin dependent diabetic patients fed low-fat/high-starch/high-fibre diets (406).

Guar supplementation does not appear to affect serum calcium concentration, but there is evidence to suggest that the urinary excretion of calcium may be reduced (407).

The quality and content of the galactomannan polysaccharide in guar gum can vary dramatically and depends upon the method of growth, final extraction and purification of the guar. The viscosity of the hydrated guar is important, because this property influences its clinical effectiveness. It is therefore vital to characterise

accurately the preparation used in any experimental work. Any delay in the rate of development of viscosity on hydration of a guar preparation may mean that the improvement in postprandial blood glucose and insulin levels will be missed and therapeutic effects attenuated. Highly viscous guar preparations are the most clinically useful but unfortunately they are also the least palatable. There is also evidence that the intimate mixing of guar with food is required for the maximum effect on blood glucose and insulin levels (483). At present, guar can be considered as a granular drug or a food product, bread being one possible future vehicle for its administration (408). The quantities of guar used in early investigational studies were very large (40g per day). It is now known that as little as 2.5g of guar incorporated into a test meal is just as effective as these larger doses (425). The dose and form of administration of guar has been investigated using bread and soup test meals (426). Guar was most active in modulating blood glucose and serum insulin levels when it was taken rapidly suspended in liquid.

The first part of the present study (Part A) was initiated to investigate whether guar (Guarem(R), Remeda Pharmaceuticals, Kuopio, Finland) was an effective and acceptable form of treatment for poorly controlled non-compliant non-insulin dependent diabetic patients. This study involved the determination of erythrocyte insulin receptor status, oral glucose tolerance, plasma glucose, insulin and calcium concentrations, body weight, blood pressure and a biochemical profile including glycosylated haemoglobin (HbA1). This work was accompnaied by animal study (part B) involving the effect of guarem on adipocyte insulin receptor binding, body weight, blood glucose, insulin and calcium levels of genetically obese diabetic (ob/ob) mice from Aston colony.

Materials and Methods

Part A: The effect of guarem on erythrocyte insulin receptor binding in poorly controlled non-insulin dependent diabetics.

Patients:

Patients with poorly controlled non-insulin dependent diabetes and a history of non-compliance were recruited from the Diabetic Clinic at Dudley Road Hospital, Birmingham. All patients were less than 65 years of age and more than 110% ideal body weight with poor glycaemic control over the last three clinic visits i.e. with a clinic blood glucose of >9.9 mmol/l and a HbA1 level greater than 11%. As far as possible, patients were dietary controlled throughout and only postmenopausal women were selected to avoid the effect of the menstrual cycle on insulin receptor binding. Baseline recordings were made at the initial clinic visit. Patients presented at 09.00 hours in the clinic after a 12 hour overnight fast and the following parameters were determined dietary intake, oral glucose tolerance, body weight and blood pressure (taken seated using a Hawksley random zero sphygmomanometer). Venous blood was taken for insulin receptor binding, serum was separated for glucose and insulin and whole blood was taken for the determination of glycosylated haemoglobin (HbA1) and the biochemical profile. Details of the oral glucose tolerance test and biochemical determinations have been described previously, pages 119 and 120, respectively. Studies were initially performed on a total of 8 patients but only 7 completed the study, 3 postmenopausal women and 4 men, average age 58 $\pm$ 4.2 years.

Study protocol:

At the first clinic visit, diet or oral hypoglycaemic therapy was maintained and patients were reviewed one month later providing a second baseline recording.

Guarem treatment commenced at the second visit and patients were maintained on the agent for one month. The subjects were asked to take one sachet (5g) of the granules in 200 ml of flavoured water just prior to each main meal. The drink was consumed within 1-2 minutes prior to gelling (total dose 15g per day).

Patients were then reviewed after one month (providing the first treatment recordings) and subsequently asked if they would like to continue with guarem treatments. Any subjective opinions of the patients were recorded (i.e. horrible, distasteful, unsociable etc.).

Erythrocyte insulin receptor binding was determined by the method of Gambhir and colleagues (121). Non-specific binding in the presence of an excess of unlabelled insulin (10^5 ng/ml) averaged 14% of the total insulin binding.

The effect of guarem treatment on the percentage degradation of ^{125}I-insulin by isolated erythrocytes from non-insulin dependent diabetic patients was determined as described previously, page 110.

Part B: The effect of guarem administration on adipocyte insulin receptor binding in genetically obese diabetic (ob/ob) mice.

Animals:

Obese hyperglycaemic mice of both sexes (mean body weight 90.1 ± 2.4 g, and mean age 19.2 ± 0.5 weeks) from the Aston colony were fed a standard pellet diet (Heygate and Sons Ltd., Northampton, U.K.) and tap water ad libitum.

Technique for the administration of guarem.

Guarem was rapidly administered orally as a suspension in water with the aid of a 2ml syringe connected to a sterilised bent serum needle with a small bubble of epoxy resin on the end. The needle was gently eased down the pharynx of the mouse and the dose of guarem expelled into the stomach. Generally a guarem dose sufficient for six mice was made up and maintained in suspension with the aid of a magnetic stirrer. Each mouse received its dose of guarem in a final volume of 0.5 ml of double distilled water.

Experimental protocol for animal studies.

Initial experiments were carried out to determine the optimal dose and exposure time for guarem treatment. Assessment was made of body weight, plasma glucose, insulin and calcium after 4 days treatment with a range of guarem concentrations (1.1 - 19.3 mg/day) as summarised in the experimental protocol below:

Experimental protocol

Obese mice	Time of exposure	Parameters measured	Concentration of guarem (mg/day)	gm/70 kg human equivalent
13 Mean age 18.91 ± 0.62 weeks Mean body weight 91.6 ± 2.46g)	4 days	Body weight Plasma: glucose insulin Ca^{2+}	19.3	15/70
16 Mean age 18.06 ± 0.47 weeks Mean body weight 82.6 ± 5.1g) Split into 4 dosage groups	4 days	Body weight Plasma: glucose insulin Ca^{2+}		
4			9.1	8/70
4			4.6	4/70
4			2.3	2/70
4			1.1	1/70

In a second series of experiments the time of exposure was reduced to 2 days and obese mice were administered either a low dose of guarem (2.3 mg/day) or a high dose of guarem (19.3 mg/day). Assessment was made of body weight, plasma glucose, insulin, calcium and insulin receptor binding using adipocytes from the subcutaneous femoral region of obese mice.

Isolated fat cells were prepared according to the method of Pedersen and colleagues (130) as described previously (page 89). Insulin receptor binding data were analysed by the method of Scatchard (267) and interpreted using both the two class model and the negatively cooperatively model of insulin receptor binding (page 102 for details). Binding was expressed in terms of the concentration of receptor per cell and per square micrometer of the cell surface area (μm^2). Non-specific counts (10^5 ng/ml) were substracted from total to provide the amount of ^{125}I-insulin specifically bound.

^{125}I-insulin degradation by isolated adipocytes and fat segments from low and high guarem treated obese mice was estimated in the presence and absence of a high concentration of unlabelled insulin (10^5 ng/ml) (see pages 111 and 112 for details).

Results

A) Clinical study

The effect of guarem treatment for 1 month on the plasma glucose, insulin, calcium, general biochemistry and blood pressure of non-insulin dependent diabetics has been summarised in Table 19. All patients maintained their body weights throughout the study. Guarem

Table 19. The effect of guarem treatment for one month on poorly controlled non-insulin dependent diabetics (mean values ± SEM).

	n	First visit (1st baseline)	Second visit (2nd baseline)	Third visit (1 month guarem treatment)
Body weight (kg)	7	86.7 ± 5.3	88.1 ± 6.7	88.4 ± 6.9
Plasma glucose level (mmol/l)	7	10.4 ± 1.3	10.8 ± 0.7	8.1 ± 0.7*
Plasma insulin level (µU/ml)	7	48.2 ± 8.6	40.2 ± 9.9	23.8 ± 6.3+
Glycosylated haemoglobin (%)	7	13.8 ± 0.8	13.1 ± 0.7	12.2 ± 0.7
Cholesterol (mmol/l)	7	6.1 ± 0.4	6.1 ± 0.6	5.6 ± 0.6
Triglycerides (mmol/l)	7	1.8 ± 0.3	1.4 ± 0.3	1.4 ± 0.3
Urea (mmol/l)	7	5.8 ± 0.6	5.6 ± 0.6	5.1 ± 0.7
Uric acid (mmol/l)	7	0.231 ± 0.03	0.232 ± 0.03	0.214 ± 0.04
Sodium (mmol/l)	7	137.7 ± 1.0	138.6 ± 1.0	137.6 ± 1.7
Potassium (mmol/l)	7	4.4 ± 0.2	4.5 ± 0.3	4.4 ± 0.1
Calcium (mmol/l)	7	2.4 ± 0.04	2.3 ± 0.08	2.2 ± 0.04
Systolic blood pressure (mmHg)	7	140 ± 7.9	146.7 ± 4.4	138.3 ± 18.6
Diastolic blood pressure (mmHg)	7	76.8 ± 14.3	81.7 ± 10.9	81.7 ± 7.3

* $p < 0.05$ compared with second visit.

\+ $p < 0.05$ compared with first and second visits.

treatment significantly lowered plasma glucose and insulin levels, Table 19 and marginally improved oral glucose tolerance, Figure 46. Guarem treatment had no significant effect on either blood pressure or the various components of the biochemical profile.

In addition, guarem treatment had no significant effect on either erythrocyte insulin receptor binding, Table 20, or on the total amount of insulin bound in the presence of a range of low and high insulin concentrations, Figure 47. Competition-inhibition curves for insulin binding to erythrocytes were nonlinear, Figure 48. All treatments caused a displacement of bound ^{125}I-insulin as the concentration of unlabelled insulin increased. Analysis of the data by this method was not dependent on the maximum binding capacity (Ro) and reflected receptor affinity at physiological insulin concentrations. Guarem treatment did not significantly affect receptor affinity, Table 20 and Figure 48, and this observation was supported by the non-linear parallel Scatchard plots of the insulin binding data, Figure 49, indicating no change in either insulin receptor concentration or affinity.

Guarem treatment did not significantly affect the percentage degradation of ^{125}I-insulin by isolated erythrocytes from non-insulin dependent diabetic patients whether estimated in the presence or absence of unlabelled insulin.

B) Guarem treatment of obese mice.

The treatment of obese mice with a high concentration of guarem (human dose equivalent) for four days produced a high mortality (50%). In surviving animals the body weight, plasma glucose and plasma insulin levels were significantly reduced after guarem

Figure 46. The effect of guarem treatment on the 75 g oral glucose tolerance of poorly controlled non-insulin dependent diabetics. (mean of 7 determinations ± SEM).

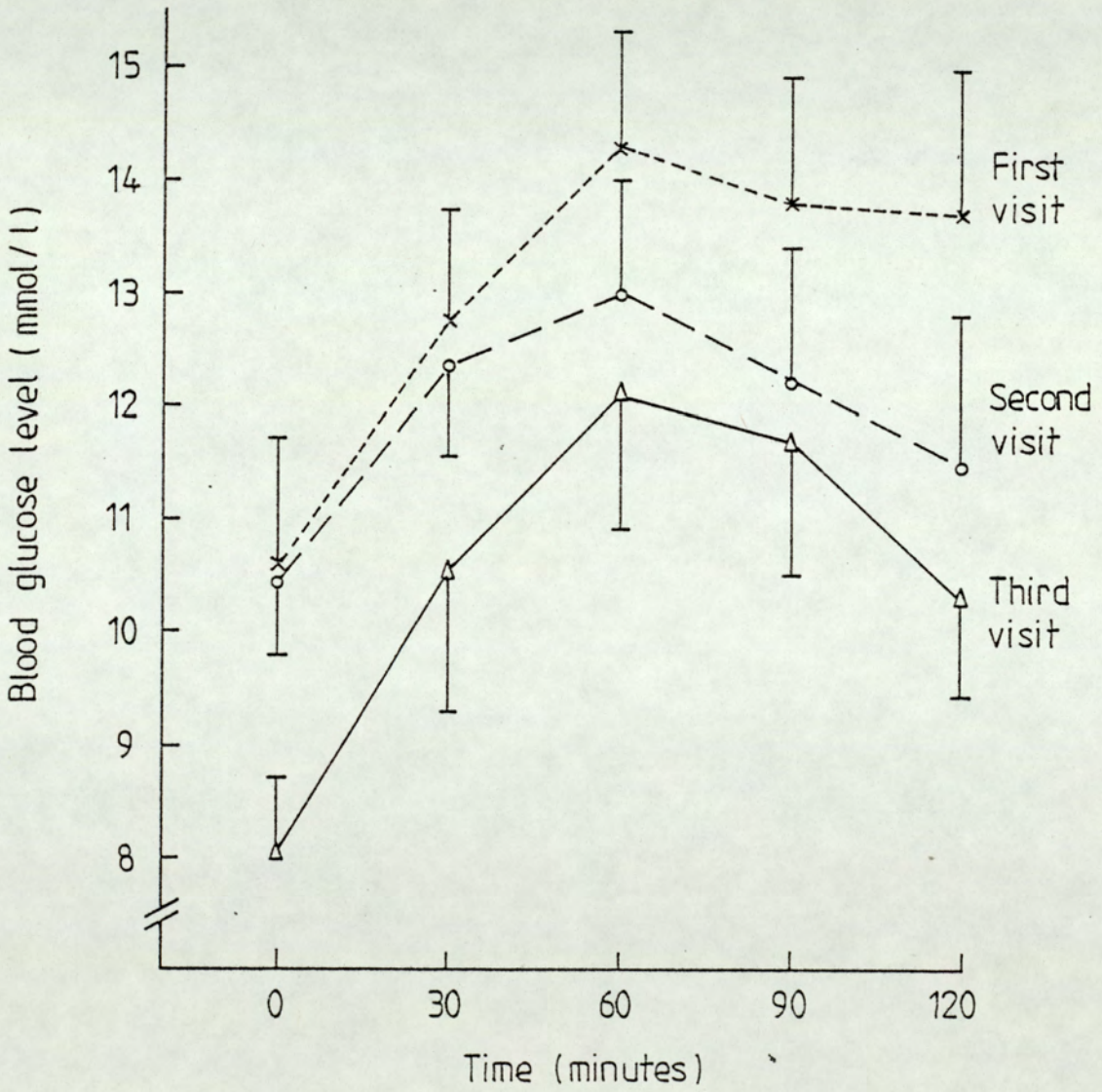


Table 20. Erythrocyte insulin receptor binding in poorly controlled non-insulin dependent diabetics treated for one month with guarem (mean values ± SEM).

	n	Specfic binding (%)	High affinity receptor concentration per cell	Low affinity receptor concentration per cell	Total affinity receptor concentration per cell	$\bar{K}_e$, affinity constant of empty sites (nM^{-1})	$\bar{K}_f$, affinity constant of full sites (nM^{-1})	$\bar{K}_m$, apparent affinity constant (nM^{-1})
First visit (1st baseline)	6	5.67 ± 0.43	11.82 ± 1.55	82.9 ± 6.5	94.72 ± 7.2	0.11685 ± 0.013	0.01478 ± 0.0017	0.06194 ± 0.0067
Second visit (2nd baseline)	6	5.76 ± 0.21	12.35 ± 1.29	87.78 ± 7.8	100.13 ± 8.5	0.11426 ± 0.014	0.01494 ± 0.001	0.06099 ± 0.0066
Third visit (one month guarem treatment)	6	5.95 ± 0.3	13.07 ± 1.84	85.82 ± 8.3	98.89 ± 9.8	0.12115 ± 0.0146	0.01799 ± 0.002	0.06495 ± 0.0077

Figure 47. Binding curves showing the effect of guarem treatment on the specific binding of insulin to erythrocytes in the presence of a range of (a) low and (b) high insulin concentrations. Each point represents the mean of 6 determinations.

×-------× First visit.

o– – – –o Second visit.

Δ———Δ Third visit.

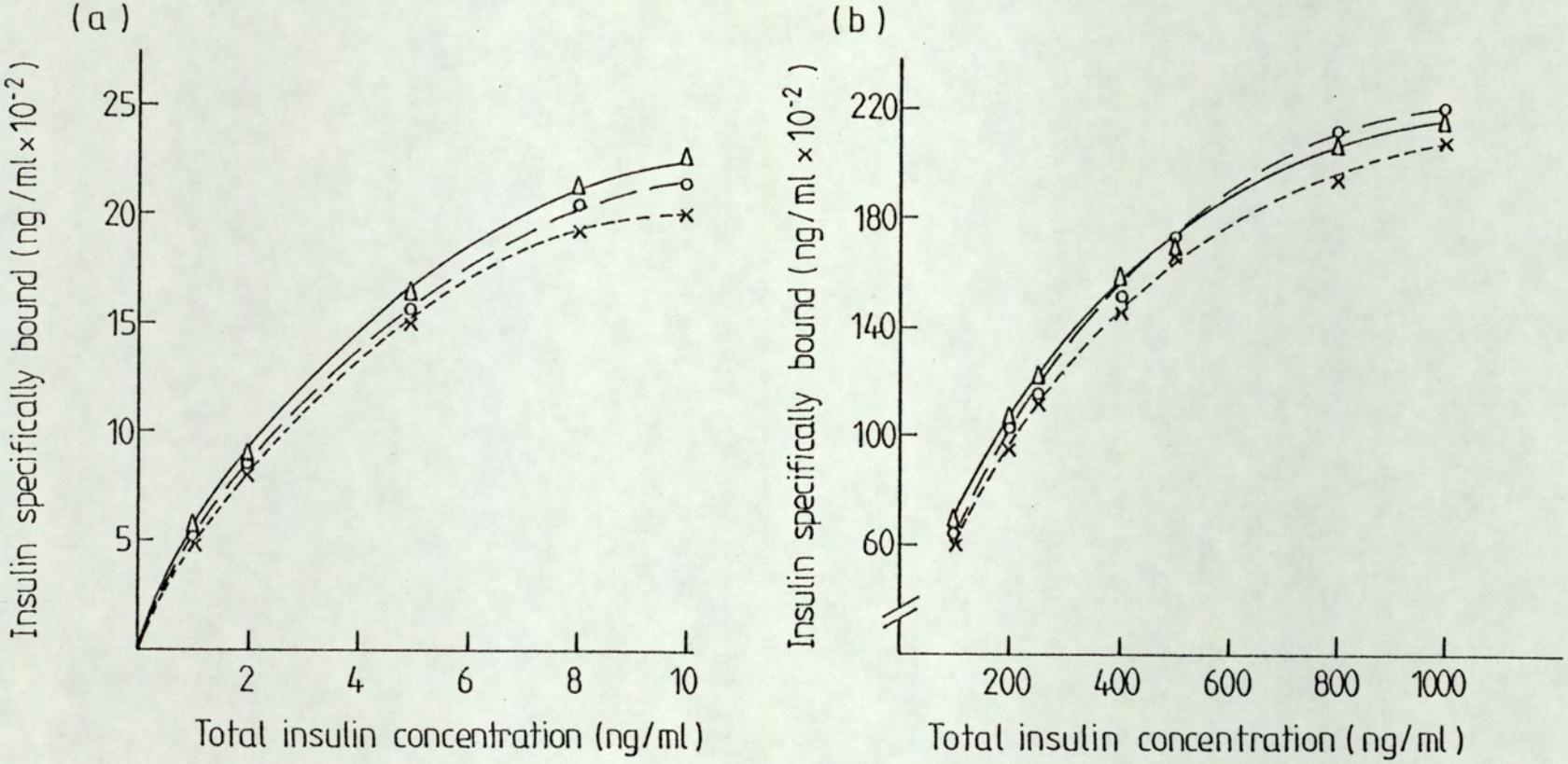


Figure 48. Competition - inhibition curves showing the effect of guarem treatment on the percentage ^{125}I - insulin specifically bound to erythrocytes from poorly controlled non-insulin dependent diabetics. Each point represents the mean of 6 determinations.

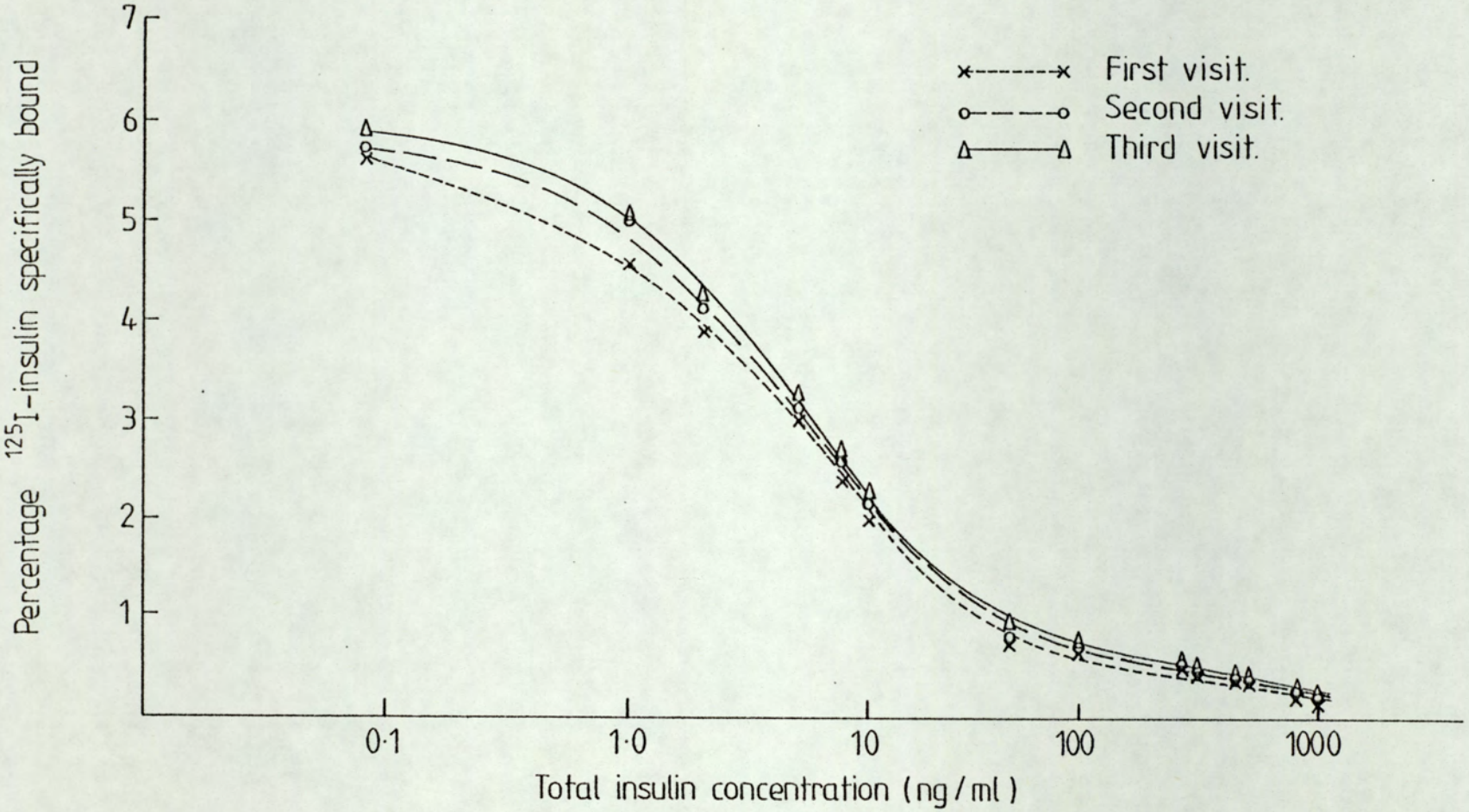
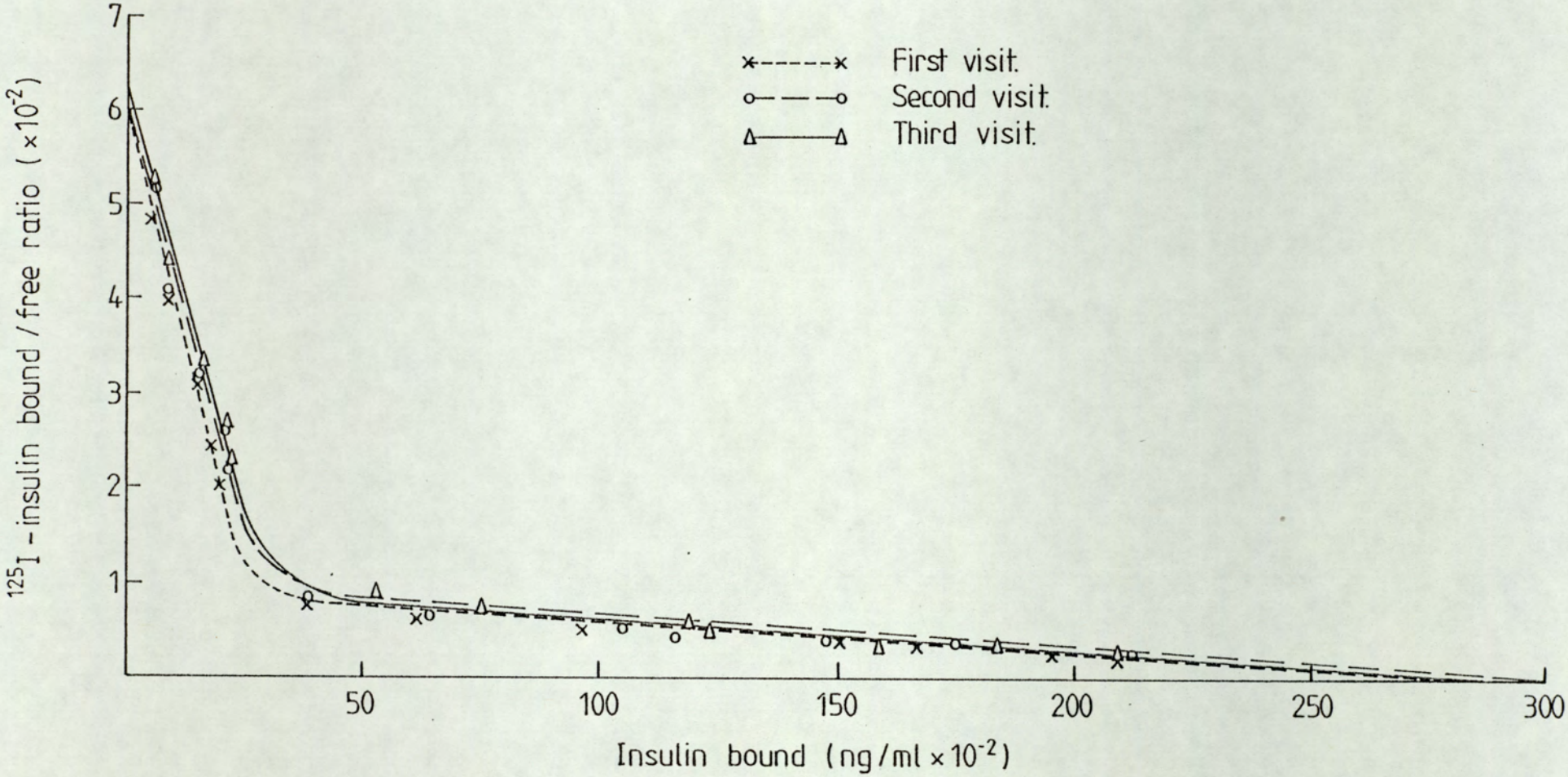


Figure 49. Scatchard analysis of insulin binding to erythrocytes from poorly controlled non-insulin dependent diabetics.



treatment, Table 21, although plasma calcium levels remained unchanged. Postmortem examination of animals that had died during guarem treatment showed evidence of intestinal blockage, large amounts of stomach gas, enhanced bile production and morbidity of the small intestine.

When obese mice were treated for four days with a range of lower guarem concentrations, Table 22, body weights were significantly reduced. In addition plasma glucose was decreased after treatment but only achieved statistical significance with guarem concentrations of 4.6 and 2.3 mg/day. Plasma insulin levels tended to decrease after guarem treatment but did not achieve statistical significance.

The treatment of obese mice for 2 days with low and high concentrations of guarem significantly lowered both the body weight and plasma glucose levels of obese mice, Table 23. However, only the high concetration of guarem significantly lowered the plasma insulin level. Plasma calcium levels were not affected by guarem treatment, Table 23. The mean adipocyte diameter was significantly reduced when obese mice were treated with a high concentration of guarem for 2 days, Table 24A.

The low concentration of guarem had no significant effect on either adipocyte insulin receptor concentration or affinity, Tables 24A and B. This observation was supported by the similarity in the binding curves obtained, Figure 50. Scatchard analysis of the binding data generated similarly shaped curviliner plots, Figure 51. Since the slopes of the Scatchard plots at the same fractional occupancy were not significantly different there appeared to be no change in adipocyte insulin receptor affinity when obese mice were treated with a low concentration of guarem. The high concentration

Table 21. The effect of four days high guarem treatment (19.3 mg/day) on the body weight, plasma glucose, plasma insulin, and plasma calcium levels of obese mice (mean values ± SEM).

	n	Values for all obese mice before guarem treatment	n	Pretreatment values for surviving obese mice	n	Values for obese mice after guarem treatment
Body weight (g)	13	91.6 ± 2.46	6	88.9 ± 3.81	6	80.1 ± 5.53 +*
Glucose level (mmol/l)	13	24.1 ± 1.64	6	22.1 ± 1.88	6	12.38 ± 2.99 +*
Insulin level (ng/ml)	13	47.01 ± 4.33	6	48.9 ± 7.81	6	29.88 ± 6.74 +
Calcium level (mmol/l)	13	2.428 ± 0.04	6	2.460 ± 0.05	6	2.625 ± 0.05

* $p < 0.05$ compared with pretreatment values of surviving obese mice.

\+ $p < 0.05$ compared with values for all obese mice before guarem treatment.

Table 22. The effect of four days treatment with a range of guarem concentrations (1.1 - 9.1 mg/day) on the body weight, plasma glucose, insulin and calcium levels of obese mice (mean values ± SEM).

	n	Body weight (g)	Glucose level (mmol/l)	Insulin level (ng/ml)	Calcium level (mmol/l
Before treatment:					
Mean values	16	82.6 ± 5.1	22.31 ± 1.93	46.4 ± 9.7	2.32 ± 0.06
First group	4	82.2 ± 3.7	-	-	-
Second group	4	81.7 ± 1.7	-	-	-
Third group	4	89.5 ± 8.2	-	-	-
Fourth group	4	77.0 ± 8.5	-	-	-
After treatment with guarem:					
Mean values for surviving mice	11	74.6 ± 3.2 *	14.23 ± 2.63	24.9 ± 4.69	2.51 ± 0.04
First group (9.1 mg/day)	2	74.2 ± 1.6 *	14.65 ± 3.75	21.5 ± 6.65	2.61 ± 0.06
Second group (4.6 mg/day)	3	73.8 ± 1.9 + *	11.22 ± 2.11 *	19.1 ± 8.65	2.43 ± 0.09
Third group (2.3 mg/day)	3	83.8 ± 8.4 + *	12.17 ± 3.65 *	30.5 ± 14.7	2.51 ± 0.08
Fourth group (1.1 mg/day)	3	66.6 ± 6.1 + *	19.03 ± 9.29	27.3 ± 7.5	2.53 ± 0.06

* $p < 0.05$ compared with mean value before treatment.

\+ $p < 0.05$ compared with individual group values before treatment.

Table 23. The effect of two days treatment with low and high concentrations of guarem on the body weight, plasma glucose, insulin and calcium levels of obese mice (mean values ± SEM).

	Low concentration of guarem (2.3 mg/day)			High concentration of guarem (19.3 mg/day)		
	n	Before treatment	After treatment	n	Before treatment	After treatment
Body weight (g)	10	96.62 ± 2.41	94.61 ± 2.27 *	12	92.95 ± 3.51	88.54 ± 3.26 *
Glucose level (mmol/l)	10	23.38 ± 1.51	19.44 ± 0.74 *	12	24.54 ± 1.17	20.11 ± 1.57 *
Insulin level (ng/ml)	9	48.48 ± 5.1	39.5 ± 6.01	10	51.81 ± 4.41	37.38 ± 4.3 *
Calcium level (mmol/l)	10	2.30 ± 0.05	2.39 ± 0.09	12	2.32 ± 0.04	2.36 ± 0.06

* $p < 0.05$ significantly reduced compared with values before guarem treatment.

Table 24A. The effect of two days treatment with low and high concentrations of guarem on the insulin receptor binding data of adipocytes from obese mice (mean values ± SEM).

	n	Specific binding (%)	Adipocyte diameter (µm)	High affinity receptor concentration per cell ($x10^5$)	Low affinity receptor concentration per cell ($x10^5$)	Total affinity receptor concentration per cell ($x10^5$)
Untreated control obese mice	7	1.831 ± 0.16	64.53 ± 1.72	0.606 ± 0.13	2.7 ± 0.18	3.304 ± 0.15
Treated with low concentration of guarem (2.3 mg/day)	5	2.134 + ± 0.19	61.34 ± 1.34	0.624 ± 0.05	2.49 ± 0.184	3.12 ± 0.19
Treated with high concentration of guarem (19.3 mg/day)	6	2.623 * ± 0.06	58.15 * ± 1.54	0.658 ± 0.04	2.54 ± 0.33	3.2 ± 0.29

* $p < 0.05$ compared with untreated control values.

\+ $p < 0.05$ compared with obese mice treated with a high concentration of guarem.

Table 24B.

	n	High affinity receptor concentration per μm^2	Low affinity receptor concentration per μm^2	Total affinity receptor concentration per μm^2	$\bar{K}_e$, affinity constant of empty sites (nM^{-1})	$\bar{K}_f$, affinity constant of full sites (nM^{-1})	$\bar{K}_m$, apparent affinity constant (nM^{-1})
Untreated control obese mice	7	4.55 $\pm$ 0.88	21.15 $\pm$ 2.29	25.7 $\pm$ 2.04	0.08053 $\pm$ 0.006	0.02450 $\pm$ 0.004	0.04422 $\pm$ 0.004
Treated with low concentration of guarem (2.3 mg/day)	5	5.3 $\pm$ 0.5	21.01 $\pm$ 1.08	26.31 $\pm$ 0.97	0.09718 + $\pm$ 0.003	0.02888 + $\pm$ 0.002	0.05556 + $\pm$ 0.003
Treated with high concentration of guarem (19.3 mg/day)	6	6.28 $\pm$ 0.58	24.31 $\pm$ 3.441	30.6 $\pm$ 3.4	0.1255 * $\pm$ 0.008	0.04263 * $\pm$ 0.005	0.07067 * $\pm$ 0.006

* $p < 0.05$ compared with untreated control values.

\+ $p < 0.05$ compared with obese mice treated with a high concentration of guarem.

Figure 50. Binding curves for obese mouse adipocytes. Data are presented as the amount of specifically bound insulin plotted against the total insulin concentration.

×-------× Untreated controls.

o— — —o Treated with low guarem concentration.

Δ———Δ Treated with high guarem concentration.

* $p < 0.05$ compared with both untreated control and low guarem treated values.

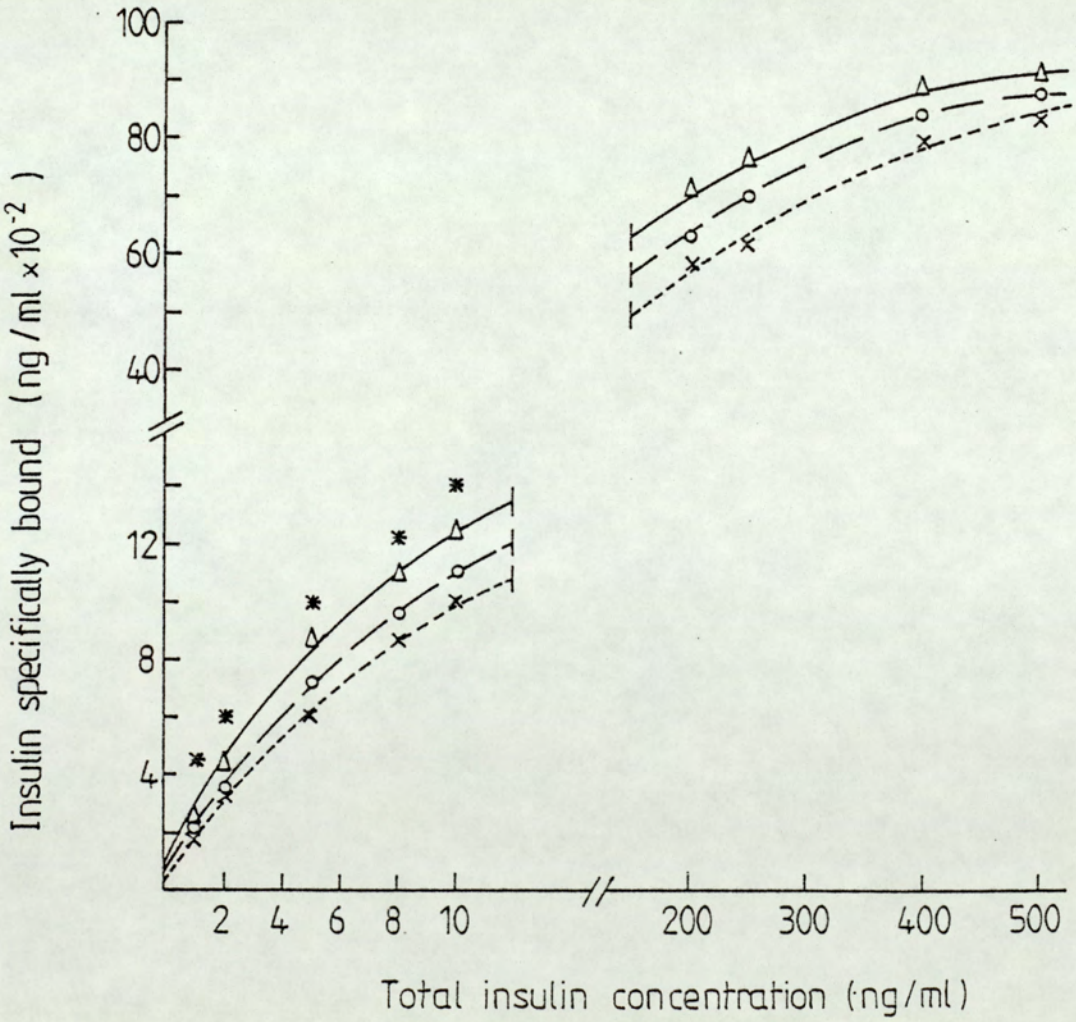
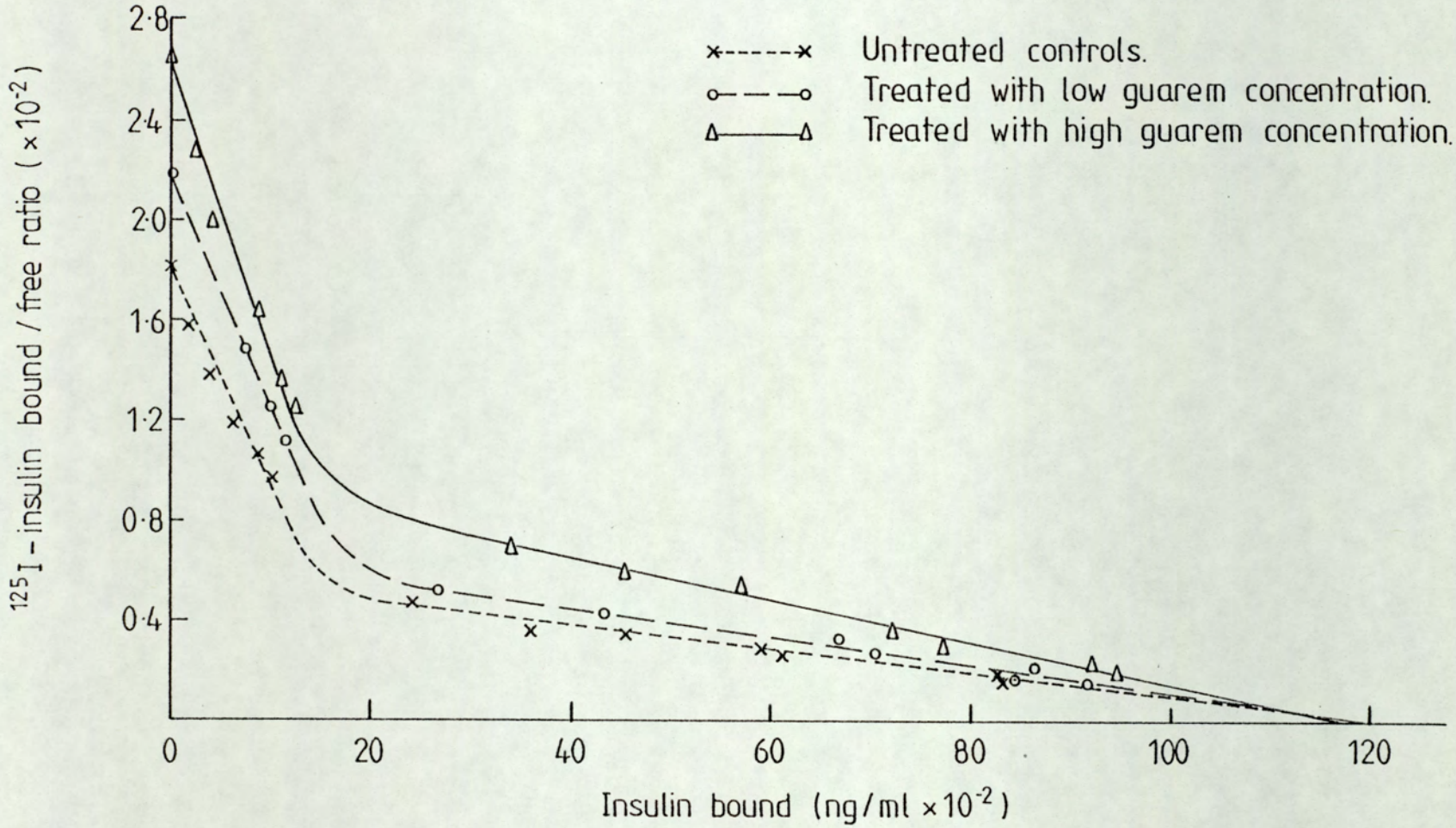


Figure 51. Scatchard analysis of insulin receptor binding data for adipocytes from obese mice treated with low and high concentrations of guarem.



of guarem had no effect on adipocyte insulin receptor concentration whether expressed per cell or μm^2 of cell surface area, but significantly increased both the percentage of insulin specifically bound to adipocytes and the values for the receptor affinity constants $\bar{K}_e$, $\bar{K}_f$ and $\bar{K}_m$, Table 24A and B. In the presence of low insulin concentrations (0-10 ng/ml) the ability of adipocytes from obese mice treated with the high concentration of guarem to specifically bind insulin was significantly increased compared with adipocytes from untreated control obese mice, Figure 50. However, no change in the specific insulin binding to adipocytes from obese mice treated with high concentration of guarem was observed in the presence of high concentrations of insulin (200-500 ng/ml), Figure 50. These observations suggest that the increased adipocyte insulin binding resulting from the treatment of obese mice with a high concentration of guarem was not due to a change in receptor number but rather to an increase in receptor affinity. This observation was also supported by the Scatchard plots obtained, Figure 51. At the same fractional occupancy two upwardly concave curves were obtained with different slopes and different origins from the bound/free axis but both intersected the abscissa at almost the same point.

When the effects of low and high guarem concentrations on insulin receptor binding were compared, the percentage of insulin specifically bound to adipocytes was significantly higher after treatment with the high guarem concentration, Table 24A, and this was due to an increase in adipocyte insulin receptor affinity, Table 24B, Figures 50 and 51 with no effect on insulin receptor concentration.

The effect of treating obese mice with low and high concentrations of guarem on the percentage degradation of ^{125}I-insulin by

isolated adipocytes and isolated fat segments has been summarised in Table 25. Fat segments produced a significantly greater degradation of ^{125}I-insulin than isolated adipocytes. The addition of a high concentration of unlabelled insulin (10^5 ng/ml) significantly reduced ^{125}I-insulin degradation by both adipocytes and fat segments. Guarem treatment had no significant effect on the degradation of ^{125}I-insulin by either adipocytes or fat segments. The percentage of ^{125}I-insulin degradation by both adipocytes and fat segments from guarem treated obese mice was not significantly affected by the addition of 10^5 ng/ml of unlabelled insulin.

Discussion

The treatment of poorly controlled non-insulin dependent diabetics with guarem for one month did not significantly alter their body weight. This observation is at variance with the results of studies by Tuomilehto and colleagues who showed that the daily ingestion of 15g of guarem by female hypercholesterolaemic patients resulted in a permanent reduction in body weight (413). On the other hand the present results are consistent with studies carried out by several other groups who have been unable to demonstrate a reduction in body weight in healthy obese and non-insulin dependent diabetic patients treated with guar gum (414,429).

Treatment of obese mice with relatively low concentration of guarem produced a significant reduction in body weight. However in this case the guar may have been having a toxic rather than physiological effect. It is clear that man and obese mice deal with guarem in different ways. In man guarem is not broken down to any

Table 25. The effect of treatment with low and high guarem concentrations on the percentage degradation of ^{125}I-insulin by isolated adipocytes and fat segments from obese mice (mean values ± SEM).

	% degradation of ^{125}I-insulin							
	Adipocytes				Fat segments			
	n.	No insulin	Insulin (10^5 ng/ml)		n.	No insulin	Insulin (10^5 ng/ml)	
Obese untreated control mice	7	2.1 ± 0.55	1.1 ± 0.24	*	7	6.7 ± 0.58	1.9 ± 0.29	* ǂ
Obese mice treated with low concentration of guarem (2.3 mg/day)	5	1.8 ± 0.47	1.0 ± 0.33	*	5	8.0 ± 1.15	2.4 ± 0.6	* ǂ
Obese mice treated with high concentration of guarem (19.3 mg/day)	6	2.2 ± 0.51	1.2 ± 0.24	*	6	6.9 ± 0.75	1.9 ± 0.37	* ǂ

* $p < 0.05$ compared with the % degradation in the absence of unlabelled insulin.

\+ $p < 0.05$ compared with the % degradation by adipocytes in the absence of unlabelled insulin.

ǂ $p < 0.05$ compared with the % degradation by adipocytes in the presence of 10^5 ng/ml unlabelled insulin.

extent in the small intestine but is thought to be fermented by the bacterial flora of the lower bowel producing excessive amounts of gas - an unsociable side affect of guarem consumption. Obese mice clearly utilise guarem differently, fermenting it in the stomach and duodenal regions producing excessive stomach gas. Also because of a reduction in motility the intestine of the obese mouse cannot cope with solidified guarem and is prone to blocking. The back pressure caused by blockage presumably ruptures the gall bladder and leaking bile then permeates the majority of the viscera. Obese mice therefore lose weight essentially by starvation with a high mortality rate.

In the present study as far as patient acceptability was concerned, few patients were prepared to continue guarem treatment longer than one month complaining of nausea, vomitting, "horrible taste", "feeling bloated" and excessive gas production. The treatment of non-insulin dependent diabetics with guarem significantly reduced fasting plasma glucose and plasma insulin levels and marginally improved oral glucose tolerance although in this case statistical significance was not achieved. Similar effects on plasma glucose (405-407) and plasma insulin (388,430) have been well documented, although Karlström and colleagues were unable to demonstrate a reduction in the plasma insulin concentration in non-insulin dependent diabetic patients after supplementation of the diet with guar (405).

The treatment of obese mice with relatively high concentrations of guarem also resulted in a reduction in both plasma glucose and plasma insulin levels. The mechanism by which guarem gelation lowers the blood glucose and insulin levels probably involves a reduction in

glucose absorption in the small intestine. This may be effected by an increase in the dimension of the unstirred layer and a compensatory reduction in insulin release facilitated by a reduction in the release and effectiveness of insulinotropic gastrointestinal hormones, i.e. GIP associated with the enteroinsular axis. It has been suggested that the release of GIP from the gut in response to oral glucose may be regulated by the rate of absorption of glucose (431). The postprandial secretion of GIP has been shown to be reduced by the addition of guar to test meals, a possible mechanism being a reduction in the rate of absorption of nutrients (397,398). In these studies both GIP and gut glucagon-like immunoreactivity (GLI) were shown to stimulate insulin release in the presence of hyperglycaemia. Therefore, it seemed probable that the reduction in the insulin response to test meals by the addition of guar was at least in part due to diminished gut hormone secretion (398).

Whether the effects of guarem described above could have been facilitated by a delay in the rate of gastric emptying, slowing the delivery of carbohydrates to the small intestine, seems unlikely.

Studies carried out in rats using sacs rings (432) and perfused loops of intestine <u>in vivo</u> (393) strongly suggest that solutions of guar and other viscous polysaccharides inhibit intestinal glucose absorption either by forming an intraluminal gel (394) or by increasing the resitance to diffusion of the intestinal unstrirred water layer (395). Blackburn and colleagues have suggested that viscous polysaccharides such as guar reduce the rate of glucose absorption across the small intestine by impairing the convective functions of gastrointestinal motor activity (388). Under normal circumstances it has been suggested that convection currents, induced

by intestinal movements, are largely responsible for bringing nutrients from the bulk phase to the epithelial surface, with diffusion playing an important role only in the unstirred region just adjacent to the epithelium (388). Viscous solutions probably reduced the access of nutrients to the epithelium by inhibiting convective solute movement within the lumen of the intestine. In the present study guarem treatment had no significant effect on the plasma calcium levels of either poorly controlled non-insulin dependent diabetics or obese hyperglycaemic mice and these observations were consistant with previous studies carried out in man (407). A reduction in diastolic blood pressure has been reported in non-insulin dependent diabetic patients during guar gum supplementation (414). However, this observation was not confirmed by the present study. In addition, guarem treatment did not significantly effect the serum levels of cholesterol and triglycerides confirming the previous observation of Tuomilehto and colleagues (413). However, in rats there is some evidence to suggest that extended guarem treatment does lead to a reduction in serum cholesterol levels (415,420).

There is a wealth of evidence to show that diet greatly influences cellular insulin binding (406). Insulin sensitivity had been shown to be enhanced in patients on high fibre diets due largely to an increase in cellular insulin receptor binding. In the present work guarem treatment of poorly controlled non-insulin dependent diabetic patients did not significantly effect erythrocyte insulin receptor concentration or affinity. It may well have been that guarem treatment for one month was too short a time period for the rather slowly responding erythrocyte insulin receptor model. Nevertheless, previous studies have also been unable to demonstrate

an effect of low-fat/high-starch/high-fibre diet on erythrocyte insulin receptor binding in insulin dependent (423) and non-insulin dependent (406) diabetic patients. In contrast to the present studies using erythocytes, increased insulin receptor binding to monocytes and adipocytes has been demonstrated for both insulin dependent and non-insulin dependent diabetic patients treated with high fibre diets.

In obese mice, treatment with a low guarem concentration had no significant effect upon adipocyte insulin receptor concentration or affinity. However, treatment with a high concentration of guarem resulted in a significant reduction in adipocyte diameter and improvement in insulin receptor affinity. The improvement in insulin receptor affinity may have been the result of the marked reduction in body weight. A positive correlation exists between adipocyte size and body weight in the rat (433). Furthermore, weight loss has been associated with a rise in cellular insulin binding in both man (330) and rat (302).

The treatment of obese mice with gaurem had no significant effect upon the inate capacity of either isolated adipocytes or fat segments to degrade insulin. In addition isolated subcutaneous adipocytes from obese mice treated with a high concentration of guarem bound more ^{125}I-insulin when exposed to low concentrations of insulin (0-10 ng/ml), than adipocytes from either untreated control mice or mice treated with a low concentration of guarem. In the presence of higher concentrations of insulin (200-500 ng/ml), treatment with high concentration of guarem did not significantly influence ^{125}I-insulin binding. This suggested that treatment with high concentration of guarem significantly increased adipocyte

insulin receptor affinity. It is now well established that Scatchard analysis of adipocyte insulin receptor binding is characterised by curvilinear (upwardly concave) plots that result from binding site heterogeneity (132), negative cooperativity (312) and perhaps a combination of the two (434). In the present study with obese mouse adipocytes a difference in insulin receptor binding affinity was established using non-linear least squares curve fitting of the Scatchard plots. Assuming a two-site model, the differences in the shapes of the curvilinear Scatchard plots were consistent with differences in adipocyte insulin receptor affinity. Differences in affinity were also confirmed from the slope of the line joining the B/F axis for that occupancy to the Ro intercept on the abscissa (the maximum binding capacity). The mechanism by which guarem treatment might increase adipocyte insulin receptor affinity is not understood. However, a reduction in the circulating insulin level (435) might explain the observed increase in receptor affinity. Insulin receptor affinity has been shown to be regulated by rapid fluctuations in insulin levels (334) and a reduction in plasma insulin concentrations may be the major influence regulating acute changes in receptor affinity. If this is true then modulation of insulin receptor affinity might best be considered as a post-receptor event analogous to insulin-induced down regulation (436). It should be pointed out that there are other examples where the affinity of the receptor for insulin has been regulated widely under biologically relevant conditions. Occupancy of only a small number of adipocyte receptor binding sites by insulin has been suggested to produce a marked fall in the affinity of all of the receptors because of negatively cooperative site-site interactions among the receptors

(229,312). Fluctuations in pH within the physiological range have also been shown to produce major changes in receptor affinity (196) and it is important to emphasise that receptor concentration as well as receptor affinity, are probably subject to numerous potential influences, including the effects of other hormones, metabolic alterations such as acidosis or ketosis and dietary composition (196).

CHAPTER SIX

Insulin Receptor Binding and Insulin Action in Abdominal Fat of Lean Mice and Abdominal and Subcutaneous Fat of Genetically Obese Diabetic (ob/ob) Mice.

Introduction

Obese mouse adipose tissue, although not resistant to the metabolic effects of insulin shortly after birth, becomes so with the full-blown expression of obese-hyperglycaemic syndrome (439). The body weight of the adult ob/ob mouse exceeds that of its lean littermate by more than two-fold and this difference is reflected in a markedly increased adipocyte cell size and a slightly increased adipocyte number (440). The cause of insulin resistance in the obese mouse has not proved easy to define, but since insulin resistance is a relatively early feature of the syndrome, the factor(s) responsible for its development may be of primary importance. Moreover, tissue resistance to the action of insulin underlies the manifest symptoms of non-insulin dependent diabetes mellitus in man, and it is therefore possible that the primary abnormality or abnormalities in question are associated with the link between obesity and diabetes. Genuth and colleagues (441) suggested that insulin resistance was not likely to be the basic genetic abnormality in ob/ob mice. Hyperinsulinaemia and hyperphagia either causally related or both due to the same genetic hypothalamic disturbance appeared more likely to initiate the obesity seen post weaning. The obesity would in turn further augment insulin resistance and hyperinsulinism with diabetes the ultimate outcome. Many studies have focussed attention on the role of the expanded adipose tissue in the insulin resistance of obese humans (442), mice (437) and rats (438).

It is well established that insulin binding to the plasma membrane of adipocytes and the degree of response to insulin can be

influenced by the concentration and affinity of insulin receptors. In obese mice decreased binding of insulin to liver receptors has been observed (443) a finding that correlates well with the known hepatic insulin resistance of those animals (444). A reduction in insulin binding to receptors on adipocytes (337), muscle (445) and thymic lymphocytes (205) has been reported in a variety of genetically spontaneous (ob/ob) and artificially induced obese states in mice. Furthermore, the decrease in insulin receptor binding was specific and not associated with changes in receptors for either glucagon, growth hormone or catecholamines and there were no detectable changes in plasma membrane structure or function (443). Thin mice that are heterozygous for the obese (ob) mutation have been shown to demonstrate normal insulin binding (339). Alternative and possibly more physiologically significant sites for the regulation of insulin action have been suggested to occur distal to the receptor binding event at postreceptor sites. A postreceptor defect has been identified in obese mice (208) at the level of the insulin mediated glucose transport system. Obesity had no effect on the system in adipocytes (337,478) but greatly reduced the activity of the system in skeletal muscle (446). Postreceptor abnormalities of insulin action observed in obesity appear to be heterogeneous in nature and emphasise the need for studies involving more than one major target tissue.

Studies from several laboratories have shown that adipocyte enlargement in rodents may be accompanied by changes in insulin receptor binding and glucose metabolism (302,447). Early reports suggested that insulin binding might either decrease or remain unchanged with increasing adipocyte size (31). Certainly adipose

tissue glucose metabolism and insulin sensitivity has been shown to be influenced by the cellular character of the tissue. In vitro, basal glucose metabolism (i.e. glucose metabolism in the absence of added insulin) has been reported to be related to the number of fat cells in tissue fragments, small and large adipocytes oxidizing glucose to carbon dioxide at similar rates (448). In contrast, the ability of insulin to stimulate the rate of glucose oxidation in adipose tissue was found to be related to the size of the adipocytes in the tissue. Large adipocytes from obese patients and obese rats have been found to be relatively resistant to this action of insulin compared to smaller adipocytes from non-obese controls (442,447). Weight loss in the obese and a reduction in adipocyte size were associated with the restoration of normal adipose tissue insulin sensitivity (442) and paralleled a similar decrease in the concentration of insulin in the plasma (448). It was subsequently postulated that the insulin resistance of the enlarged adipocyte might play a role in the development or perpetuation of the glucose intolerance and hyperinsulinaemia commonly seen in obesity (448). Over the past fourteen years considerable evidence has accumulated in support of the concept that the disordered glucose metabolism of obesity may be related to the presence of insulin resistance (448). Although animal models of obesity are quite diverse, they share with human obesity the common characteristic of enlarged adipocytes. Alterations in the ability of insulin to influence glucose metabolism in the enlarging adipocyte have been observed both during normal growth and obesity (449) and it has been suggested that differences in cell size rather than the age of the animals might be responsible for differences in insulin binding and insulin sensitivity (302).

According to the spare receptor concept (see page 40), only a small fraction of the total number of insulin receptors needs to be occupied by insulin to elicit a maximal insulin response (201,301). In the normal adipocyte if only 10-15% of the available insulin receptors need be occupied to elicit a maximal insulin effect (135) and sufficient insulin is present (maximally effective insulin level) to occupy this same absolute number of insulin receptors in cells, then one would expect to see a normal insulin response. Indeed such observations have been reported in obese rats (447). Livingstone and Lockwood demonstrated that maximally effective insulin levels produced comparable rates of glucose transport in both normal and enlarged adipocytes from rats (450). On the other hand, glucose oxidation has been studied more extensively and several groups have found that maximally effective insulin levels have a decreased ability to stimulate glucose oxidation in isolated adipocytes from obese rats when compared with adipocytes from lean rats (438,448). The glucose transport data of Livingstone and Lockwood (450) was consistent with a decreased number of insulin receptors on adipocytes from obese rats, while the data which demonstrated decreased maximal rates of glucose oxidation (438,448) implied the presence of additional abnormalities.

There is substantial evidence to suggest that both insulin action and degradation might be mediated through the binding of insulin to specific receptors on the adipocyte plasma membrane (190) although the precise nature of this relationship remains to be elucidated. Previous studies have shown that most of the degradation of insulin by rat liver membranes and rat adipocyte suspensions was accounted for by a protease (s) partially released into the

incubation medium unrelated to receptor binding (135,281). However, these observations did not exclude the possibility that some receptor-bound insulin might have been degraded. Gliemann and Sonne found that the treatment of adipocytes with trypsin markedly inhibited insulin degradation (304). Grofford and colleagues showed that adipocyte plasma membranes were able to degrade insulin and that this activity was abolished in membranes prepared from trypsin treated cells (485). These studies suggested that the enzyme system responsible for insulin degradation was loosely bound to the adipocyte plasma membrane. It has proved difficult to evaluate insulin binding data obtained using suspensions of cells with high protease activity because the concentration of free insulin decreases progressively (304). The finding that labelled degradation products were taken up by cells showed that the control of insulin degradation was even more critical since the accumulation of degradation products might be misinterpreted in relation to the receptor binding. In several studies the extracellular degradation of labelled insulin by adipocytes was inhibited by adding unlabelled insulin (10µmol/l) to the wash buffer (130). Using higher unlabelled insulin concentrations the percentage insulin degradation was found to be even less (135). Pedersen and colleagues have shown almost no difference in the degrdation of ^{125}I-insulin by adipocytes from normal and obese patients (330). In addition, Bolinder and co-workers have demonstrated no significant difference between human omental and human subcutaneous fat in terms of their ability to degrade ^{125}I-insulin (247). Considerable variation in the distribution of fat depots has been well documented in man (451) and gender is known to influence the distribution of subcutaneous fat

(452). Furthermore, it has been suggested that the particular anatomical site is an important determinant for the effect of nutritional factors and hormones on human adipose tissue insulin receptor binding (453). In obese men, regional differences in either the loss or formation of fat may contribute to local differences in adiposity (246). Significant regional differences in the in vitro basal metabolism, insulin receptor binding and glucose oxidation have been observed between abdominal and femoral subcutaneous adipose tissue from obese women (125). In addition significant differences have been observed between omental and subcutaneous adipose tissue from normal menopausal women as regards insulin receptor affinity and insulin action (247).

On the basis that changes in insulin receptor binding and/or postreceptor action of insulin might account for the insulin resistance of obese mice and non-insulin dependent diabetes in man, adipocyte insulin receptor binding and glucose axidation have been examined in lean (+/+) and genetically obese diabetic (ob/ob) mice.

Furthermore, regional differences in adipose tissue insulin sensitivity have been documented for obese human subjects but not for animal models of obesity. Consequently, the present study has also investigated the possibility of regional variations in insulin receptor binding, insulin action (basal and insulin stimulated glucose oxidation) and insulin degradation in different adipose tissue depots (lower abdominal and femoral subcutaneous regions) of obese mice.

Materials and Methods

Animals

Twenty-two week old obese mice (ob/ob) and their lean littermates (+/+) were obtained from the Aston colony, Plate 2, and fed standard pellet diet (Mouse breeding diet, Heygate and Sons Ltd., Northampton) and tap water ad libitum. All animals were maintained in an air conditioned room 22 ± 2°C with a 12h dark, 12h light schedule. Prior to sacrifice animals were weighed and blood collected from the cut tip of the tail. Plasma was separated for the determination of glucose, insulin and calcium levels.

Insulin binding studies.

Either 2 obese mice or 5 lean mice were used for each insulin receptor binding experiment. All animals were killed by a blow to the back of the head and subsequent cervical dislocation at 10 am on the day of study. Adipose tissue was excised from the lower abdominal and femoral subcutaneous region of obese mice and from the lower abdominal region of lean mice and retained in warm saline containing 5 mmol/l glucose at 37°C. Isolated adipocytes were prepared according to the method of Pedersen and colleagues (130) as described previously (page 89). Adipocyte concentration and diameter were determined (pages 88 and 89) with typical coefficients of variation of 3.6% and 3.5% for counting and sizing, respectively. The adipocyte concentration was adjusted to 4-5 x 10^5/ml with Hepes buffer, pH 7.4 (Appendix 4, page 292) supplemented with 5% BSA and 5 mmol/l glucose. Adipocytes were then transferred to 15ml polystyrene centrifuge tubes and insulin receptor binding assays performed as described previously (page 101). Results were corrected for non-

Plate 2 . Illustrating the large difference in body weight between obese and lean mice .

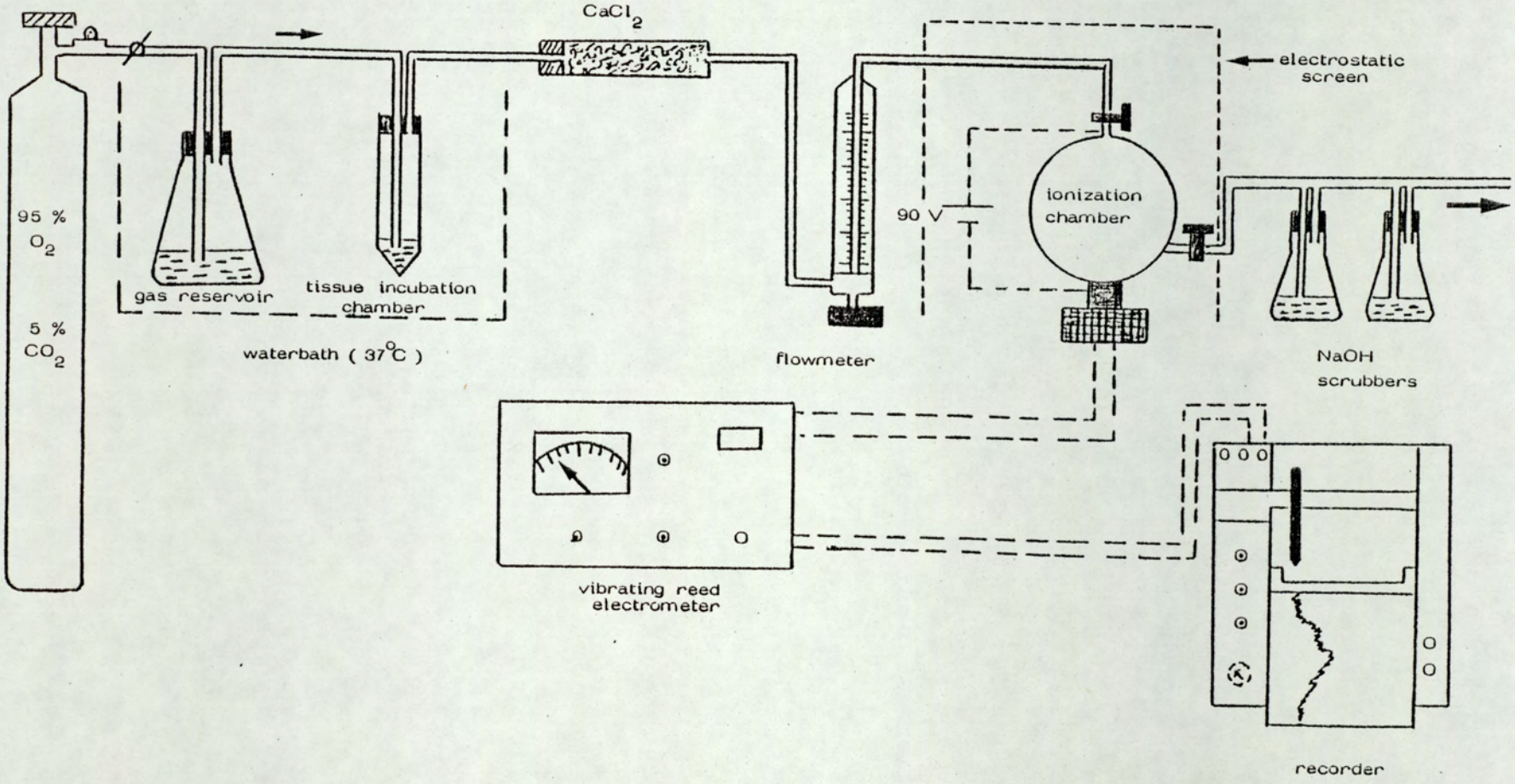
specific binding which in these experiments amounted to about 0.1% of the total radioactivity for both lean and obese mouse adipocytes. The amount of ^{125}I-insulin specifically bound to adipocytes was calculated by subtracting the non-specific counts from the total counts. All binding studies were carried out in duplicate and binding data were analysed using Scatchard plots for the estimation of receptor concentrations and affinity constants $\bar{K}_e$, $\bar{K}_f$ and $\bar{K}_m$. Since adipocytes vary in size and previous studies had indicated that most of the insulin binding activity of adipocytes could be recovered in the particulate, non-nuclear plasma membrane fraction (104), it was considered physiologically relevant to express insulin receptor concentration in terms of per square micrometer (μm^2) of cell surface area in addition to receptor concentration per cell.

The measurement of the rate of D-(U-^{14}C)-glucose oxidation.

Fat segments rather than adipocytes were used in these studies because there was some evidence to suggest that the procedures used for adipocyte isolation might artificially alter adipose tissue metabolism (454). D-(U-^{14}C)-glucose oxidation was continuously monitored with the aid of a vibrating reed electrometer (VRE) and ionization chamber (IC). This novel technique has been used for radiorespirometric studies of ^{14}C-labelled substrate oxidation to $^{14}CO_2$ (25,26,197) and offered several advantages over the conventional means of measuring glucose oxidation. Foremost of these was the ability to measure and record the rate of substrate oxidation simultaneously and continuously during the incubation of the tissue. The apparatus designed for this study consisted of a vibrating reed electrometer (Cary model 401, Cary Instruments,

Monrovia, California), an ionization chamber (IC: volume 275 ml; Varian Associates Ltd, Surrey), a gassing chamber and a small incubation vessel. The apparatus has been represented schematically in Figure 52. The vibrating reed electrometer was used without critical damping and 90 volts were applied across the terminals of the 275 ml ionization chamber. A high resistance leak method using a calibrated 10^{12} ohm resistor was used to measure the tiny ionization current generated by $^{14}CO_2$ produced from D-(U-^{14}C)-glucose by the metabolising tissue. The application of 90 volts across the electrodes of the chamber caused a tiny current to flow towards the cathode of the chamber. The vibrating reed electrometer detected and amplified this tiny current. The output of the vibrating reed electrometer was connected to a potentiometric chart recorder (Servoscribe RE 540, Smiths Industries Ltd., London). The gas flow through the system was maintained with the aid of a vacuum pump and precise control of the gas flow rate (in the range 50-60 ml/min) was achieved by means of a needle valve and a floating-ball type gas flometer (Platon Flowbits Ltd., Basingstoke). The Krebs bicarbonate buffer in the tissue incubation chamber was gassed continuously with water vapor saturated 95% O_2/5% CO_2. Water vapor was removed from the gas by means of a drying tube containing anhydrous calcium chloride. Both the tissue incubation chamber and the gas reservoir were immersed in a waterbath adjusted to 37°C. The ionization chamber was calibrated with $^{14}CO_2$ generated from 1 µCi of $Ba^{14}CO_3$ (specific activity, 0.27 µCi/mg) by reaction with few drops of 2mol/l HCI. The evolution of $^{14}CO_2$ was completed within 30 seconds and about 300 ml of carrier gas was used to carry the $^{14}CO_2$ into the ionization chamber. Carrier gas was used to purge $^{14}CO_2$ from the

Figure 52 . Apparatus for the measurement of the rate of glucose oxidation by pieces of adipose tissue .



ionization chamber into scrubbers containing 2 mol/l NaOH, Figure 53. Calibration of the ionization chamber was carried out 5 times and the mean value was used to calculate a calibration factor defined as the unit radioactivity (μCi) per unit volume (ml) per millivolt (mV) [μCi/ml/mV]. The $^{14}CO_2$ evolved from 1 μCi of $Ba^{14}CO_3$ produced a mean response of 2.51 V/μCi (range 2.4 - 2.74 V). The total volume of the system was 285 ml (assuming a dead space of 10 ml and 275 ml ionization chamber) and the calibration factor was calculated using the following formula:

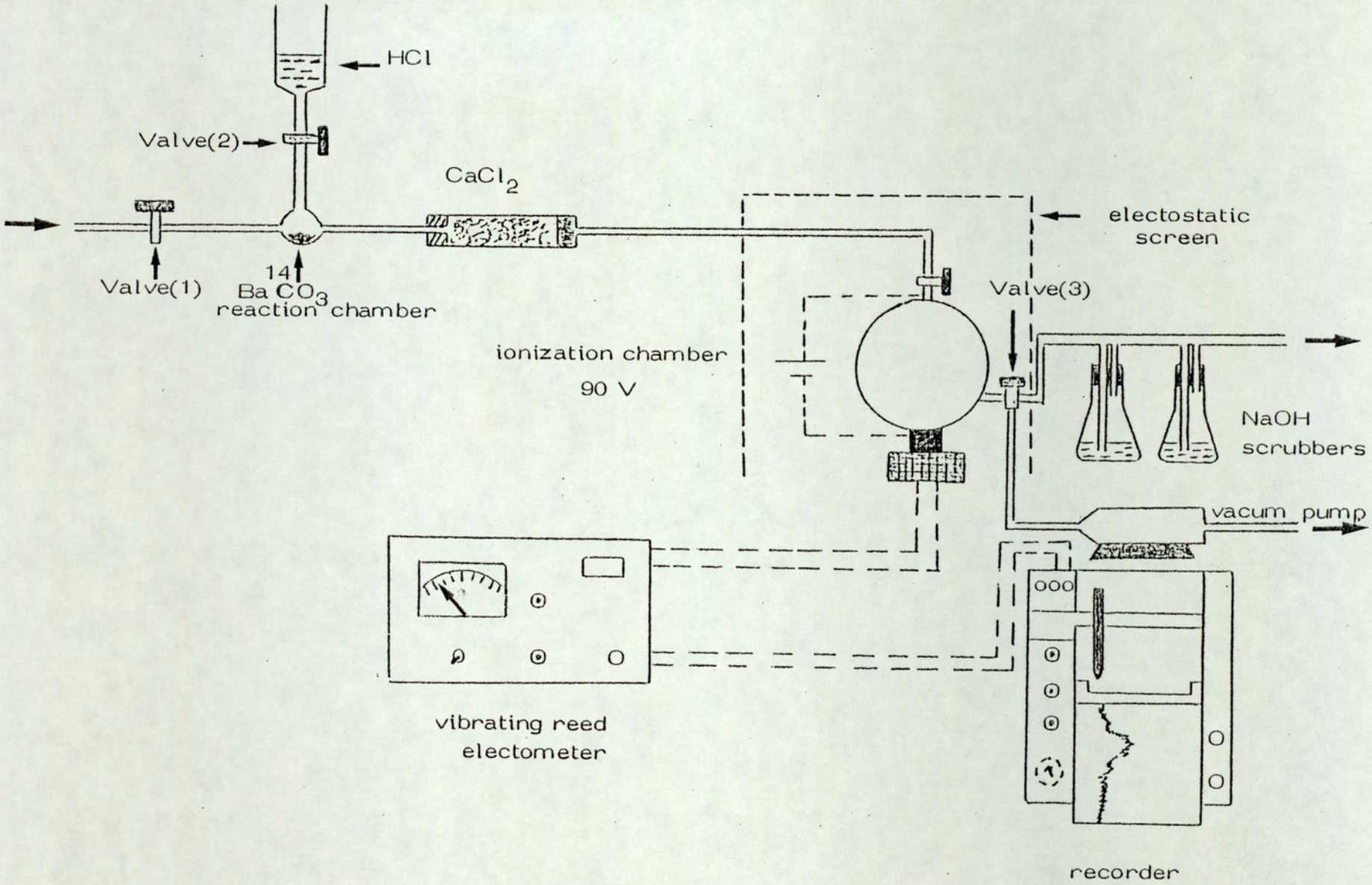
$$\text{Calibration factor (μCi/ml/mV)} = \frac{\text{Number of μCi used}}{\text{Total volume of system (volume of chamber + tubing in ml)} \times \text{Deflection in mV}}$$

Hence the calibration factor in the present study was found to be =

$$= \frac{\frac{1\ \mu Ci}{285\ ml}}{2510\ mV} = 1.39 \times 10^{-6}\ \mu Ci/ml/mV$$

Background electrometer readings with 5% CO_2/95% O_2 flowing through the incubation chamber were routinely between 0.2 and 0.5 mV. Electrometer readings obtained during the incubation of tissues were 20 to 200 times greater than background i.e. 10 ⟶ 100 mV. At any instant during incubation, the rate of glucose oxidation could be calculated using the following equation:

Figure 53 .Apparatus for the calibration of the ionization chamber .



$$\text{Rate of glucose oxidation } (\mu\text{mole/hr/g}) = \frac{\text{GFR} \times \text{SA} \times \text{CF} \times \text{ER}}{\text{Wgt. of tissue (g)}}$$

GFR = gas flow rate in ml/hr

SA = specific activity in µmole/µCi

CF = calibration factor 1.39×10^{-6} µCi/ml/mV

ER = electrometer response in mV.

The removal of labelled volatile impurities from D-(U-^{14}C)-glucose.

In preliminary experiments the D-(U-^{14}C)-glucose was found to be contaminated with small amounts of labelled volatile compounds. These were removed by rotary evaporation and subsequent reconstitution with sterile distilled water to avoid falsely high values for the rates of glucose oxidation. The labelled glucose was stored at -20°C until required.

Preliminary studies were carried with segments of mouse adipose tissue to confirm that the system was capable of measuring small changes in the rate of glucose oxidation.

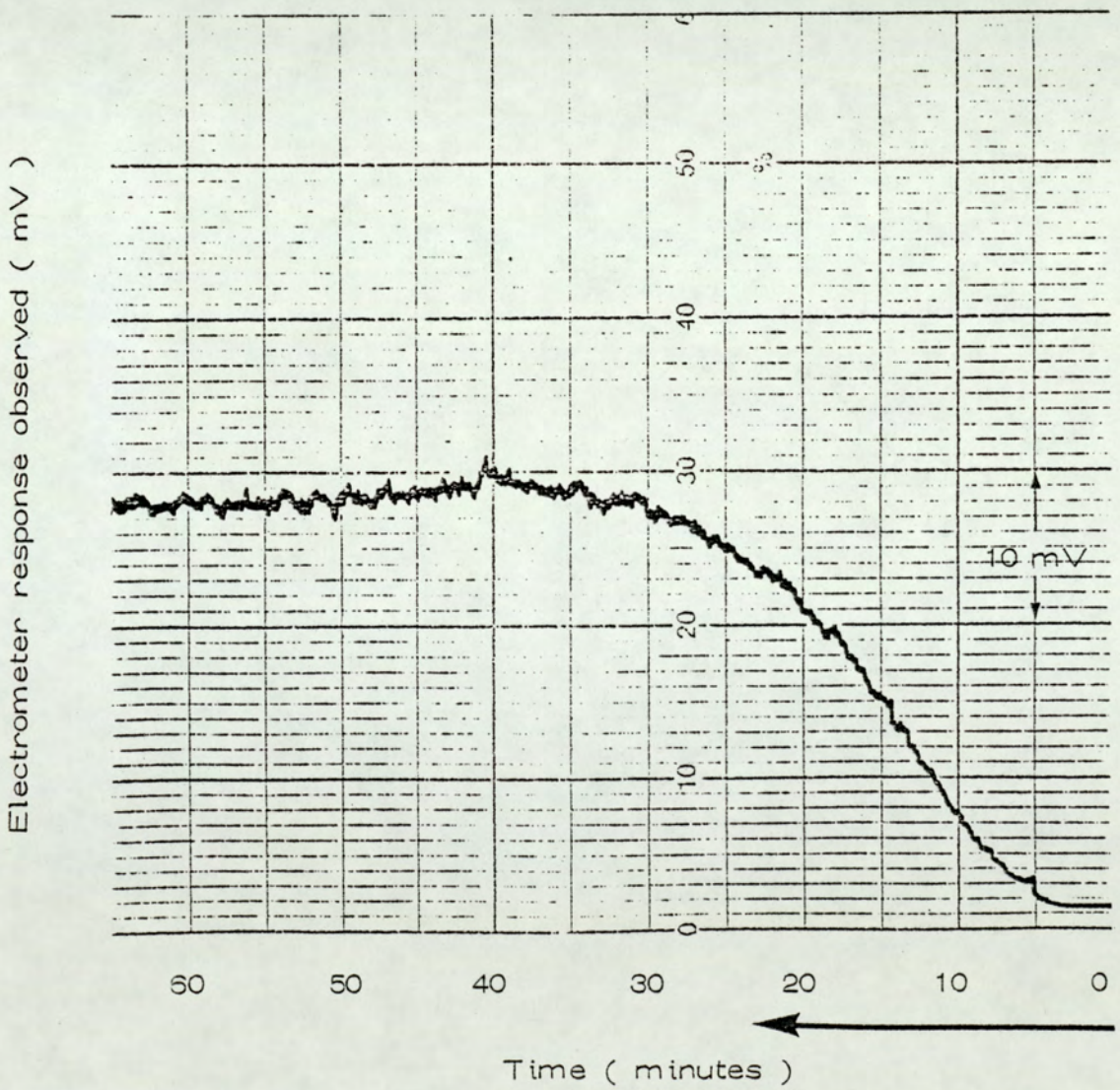
Procedure

Portions of adipose tissue were removed from the lower abdominal and femoral subcutaneous regions of obese mice and the lower abdominal regions of lean mice and retained in petridishes containing Krebs bicarbonate buffer, pH 7.4. Connective tissue, blood vessels and clotted blood were removed and each portion of tissue cut into small 30-40 mg fragments. 300 mg of adipose tissue fragments from either the lower abdominal or femoral subcutaneous regions of obese mice were either maintained statically in oxygenated Krebs bicarbonate buffer, pH 7.4 for 2 hours before the estimation of the

rate of glucose oxidation or used directly in glucose oxidation studies. In either case adipose tissue fragments were transferred to the tissue chamber and preincubated for 30 minutes at 37°C with 2 ml of Krebs bicarbonate buffer, pH 7.4 containing 5.5 mmol/l glucose. The buffer was gassed with 5% CO_2/95%/O_2 at a gas flow rate of $\simeq$ 50 ml/min to establish baseline metabolism. At the end of the pre-incubation period 2ml of fresh buffer containing 5 µCi of D-(U-^{14}C)-glucose was added and a stable plateau for the basal rate of glucose oxidation obtained after approximately 40-60 minutes. The effect of a range of different insulin concentrations (3-300 ng/ml) was subsequently investigated on the rate of glucose oxidation and a recording trace obtained, Figure 54. After each experiment a smooth continuous curve of best fit was drawn through the recorder tracing and after subtraction of the background the rate of glucose oxidation in µmole/hr/g was calculated for points at 2 minute intervals along the curve. These points were replotted on graph paper as shown in Figure 57 to illustrate the continuous recording of the rate of glucose oxidation.

^{125}I-insulin degradation by either isolated adipocytes or fat segments (pages 111 and 112) was estimated by assessing the ability of ^{125}I-insulin remaining in the incubation buffer to precipitate with either 12% or 15% TCA. In this method TCA-precipitable radioactivity was considered to be intact insulin and TCA-soluble material was assumed to be degraded insulin. Insulin degradation was expressed as a percentage of the total radioactivity in the buffer.

Figure 54. An original recorder trace showing the basal rate of glucose oxidation by abdominal adipose tissue from lean mice.



Results

The body weight, plasma glucose and plasma insulin levels of obese mice were significantly greater than the corresponding values for age matched lean mice, Table 26. Adipocytes from obese mice irrespective of origin were always significantly larger than those of lean mice, Table 27A. There was no significant difference between the diameters of subcutaneous and abdominal adipocytes from obese mice, Table 27A.

Insulin receptor binding to isolated adipocytes from obese and lean mice has been summarised in Tables 27A and B. The binding of insulin to lean mouse abdominal adipocytes was always significantly greater than the insulin binding to obese, Table 27A, in the presence of both low and high unlabelled insulin cocnentrations, Figure 55. Adipocytes from obese mice bound only half as much insulin as adipocytes from lean mice over the whole range of insulin concentrations, Figure 55. Lean and obese mouse adipocytes showed very similar values for the various insulin receptor affinity constants and the 50% inhibition constant, $\bar{K}_m$, Table 27B. Scatchard analysis of the data provided similarly shaped curvilinear plots for adipocytes from lean and obese mice, Figure 56. At the same fractional occupancy the slopes of the curves for lean and obese mouse adipocytes were the same indicating no difference in apparent receptor affinity, Figure 56. It was clear that the decrease in insulin binding observed with obese mice adipocytes could be totally accounted for by a decrease in the insulin receptor concentration obtained from the intercept of the Scatchard plot with the abscissa (455). Compared to lean, obese mouse abdominal adipocytes showed a

Table 26. Age, body weight, plasma glucose, plasma insulin and plasma calcium levels in lean and obese mice (mean values ± SEM).

	n	Obese mice	n	Lean mice
Age (weeks)	14	21.9 ± 0.3	15	22.2 ± 0.7
Body weight (g)	14	98.0 ± 3.9 *	15	45.6 ± 1.1
Glucose (mmol/l)	14	23.9 ± 2.6 *	15	12.3 ± 0.3
Insulin (ng/ml)	14	50.9 ± 3.7 *	15	2.8 ± 0.3
Calcium (mmol/l)	12	2.46 ± 0.03	12	2.33 ± 0.02

* $p < 0.05$ for obese compared with lean mice.

Table 27A. Insulin receptor binding data for adipocytes from lean and obese mice (mean values ± SEM).

Mice	Region	n	Specific binding (%)	Adipocyte diameter (μm)	High affinity receptor concentration per cell ($x10^5$)	Low affinity receptor concentration per cell ($x10^5$)	Total affinity receptor concentration per cell ($x10^5$)
Lean	Abdominal	6	3.56 ± 0.26 +	43.35 ± 1.59 +	1.26 ± 0.15 +	3.45 ± 0.22 +	4.71 ± 0.19 +
Obese	Abdominal	7	1.86 ± 0.11	62.13 ± 2.35	0.376 ± 0.01	1.872 ± 0.14	2.25 ± 0.14
	Subcutaneous	7	1.83 ± 0.16	64.53 ± 1.72	0.606 ± 0.13	2.7 ± 0.18 *	3.304 ± 0.15 *

* $p < 0.05$ for obese subcutaneous compared with obese abdominal.

\+ $p < 0.05$ for lean abdominal compared with obese abdominal.

Table 27B.

Mice	Region	n	High affinity receptor concentration per μm^2	Low affinity receptor concentration per μm^2	Total affinity receptor concentration per μm^2	$\overline{K}_e$, affinity constant of empty sites (nM^{-1})	$\overline{K}_f$, affinity constant of full sites (nM^{-1})	$\overline{K}_m$, apparent affinity constant (nM^{-1})
Lean	Abdominal	6	21.04 ± 1.31 +	59.58 ± 5.3 +	80.62 ± 4.68 +	0.1216 ± 0.004	0.0528 ± 0.005	0.0703 ± 0.002
Obese	Abdominal	7	3.16 ± 0.23	15.54 ± 1.02	18.7 ± 1.07	0.1258 ± 0.007	0.0557 ± 0.005	0.0709 ± 0.005
	Subcutaneous	7	4.54 ± 0.88	21.15 ± 2.29*	25.7 ± 2.04*	0.0805 ± 0.006*	0.0245 ± 0.004*	0.0442 ± 0.004*

* $p < 0.05$ for obese subcutaneous compared with obese abdominal.

\+ $p < 0.05$ for lean abdominal compared with obese abdominal.

Figure 55. Insulin binding curves for lean and obese mouse adipocytes. Data are presented as the amount of specifically bound insulin plotted against the total insulin concentration.
Mean values, SEM omitted for clarity (number of determinations in brackets).

o------o Lean abdominal (6).
●——● Obese subcutaneous (7).
×——× Obese abdominal (7).
* $p < 0.05$ compared to obese abdominal.
+ $p < 0.05$ compared to obese abdominal.

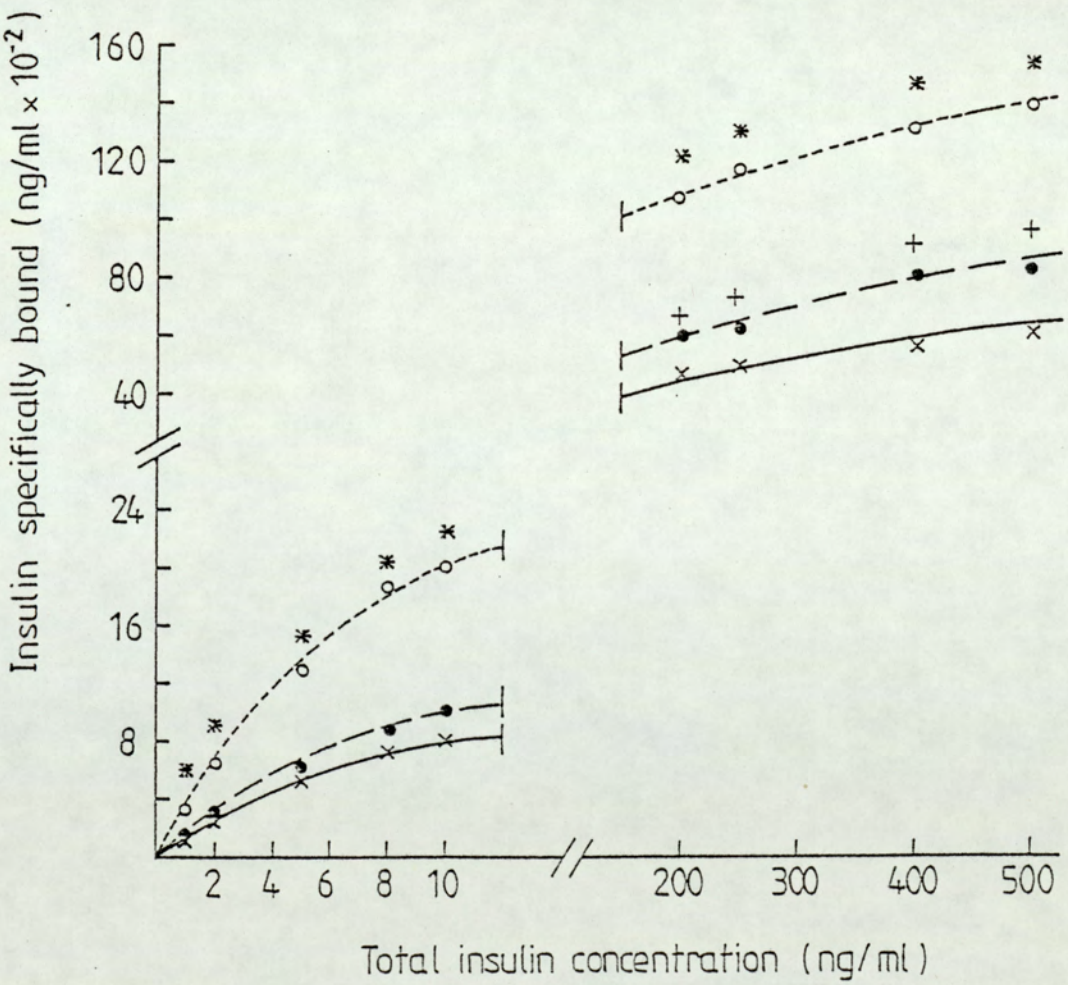
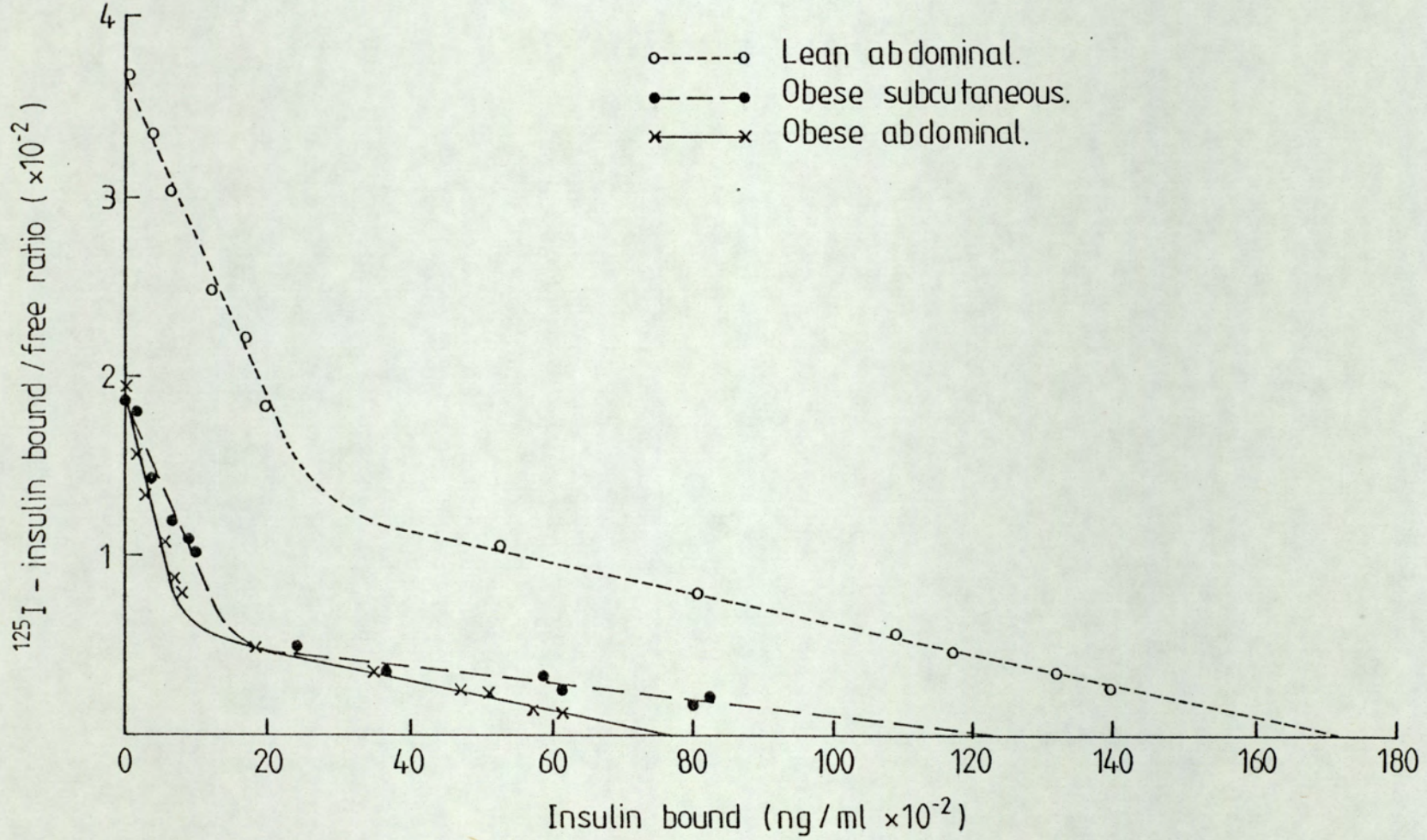


Figure 56. Scatchard analysis of binding data for lean and obese mouse adipocytes.



significant reduction in the total receptor concentration per cell which was largely accounted for by a 70% reduction in the concentration of high affinity low capacity receptors and 46% reduction in the concentration of low affinity high capacity receptors, Table 27A. When insulin receptor binding was expressed in terms of the receptor concentration per μm^2 cell surface area obese mouse adipocytes again showed a significant reduction in total receptor concentration which was largely accounted for by an 85% reduction in the concentration of high affinity low capacity receptors and a 74% reduction in the concentration of low affinity high capacity receptors, Table 27B. Abdominal and femoral subcutaneous adipocytes from obese mice showed very similar specific insulin binding capacities in the presence of low insulin concentrations, Figure 55, while in the presence of high insulin concentrations (>100ng/ml) abdominal adipocytes from obese mice bound significantly less insulin than femoral subcutaneous adipocytes, Figure 55. In addition femoral subcutaneous adipocytes from obese mice showed significantly lower values for $\bar{K}_e$ and $\bar{K}_f$ and the $\bar{K}_m$ when compared with abdominal adipocytes, Table 27B. The fact that at low insulin concentrations both femoral subcutaneous and abdominal adipocytes from obese mice bound similar amounts of insulin even though abdominal adipocytes showed a reduced insulin receptor concentration suggested that abdominal adipocytes were endowed with an increased insulin receptor binding affinity. Scatchard analysis revealed that the total binding capacity of abdominal adipocytes was significantly lower than that of femoral subcutaneous adipocytes, Figure 56. The reduction in abdominal adipocyte total insulin receptor concentration was largely accounted for by a 31% reduction

in the low affinity receptor concentration per cell (27% when expressed per μm^2 cell surface area).

The basal rate of glucose oxidation by lean mouse abdominal fat segments was almost twice that of obese mouse, Table 28 and Figure 57. The rate of insulin stimulated glucose oxidation by obese mouse abdominal fat segments was significantly reduced compared to that of lean, Figure 57. The dose response curve for the rate of obese mouse abdominal fat segment insulin stimulated glucose oxidation was displaced to the right compared to lean, Figure 58, indicating that lean abdominal fat segments were significantly more sensitive to insulin than obese. The rate of glucose oxidation by lean mouse abdominal fat segments was maximally stimulated by 30 ng/ml of insulin. On the other hand, this dose of insulin together with 60 and 300 ng/ml insulin only marginally increased the rate of glucose oxidation by obese mouse fat segments, Figure 58. Femoral subcutaneous and abdominal fat segments from obese mice showed similar rates of both basal and insulin stimulated glucose oxidation, Figure 57, indicating no regional differences in insulin sensitivity. Static incubation for 2 hours (carried out in order to retain tissue for subsequent estimation of the rate of glucose oxidation) significantly reduced the rate of basal glucose oxidation and marginally reduced the rate of insulin stimulated glucose oxidation by femoral subcutaneous and abdominal fat segments from obese mice, Figure 59.

The percentage degradation of ^{125}I-insulin by lean and obese mouse abdominal fat segments was significantly higher than the respective degradation by lean and obese mouse abdominal adipocytes, Table 29. The addition of a high concentration of unlabelled insulin (10^5 ng/ml) significantly reduced the percentage of degradation of

Table 28. The rate of D-(U-^{14}C) - glucose oxidation by abdominal and subcutaneous adipose tissue from obese mice and abdominal adipose tissue from lean mice in the absence and presence of insulin (mean values ± SEM, number of determinations in brackets).

	Lean mice Abdomonal	Obese mice			
		Abdominal		Subcutaneous	
		Fresh tissue	After 2 hours incubation	Fresh tissue	After 2 hours incubation
Rate of glucose oxidation in the absence of insulin (basal rate) (u moles CO_2/hr/g)	0.806 ± 0.054 (n=6)	0.404 * ± 0.037 (n=10)	0.184 + ± 0.02 (n=5)	0.413 ± 0.038 (n=8)	0.225 ǂ ± 0.021 (n=5)
Rate of glucose oxidation in the presence of insulin (u mole CO_2/hr/g)					
3 ng/ml insulin	1.409 ± 0.127 (n=6)	0.411 * ± 0.041 (n=5)	-	0.40 ± 0.058 (n=5)	-
7.5 ng/ml insulin	1.987 ± 0.152 (n=5)	0.40 * ± 0.043 (n=7)	-	-	-
30 ng/ml insulin	2.135 ± 0.152 (n=5)	0.588 * ± 0.088 (n=7)	0.384 ± 0.063 (n=5)	0.572 ± 0.025 (n=5)	0.515 ± 0.041 (n=5)
60 ng/ml insulin	-	0.681 ± 0.16 (n=6)	-	-	-
300 ng/ml insulin	-	0.731 ± 0.150 (n=5)	-	-	-

* $p < 0.05$ compared with lean abdominal adipose tissue.
\+ $p < 0.05$ compared with fresh obese abdominal adipose tissue.
ǂ $p < 0.05$ compared with fresh obese subcutaneous adipose tissue.

Figure 57. Basal and insulin stimulated rate of glucose oxidation by adipose tissue segments from lean and obese mice. (number of determinations in brackets).

o--------o Lean abdominal.
●— — —● Obese subcutaneous.
×———× Obese abdominal.

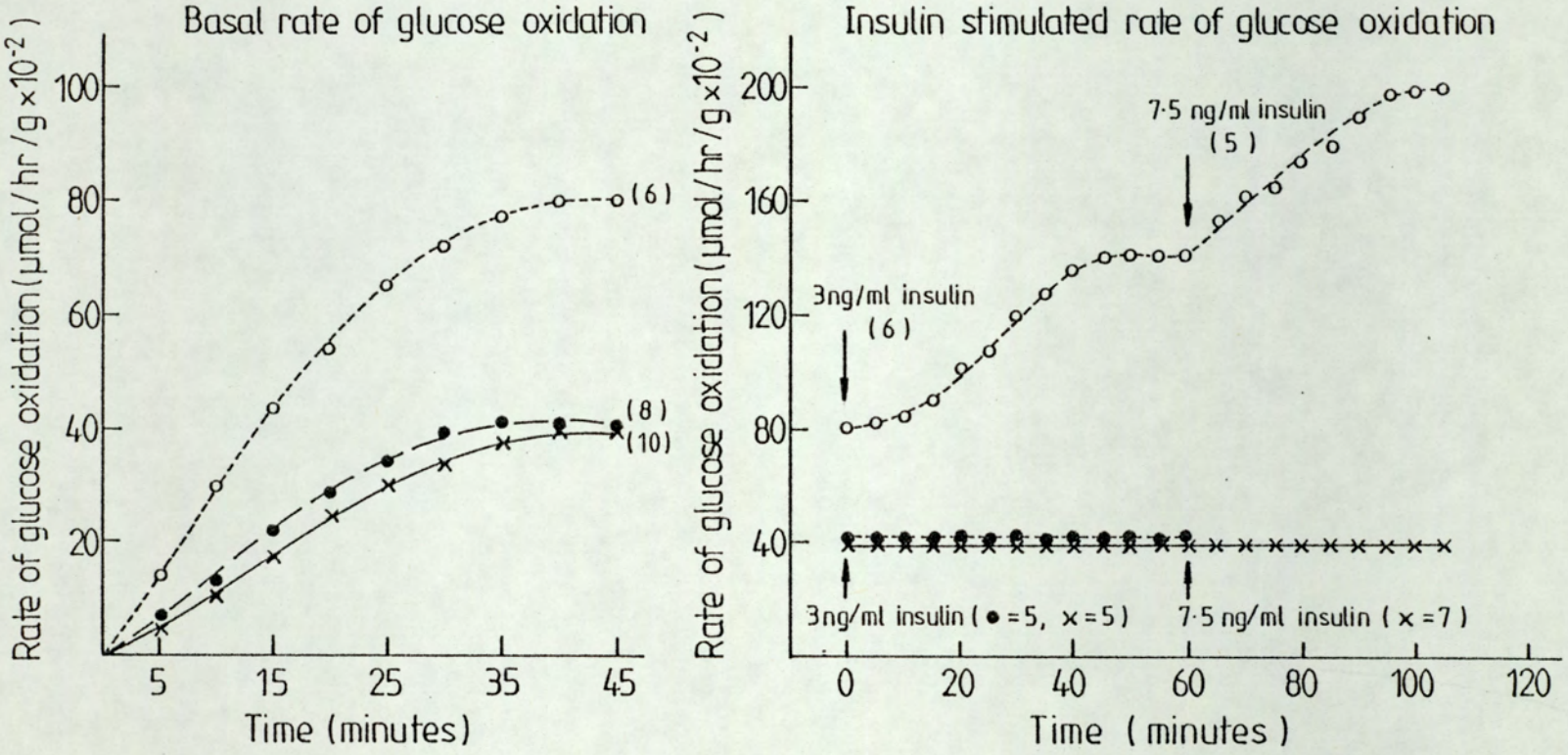


Figure 58. Basal and dose response for the insulin stimulated rate of glucose oxidation by adipose tissue from lean and obese mice. Mean values ± SEM (number of determinations in brackets).

× Obese abdominal.
• Obese subcutaneous.
* $p < 0.05$ compared to obese abdominal.
o Lean abdominal.

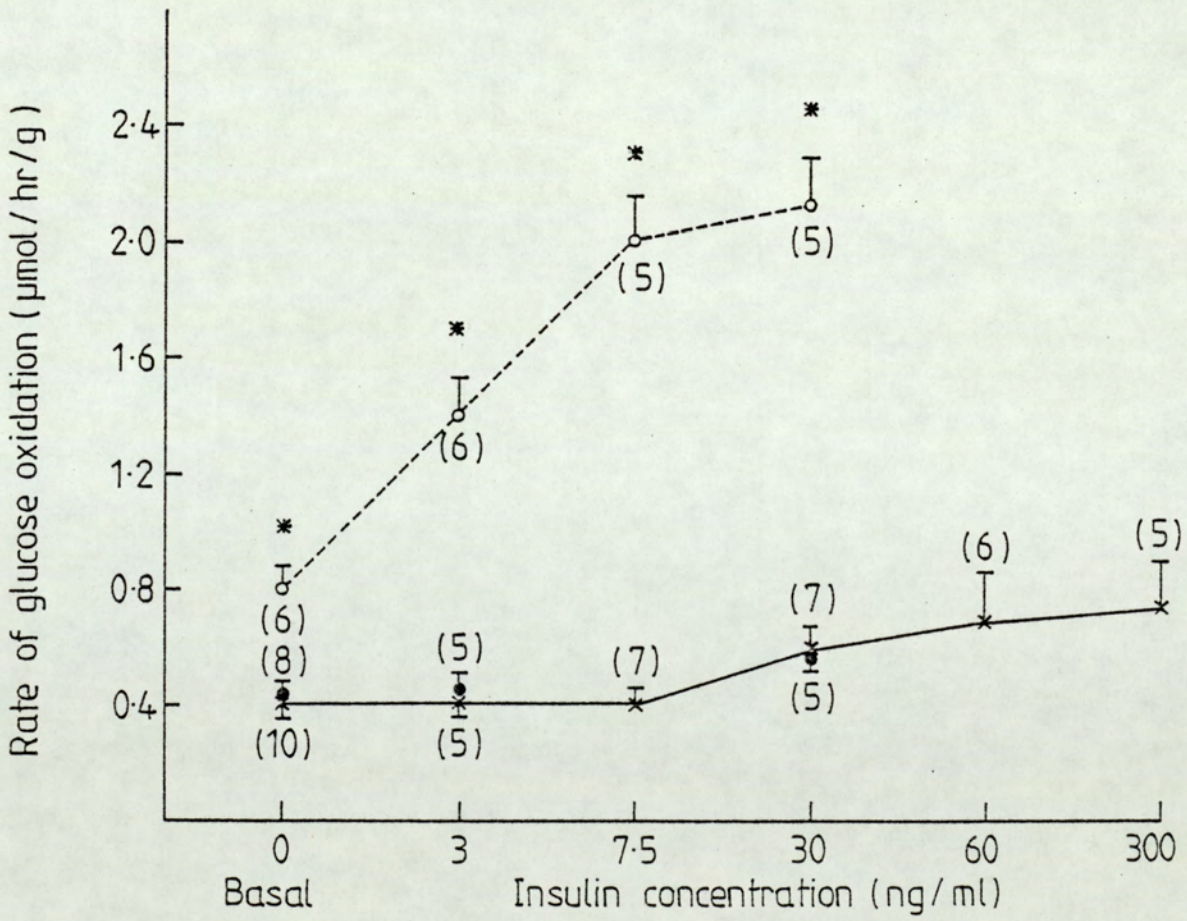


Figure 59. Basal and insulin stimulated rate of glucose oxidation by segments of abdominal and subcutaneous adipose tissue from obese mice before and after incubation. (number of determinations in brackets).

●-----● Obese subcutaneous before incubation.
□----□ Obese subcutaneous after incubation.
×——× Obese abdominal before incubation.
+——+ Obese abdominal after incubation.

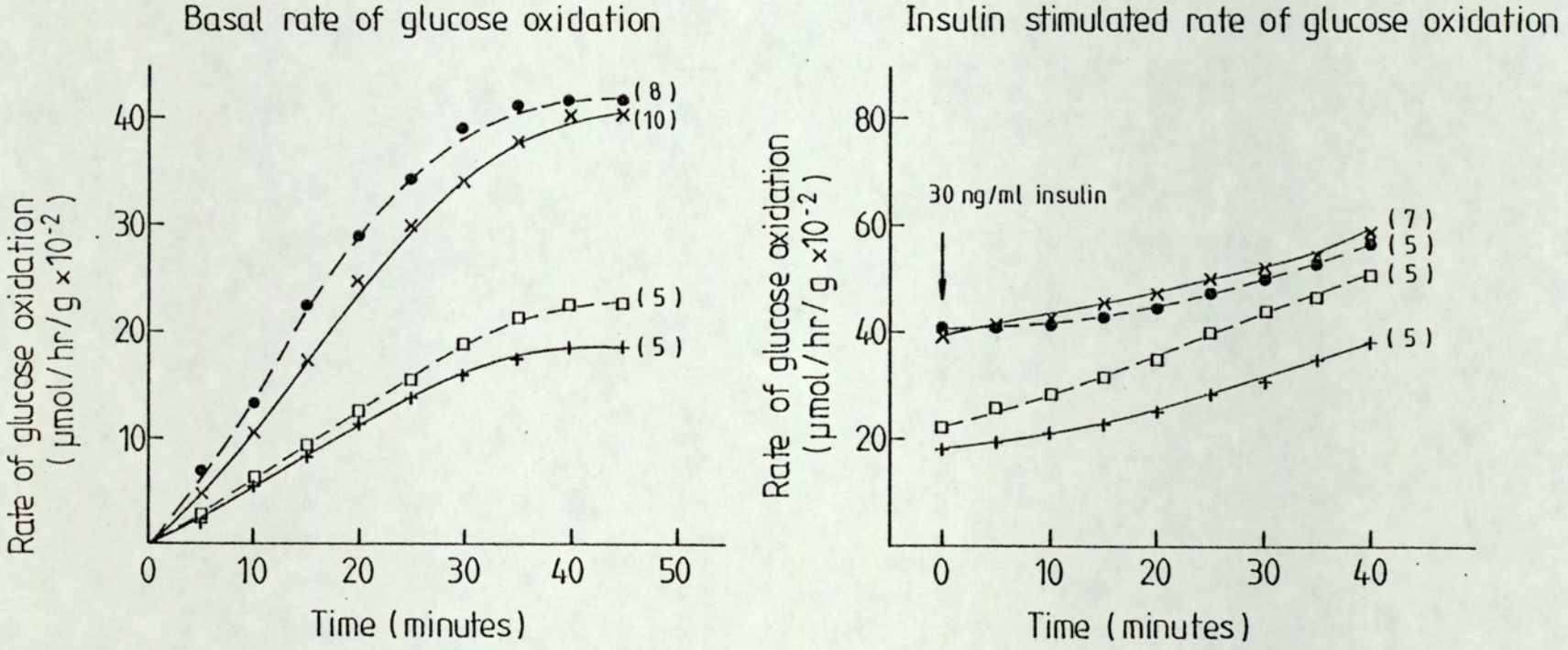


Table 29. Percentage degradation of ^{125}I-insulin by fat segments and isolated adipocytes in the absence and presence of unlabelled insulin (10^5 ng/ml). Values are means ± SEM.

Mice	Region	% degradation of ^{125}I-insulin					
		Adipocytes			Fat segments		
		n	No insulin	Insulin (10^5 ng/ml)	n	No insulin	Insulin (10^5 ng/ml)
Lean	Abdominal	6	1.6 ± 0.34	0.8 ± 0.13*	6	5.9 ± 0.89 +	1.6 ± 0.29 *ǂ
Obese	Abdominal	7	1.8 ± 0.35	0.9 ± 0.1*	7	7.5 ± 0.76 +	2.2 ± 0.45 *ǂ
	Subcutaneous	7	2.1 ± 0.55	1.1 ± 0.24*	7	6.7 ± 0.58 +	1.9 ± 0.29 *ǂ

* $p < 0.05$ compared to the % degradation in the absence of unlabelled insulin.

\+ $p < 0.05$ compared to the % degradation by adipocytes in the absence of unlabelled insulin.

ǂ $p < 0.05$ compared to the % degradation by adipocytes in the presence of 10^5 ng/ml unlabelled insulin.

^{125}I-insulin by abdominal fat segements and abdominal adipocytes from lean and obese mice. There was no significant difference in the percentage degradation of ^{125}I-insulin by abdominal fat from lean and obese mice whether using fat segments or isolated adipocytes either in the absence or in the presence of a high concentration of unlabelled insulin. However, the suppression of ^{125}I-insulin degradation by 10^5 ng/ml of unlabelled insulin was marginally more effective with fat segments than with adipocytes irrespective of origin.

Discussion

Decreased insulin binding has been suggested to be a common feature of the insulin resistance of the obese state. In the present study specific binding to obese mouse abdominal adipocytes was reduced compared to lean and this was largely due to a reduction in the concentration of insulin receptors and a markedly reduced sensitivity of the rate of glucose oxidation to insulin. These observations suggested the presence of defects at both the receptor and postreceptor level in obese mouse abdominal fat. Abdominal adipocytes from lean mice bound significantly larger amounts of ^{125}I-insulin than abdominal adipocytes from obese mice when exposed to both low and high insulin concentrations. This observation was consistent with the increased insulin receptor concentration determined for lean mouse abdominal adipocytes. Adipose tissue samples taken from different sites in obese mice for the continuous monitoring of the rate of glucose oxidation could not all be utilised at the same time because of the nature of the experimental system and

there was a need to maintain adipose tissue samples in static incubation for 2 hrs prior to use. This incubation led to a significant reduction in the basal rate of glucose oxidation presumably the result of flushing out high concentrations of endogenous insulin. However, this period of incubation did not detrimentally affect the subsequent rate of insulin stimulated glucose oxidation. There is some evidence to suggest that a relationship might exist between the basal rate of adipocyte glucose oxidation and adipocyte size (448). In rats, basal rates of $^{14}CO_2$ production were found to be similar over a wide range of adipocyte size (448). In obese humans the basal rate of glucose oxidation to $^{14}CO_2$ by large femoral adipocytes was almost twice that of abdominal adipocytes (125). In the present work the basal rate of glucose oxidation was significantly greater for lean mouse fat segments than for obese irrespective of region. This difference was probably due to the greater number of adipocytes in lean mouse fat segments, since lean mouse adipocytes were always significantly smaller than adipocytes from obese mice. Smith has demonstrated a reduction in the ability of insulin to stimulate glucose oxidation in large human fat cells (456). In rat adipose tissue, insulin sensitivity has been shown to be related to the size of the component fat cells, the larger the fat cells, the less insulin sensitive the adipose tissue.

For lean mouse fat segment the maximum rate of glucose oxidation was observed at an insulin concentration of 30 ng/ml while for obese mouse fat segments very high insulin concentrations of the order of 300 ng/ml only marginally increased the rate of glucose oxidation. Maximal insulin stimulated rates of glucose oxidation have been suggested to occur when only a minority (10-15%) of the available

insulin receptors are occupied (202). On this basis a decrease in insulin action observed with insulin concentrations great enough to saturate this absolute number of receptors (maximally effective insulin level) could not be due to a decreased number of insulin receptors. This line of reasoning implies that the defect in glucose oxidation in large adipocytes is distal to the insulin receptor i.e. either at the level of the glucose transport system or at one or more of the intracellular steps in glucose metabolism. This hypothesis is supported by the observation that, in large adipocytes, maximal rates of gluocse oxidation (at glucose concentrations > 5mM) are decreased in the absence of insulin (447). A diminished responsiveness of adipose tissue glucose oxidation to insulin has been demonstrated for old obese rats (438) and experimentally obese mice (437). The nature of adipose tissue insulin resistance in obesity and the mechanism by which enlarged fat cells become insulin resistant remains unknown. One possibility is that the insulin senstivity of adipose tissue is related to its cellular character. This was suggested by the observation that the diminished insulin responsiveness of human adipose tissue was associated with the presence of enlarged fat cells, an abnormality reversible by weight loss and reduction of fat cell size (442).

In man regional differences in insulin binding and insulin action have been demonstrated for adipose tissue (125,247). The present study has shown that regional differences do exist in insulin receptor binding and insulin action in adipose tissue from obese hyperglycaemic mice. In the presence of low concentrations of insulin abdominal and femoral subcutaneous adipocytes from obese mice bound similar amount of insulin. But at high concentrations of

insulin (>100 ng/ml) there was an increased binding to femoral subcutaneous adipocytes. Femoral subcutaneous fat had a greater total concentration of insulin receptors but a lower insulin receptor affinity than abdominal fat and these characteristics could not be attributed to differences in the percentage degradation of ^{125}I-insulin. Although any differences in ^{125}I-insulin degradation were small compared to differences in insulin receptor binding, the percentage ^{125}I-insulin degradation by fat segments was always significantly higher than that by isolated adipocytes and could be markedly reduced by the addition of a high concentration of unlabelled insulin.

Femoral subcutaneous and abdominal fat segments from obese showed similar rates of basal and insulin stimulated glucose oxidation and this may have been due to the homogeneity of adipocyte size in the two adipose tissue regions (442). Whilst the basal rate of femoral fat glucose oxidation has been shown to be twice that of abdominal fat in obese women (125) differences between femoral subcutaneous and abdominal fat of obese mice appear to be restricted to the level of insulin receptor binding with little evidence of differences in postreceptor activity.

CHAPTER SEVEN

General Discussion

The present study has been concerned with aspects of erythrocyte and adipocyte insulin receptor binding both in the normal situation and in disease, in particular mild hypertension and non-insulin dependent diabetes mellitus in man and spontaneous obesity with hyperglycaemia in mice. These studies have been facilitated by the introduction of techniques for the radio-iodination of porcine monocomponent insulin and the generation of monoiodinated ^{125}I-insulin for use in radioreceptor assays and radio-immunoassays. The ^{125}I-insulin synthesised was characterised by a high specific activity and percentage incorporation and low percentage damage. The validity of insulin binding data depended upon the degree of purity and homogeneity of the tracer and the distribution of ^{125}I within the insulin molecule. Pure preparations of monoiodoinsulin labelled at residue 14 of the A chain were stable and had the same receptor binding capacity and biological potency as native insulin. A major question in clinically orientated insulin receptor binding studies is whether changes in insulin receptor binding in one type of cell reflect those in other insulin sensitive tissues? Few studies have compared variations in insulin receptor binding in several cell types from the same individual or group of individuals simultaneously (216,466). It is controversial as to whether correlations really exist between the insulin receptor binding status of different cell types in the same individual. However, insulin receptors would theoretically be expected to exhibit a differentiated tissue - specific, rather than a uniform, overall behaviour due to the great number of different functions of various insulin binding tissues. For example, since monocytes have a much shorter turnover time in the circulation than erythrocytes the two cell types might be affected

differently by circulating receptor regulating factors. In addition, since mature erythrocytes have no nuclei and are incapable of protein synthesis, any acute alterations in insulin binding that require protein synthesis will not be reflected in these cells. Consequently, generalisation of insulin receptor binding data from one insulin sensitive type of cell to another may not always be justified. The physiological significance of insulin receptors on mature human erythrocytes remains unclear although experimental data suggests that insulin may have effects on allosteric properties of intracellular haemoglobin (467) the turnover of erythrocyte phosphorus (468) and the transmembrane oxidation-reduction potential of these cells (469).

Since the majority of clinical studies are carried out on 12 hour or overnight fasted patients. Preliminary studies in the present work set out to establish the effect of overnight fasting on erythrocyte insulin receptor binding in age and weight matched normal volunteers. Investigation confirmed that overnight fasting did not significantly influence either erythrocyte insulin receptor concentration or affinity, despite some evidence to the contrary (329).

Perhaps the most common clinical condition which reflects some malady in insulin receptor binding and insulin action is that of non-insulin dependent diabetes mellitus and evidence of a reduced insulin receptor concentration in these patients and the hint of possible changes in insulin receptor affinity has been well docuemnted (107). However, a high proportion of these patients are either prone to or have a mild to severe hypertension that complicates their diabetes and are often prescribed thiazide treatment despite the

possibility of aggravated glucose intolerance. The treatment of mildly hypertensive patients with and without non-insulin dependent diabetes with the thiazide bendrofluazide revealed a marginal deterioration in glucose tolerance as evidenced by the rise in fasting blood glucose levels and the increased areas under the oral glucose tolerance curves. These effects were more noticeable in the diabetic patients. There was no evidence for a decreased tissue sensitivity to insulin, since the glucose/insulin ratio did not diminish. It has been suggested that this deficiency in insulin secretion might result from thiazide induced hypokalaemia (346). Potassium appears to be an essential cation for insulin release from pancreatic B-cells (375) and thiazides may increase the permeability of B-cell membrane to potassium (223). This might then inhibit the influx of calcium ions upon which insulin secretion is dependent (377). There is great doubt that the administration of bendrofluazide decreased serum potassium. However, although this correlated with the glucose level, it did not relate to the insulin level. This may merely reflect the poor correlation of serum potassium with total body levels of the ion (79). Alternatively potassium may not be as important in determining insulin release as previously supposed. Thiazide diuretics are known to affect other cations involved in insulin release and these may be more important determinants of insulin secretion. In non-diabetic patients, the observed increase in insulin receptor concentration would help the maintenance of normal glucose tolerance. Diabetics did not show this rise and the insulin receptor/insulin ratio deteriorated. These effects could explain why the glucose tolerance in diabetic patients administered bendrofluazide deteriorated rapidly, whereas in non-

diabetic patients, glucose intolerance might need some time to develop. Erythrocyte insulin receptor concentrations obtained in the present study correlated well with insulin receptor concentrations reported in the literature for monocytes, although these do not necessarily correlate well with receptor concentrations reported for other insulin target tissues. To date the effects of thiazides upon insulin receptor binding have only been reported for the drug diazoxide, a particularly diabetogenic thiazide vasodilator (256,370). Wigand and Blackard have shown an increase in insulin receptor concentration in obese men after 7 days treatment with diazoxide (370) while Olczak and colleagues were unable to demonstrate any change in insulin receptor binding with the drug in either normal or non-insulin dependent diabetic patients (256).

In the present study treatment with bendrofluazide and potassium supplementation halted the fall in serum potassium, but did not restore the latter to pre-treatment values. Fasting blood glucose decreased significantly in diabetic patients during this period. It was evident, however, that this decrease was not due to a restoration of insulin levels which also continued to decrease during this phase. This observation contrasts with previous studies in which potassium supplementation has been shown to correct the deficiency in insulin secretion (87). Since the insulin receptor concentration in the diabetic group remained unchanged, the effect of potassium was likely to be mediated via an action upon intracellular glucose metabolism. On the other hand, potassium might equally have affected other mechanisms involved in the maintenance of carbohydrate tolerance (421,476).

The present study also demonstrated changes in certain biochemical parameters as a result of bendrofluazide treatment. These included significantly increased levels of urea, urate and cholesterol. Potassium supplementation failed to correct these changes. Despite making attempts to administer large amounts of potassium in the present study, serum potassium was never restored to pretreatment values. This was largely the result of patient resistance. A better method of potassium supplementation might have been via potassium sparing diuretics. However, even these are suspected of inducing some glucose intolerance further substantiating the proposal that changes in glucose tolerance may not be a consequence of potassium depletion. One can speculate as to how bendrofluazide might influence insulin receptor concentration. The drug might induce conformational changes in insulin receptors, leading to increased cross-linking between hormone-receptor complexes and thus an increased biological activity of insulin. As the early effect of bendrofluazide was not mediated via a reduction in insulin concentration, the increased insulin receptor number observed after the administration of the drug to non-diabetic patients could not be explained by an effect on up-regulatory mechanisms. Bendrofluazide might affect insulin receptor turnover by slowing the internalisation process, possibly by sterically hindering the movement of insulin receptor complexes into coated pits.

The cause of the reduced concentration of insulin receptor sites in non-insulin dependent diabetics remains a controversial issue. Is the receptor defect a primary lesion, or is it secondary to the diabetic state? Not all individuals with a reduced concentration of insulin receptor sites become diabetic, as evidenced by the loss of

insulin receptor sites in non-diabetic obese patients. Hyperinsulinaemia at least partly offsets the insulin receptor loss in non-diabetic obese subjects, although it has been argued that a raised insulin concentration is the cause of the insulin receptor loss (474). Down-regulation is likely to operate in non-insulin dependent diabetic patients with raised basal insulin concentrations. However, this mechanism does not explain the insulin receptor loss in patients with normal or reduced insulin concentrations. Ginsberg and Rayfield have described the effects of insulin treatment for 1 to 8 weeks in a group of insulin resistant hypoinsulinaemic non-obese non-insulin dependent diabetics (475). In one third of these patients a normal biological action of insulin was re-established after insulin treatment. Gross changes in levels of insulin antagonists were not observed, suggesting that the one-third who benefited from the insulin treatment might well have corrected a previous insulin receptor or postreceptor defect. This observation suggested the possibility that insulin deficiency might adversely affect insulin receptor function in some non-insulin dependent diabetics and emphasised the heterogeneity of the condition. Non-insulin dependent diabetics who are unable to modulate insulin receptor number may be more likely to suffer further impairment of glucose tolerance. The present studies with erythrocytes did not allow the investigation of meaningful postreceptor mechanisms. It might well have been of value to evaluate the effects of bendrofluazide on a natural target cell for insulin such as the adipocyte. Then both insulin receptor binding and insulin action could have been evaluated in the same cell preparation. However, gluteal fat biopsies used currently by Taylor were considered too invasive for the present studies (486).

Nifedipine, a new calcium antagonist, has recently been evaluated for the treatment of hypertensive patients with non-insulin dependent diabetes mellitus (470). Concern has been expressed regarding the possibility that nifedipine, acting as a calcium antagonist, might inhibit insulin release and precipitate glucose intolerance in hypertensive patients and further aggravate glucose intolerance in diabetic patients with coronary heart disease whose endogenous insulin secretory capacity may already be compromised. The present work indicated that any deleterious effects of nifedipine (40 mg/day) on glucose tolerance were minimal and diabetic patients showed a paradoxial increase in plasma insulin levels with continued treatment. On this basis, nifedipine can be expeditiously used for the treatment of hypertension in both non-diabetic and non-insulin dependent diabetic patients without any significant adverse effect on glucose tolerance.

A great deal of literature to date in the diabetes field has commented on the possible role viscous dietary fibre (guar gum) might play in the control of non-insulin dependent diabetes. There is evidence to suggest that guar gum may be capable of reducing post-prandial blood glucose and insulin levels and at the same time facilitating some reduction in body weight. Work presented in this thesis has largely confirmed these observations and extended our understanding of the way dietary fibre might influence insulin receptor binding using both human erythrocytes and obese mouse adipocytes. Guarem treatment, significantly reduced fasting blood glucose and insulin levels in non-insulin dependent diabetic patients. Although there was some evidence of an improvement in glucose tolerance it never achieved statistical signifance. When

obese mice were treated with high doses of guarem, body weight, fasting blood glucose and insulin levels were all significantly reduced. The treatment of obese mice with low doses of guarem was largely ineffective. The reduced plasma insulin levels seen in both man and obese mouse after guarem treatment may well have resulted from the lowered blood glucose levels but might also have resulted from a reduction in the release of gastric inhibitory polypeptide from the duodenum and jejunum (398). In non-insulin dependent diabetic patients guarem treatment did not significantly influence either erythrocyte insulin receptor concentration or affinity. However, the treatment of obese mice with a high concentration of guarem significantly increased both specific ^{125}I-insulin binding to adipocytes and insulin receptor affinity without affecting insulin receptor concentration, Also, guarem treatment of obese mice was associated with a significant reduction in body weight and this observation may have contributed to the improvement in insulin receptor binding (330). The mechanism by which guarem lowers the blood glucose remains controversial, one hypothesis suggests that viscous polysaccharides like guarem act by delaying gastric emptying, hence slowing the delivery of carbohydrates to the small intestine (389). An alternative hypothesis suggests that viscous polysaccharides improve glucose tolerance by reducing the rate at which glucose is absorbed from the small intestine, probaly by increasing the resistance to diffusion of the intestinal unstirred water layer (395).

The association of non-insulin dependent diabetes with obesity is a frequent observation both in man and animals (471,472). However, the factor(s) underlying the development of obesity and

diabetes remain unknown. In the present work the use of genetically obese diabetic mice (ob/ob) and their lean (+/+) littermates allowed investigation of the impact of obesity and non-insulin dependent diabetes on adipocyte insulin receptor binding and insulin action. Obese mice showed an increased body weight, plasma insulin and plasma glucose compared with age matched lean mice. In addition, specific ^{125}I-insulin binding to lean mouse adipocytes was significantly greater than the specific binding to obese mouse adipocytes in the presence of both low and high concentrations of insulin. This observation was reflected in the significantly higher insulin receptor concentration in lean mouse adipocytes compared to obese and confirmed the previous work of Soll and colleagues (339). Differences in insulin binding to adipocytes may be represented by changes at the level of the insulin receptor or at some insulin sensitive postreceptor site. In the present study the rate of D-(U-^{14}C)-glucose oxidation was used as a parameter of postreceptor insulin action. Lean mouse abdominal fat segements demonstrated a significantly higher basal rate of glucose oxidation than obese. This higher rate could be ascribed to the greater energy requirement of the large number of intrinsically small adipocytes in each lean mouse fat segement. Indeed abdominal and femoral subcutaneous obese mouse adipocytes were always significantly larger than lean mouse abdominal adipocytes. In addition, the rate of insulin stimulated glucose oxidation by lean mouse abdominal fat segments was markedly greater than that by obese. Maximal rates of insulin stimulated glucose oxidation were obtained at around 30 ng/ml for lean mouse abdominal fat segements whereas marginal increases in the rate of insulin stimulated glucose oxidation were only observed at insulin

concentrations of 60-300 ng/ml for obese mouse fat segments. In this regard, the rate of glucose oxidation by obese mouse adipose tissue showed a markedly reduced sensitivity to insulin. However, it has been shown that the maximum rate of insulin stimulated glucose oxidation occurs when about 10-15% of the available insulin receptors are occupied (201). Therefore, a decrease in the rate of glucose oxidation observed at insulin concentrations great enough to saturate this absolute number of receptors (maximally effective insulin concentration) cannot be solely due to a decrease in the number of insulin receptors. This line of reasoning suggests that the defect in glucose oxidation in large adipocytes from obese mice resides distal to the insulin receptor, perhaps at the level of glucose transport or at some enzymic step in the oxidation process. In fact, studies on large adipocytes from obese rats have demonstrated an enhanced glucose oxidation via the pentose phosphate pathway in the presence of insulin. At the same time the insulin stimulated oxidation of glucose via the tricarboxylic acid cycle appeared normal in these cells (473). The nature of the insulin resistance of obese mouse adipose tissue and the mechanism by which the enlarged adipocytes become insulin resistant is not understood.

Whilst regional differences in adipose tissue insulin sensitivity have been documented for obese women (125) little information is available regarding possible regional differences in adipose tissue sensitivity in animal models of obesity. The present work is the first to demonstrate regional differences in insulin receptor binding in adipose tissue from obese mice. Obese mouse subcutaneous adipocytes had a significantly higher insulin receptor concentration but a significantly lower insulin receptor affinity than obese mouse

abdominal adipocytes. A similar phenomenon has been observed between abdominal subcutaneous and femoral subcutaneous fat from obese women (125). An explanation for such parallel but opposite changes in insulin receptor concentration and affinity has not previously been offered for animal studies. However, it seem plausible to suggest that an increase in receptor affinity might be the product of an immediate reduction in insulin receptor concentration brought about by a reduction in site-site interactions. On a physiological level, an increase in adipocyte insulin receptor affinity, occurring in parallel with a reduction in insulin receptor concentration might serve to maintain adipocyte sensitivity to insulin following insulin induced down regulation of its receptors. Such an hypothesis has been strengthened by the observation that both subcutaneous and abdominal fat segements from obese mice showed similar basal and insulin stimulated rates of glucose oxidation. Hence, differences between subcutaneous and abdominal fat of obese mice appeared to be restricted to the level of insulin receptor binding with little evidence of differences in postreceptor activity.

It should be emphasised that an increase in insulin receptor binding does not necessarily lead to an increase in the biological action of insulin (477). More importantly, it is thought to be those agents than can affect either insulin dimer aggregation, receptor clustering or the second messenger producing stages of the mechanism of insulin action, that can significantly alter insulin bioactivity. Our present understanding of insulin receptor function is governed by the work of Van Obberghen and colleagues who have suggested that the insulin receptor is an insulin-activated protein kinase (479). They have based this observation on the fact that the

receptor β-subunit isolated by immunoprecipitation is a substrate for insulin-stimulated phosphorylation, and that an ATP binding site can be identified on this same receptor subunit. The insulin receptor is not only suggested to be a kinase capable of self-phosphorylation, but it is also thought capable of catalysing the phosphorylation of exogenous substrates. An insulin receptor kinase complex is envisaged with two separate protein kinases, a tyrosine and a serine specific kinase. The tyrosine would be a constituent of the insulin receptor and supported by the existence of an ATP binding site on the receptor β-subunit. This kinase would be contained in the receptor β-subunit itself. In contrast, the serine kinase would be associated with the insulin receptor in the cell membrane, but would not form an integral part of the receptor. The biological relevance of insulin receptor phosphorylation has yet to be established, but it may play a role in biologically relevant phenomena such as receptor affinity modulation, hormone and receptor internalisation and signal transmission. Insulin-regulated covalent receptor modification may be an early step in insulin action and the insulin receptor may be envisaged as an integrated system for transmembrane hormone signalling. The increased kinase activity of the insulin receptor evoked by insulin binding could lead to the phosphorylation-dephosphorylation of other cellular proteins and through the generation of a cascade of reactions this would result in the final actions of insulin. The relationship between the two protein kinase activities of the insulin receptor, and their individual roles remain to be established. At least two situations are possible, one in which both kinases serve separate cellular functions and another which involves sequential activation of the kinases. It is an

appealing idea to conceive that tyrosine kinases may be involved in the generation of signals that might regulate the mitogenic action of insulin. While in contrast, the serine kinase might play a role in metabolic processes controlled by insulin. All the known enzymes that play a role in cellular intermediary metabolism and whose activities are modulated by insulin through phosphorylation-dephosphorylation reactions, are indeed modified at serine residues. Presently available data are consistent with the idea that the insulin receptor fulfills the requirements for a system regulating transmembrane hormone signalling. A major advance for the reality of this contention will be demonstration that receptor phosphorylation is necessary and sufficient for insulin to act.

Changes in insulin receptor binding have been identified in an increasing number of metabolic and endocrine abnormalities either as an intrinsic characteristic of the condition (443,474) or the product of drug therapy for a condition (484). The treatment of non-insulin dependent diabetes in enlightened clinics now takes account of changes in insulin receptor binding and insulin action as part of diabetic control. However, since the mechanism of insulin action and the link between insulin receptor binding and postreceptor events is poorly understood, the interpretation of binding data needs to be flexible and form only part of the differential diagnosis. Certainly, information gained on the pathophysiology of insulin receptor binding and postreceptor processes may in the future influence the method of treatment of non-insulin dependent diabetes complicated by hypertension.

APPENDICES

APPENDIX 1

The preparation of phosphate buffers:

0.5 mol/l phosphate buffer, pH 7.4

73g $Na_2 HPO_4$ combined with 14.04g of $NaH_2PO_4.2H_2O$ and made up to 1 litre with 2 x distilled H_2O, pH adjusted to 7.4 with 1 mol/l NaOH.

0.05 mol/l phosphate buffer, pH 7.4

7.3g Na_2HPO4 combined with 1.404g of $NaH_2PO_4.2H_2O$ and made up to 1 litre with 2 x distilled H_2O, pH adjusted to 7.4 with 1 mol/l NaOH.

APPENDIX 2

Composition of buffer G

Buffer G was used both for the isolation of erythrocyes and the erythrocyte insulin receptor binding assay (121). Buffer G was supplemented with 1% BSA to reduce the adhesion of insulin to the incubation vessel and reduce nonspecific binding. Bacitracin (0.08%) was added to reduce extracellular insulin degradation.

Chemicals	mmol/l	g/l
Hepes	50	11.91
Tris	50	6.05
$MgCl_2.6\ H_2O$	10	1.78
EDTA	2	0.58
Glucose	10	1.8
NaCl	50	2.92
KCl	5	0.37
$CaCl_2.\ 2H_2O$	10	1.47

The pH was adjusted to 8 with 1 mol/l NaOH at 23-25°C.

APPENDIX 3

Buffers used for the isolation of adipocytes and insulin receptor binding assays using rat adipocytes.

Buffer 1

Composition of Krebs bicarbonate buffer, pH 7.4 used for the isolation of rat adipocytes (308).

Chemicals	Directly prepared buffer (g/l)	Stock solutions			
		Percentage (g%)	Molarity	g/l	Parts by volume* ml
NaCl	6.923	0.9	0.154	9.0	100
KCl	0.354	1.15	0.154	11.48	4
$CaCl_2 \cdot 2H_2O$	0.373	1.617	0.11	16.172	3
KH_2PO_4	0.162	2.11	0.154	20.96	1
$MgSO_4 \cdot 7H_2O$	0.2923	3.8	0.154	37.96	1
$NaHCO_3$	2.1	1.3	0.155	13.02	21

* Gassed with 5% CO_2.
Supplemented with collagenase (3 mg/ml) and 4% BSA (303).

Buffer 2

Composition of Tris buffer, pH 7.45 used for both the isolation of adipocytes and insulin receptor binding assays with rat adipocytes (309).

Chemicals	mmol/l	g/l
Tris	35	4.235
NaCl	120	7.013
$MgSO_4 \cdot 7H_2O$	1.2	0.296
$CaCl_2 \cdot 2H_2O$	2	0.294
KCl	2.5	0.187
Glucose	10	1.8

Supplemented with either 1% BSA or 5% BSA and 0.08% bacitracin.

APPENDIX 4

Composition of Hepes buffer, pH 7.4 used for both the isolation of adipocytes and insulin receptor binding assays using lean and obese mouse adipocytes (130).

Chemicals	mmol/l	g/l
NaCl	135	7.889
KCl	4.8	0.358
$MgSO_4 \cdot 7H_2O$	1.7	0.419
$CaCl_2 \cdot 2H_2O$	2.5	0.368
$NaH_2PO_4 \cdot 2H_2O$	1.0	0.156
Hepes	10	2.38

The buffer was supplemented with BSA (2.5%), glucose (5 mmol/l) and collagenase (0.5 mg/ml) for the digestion of fat segments. The buffer used for washing adipocytes was supplemented with 5% BSA but contained no glucose. The buffer used for the dilution of both ^{125}I-insulin and cold insulin and mouse adipocyte insulin receptor binding assays was supplemented with 5% BSA and glucose (5 mmol/l).

APPENDIX 5 Reaction protocol for the human erythrocyte insulin receptor binding assay. Reagents were added in the order indicated in the table.

Tube no.	Buffer G (µl)	Concentration of stock insulin standard (ng/ml)	Volume of unlabelled insulin standard to be added (µl)	Final insulin concentration (ng/ml)	^{125}I-insulin* (µl)	Erythrocyte suspension (µl)	Final assay volume (µl)
1 (blank)	450	0	0	0	50	0	500
2 (total binding)	50	0	0	0	50	400	500
3	-	10	50	1	50	400	500
4	-	20	50	2	50	400	500
5	-	50	50	5	50	400	500
6	-	80	50	8	50	400	500
7	-	100	50	10	50	400	500
8	-	5×10^2	50	50	50	400	500
9	-	1×10^3	50	100	50	400	500
10	-	2×10^3	50	200	50	400	500
11	-	2.5×10^3	50	250	50	400	500
12	-	4×10^3	50	400	50	400	500
13	-	5×10^3	50	500	50	400	500
14	-	8×10^3	50	800	50	400	500
15	-	10^4	50	10^3	50	400	500
16 (non specific binding)	-	10^6	50	10^5	50	400	500

* 50 µl contained 0.04 ng (10,000 cpm) of ^{125}I-insulin.

APPENDIX 6

Example calculation for erythrocyte and adipocyte insulin receptor binding assays

To calculate the amount of ^{125}I-insulin added to each tube:

Total counts (T) are the accumulated counts for 100 µl of reaction volume over 5 minutes.

1 µCi = 2.2×10^6 cpm and since the counting efficiency of the gamma counter = 57% and 1 µCi = 1.26×10^6 cpm

The amount of ^{125}I-insulin added to each tube in ng =

$$\frac{T \times 10^3}{1.26 \times 10^6 \times \text{specific activity}}$$

The ^{125}I-insulin concentration in each tube in ng/ml =

$$\frac{T \times 10^3 \times 2}{1.26 \times 10^6 \times \text{specific activity}}$$

The ordinates, (B/F) $\times 10^{-2}$ and (B) ng/ml $\times 10^{-2}$ were derived as shown in table 1A.

A graph was plotted of (B/F) $\times 10^{-2}$ against Bng/ml $\times 10^{-2}$ (Scatchard plot). Straight lines were fitted by least squares for high ($\bar{K}_e$) and low ($\bar{K}_f$) receptor affinity. $(R_o)_1$, and R_o were computed from the equation, $y = a + bx$

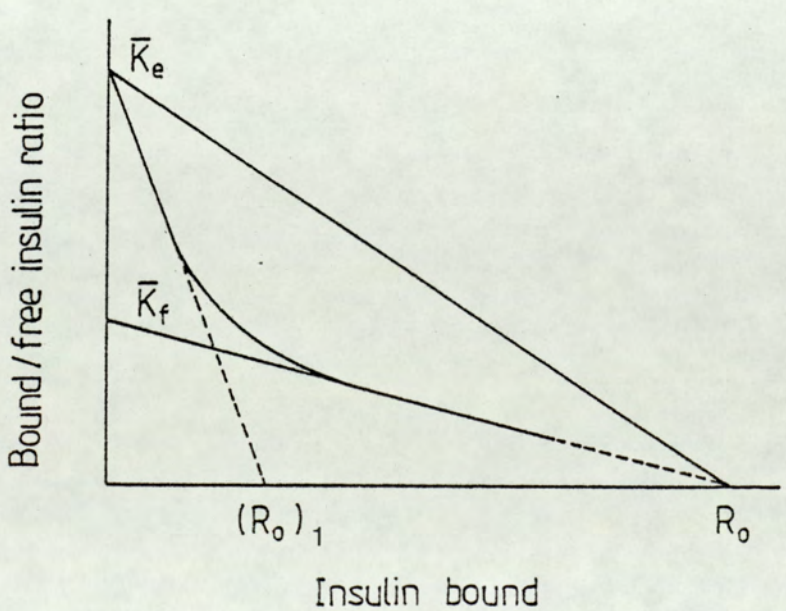


Table 1A. Calculation of ordinates for Scatchard plot.

Tubes no.	Total insulin concentration [H] ng/ml	Counts for specific binding	%B	Amount of bound insulin (B) ng/ml x 10^{-2}	Amount of free insulin (F) ng/ml x 10^{-2}	Bound/free insulin ratio (B/F) x 10^{-2}
1 2 3 n	Concentration of ^{125}I-insulin + concentration of unlabelled insulin	Total counts in each tube - counts for nonspecific binding	$\frac{\text{Counts for specific binding in each tube} \times 100}{\text{Total counts}}$	[H] x % B for each tube	[H] x 100-(B) for each tube	$\frac{(B)}{(F)} \times 100$ for each tube

The value for each abscissa intercept (R_o) was converted to moles/l. The number of receptor sites/cell was calculated from the following formula:

$$= \frac{\text{moles of insulin bound per litre} \times 6.022 \times 10^{23}*}{\text{number of cells/litre}}$$

where * is the Avagadro's number = number of molecules in one mole of insulin (220).

The following formulas were used for calculation of the affinity constants:

$\overline{K}_e$ = affinity constant of empty sites

$$= \frac{\text{Initial B/F value}}{R_o \text{ in moles}} \qquad nM^{-1}$$

$\overline{K}_f$ = affinity constant of full sites

= slope of total affinity plot $\qquad nM^{-1}$

$\overline{K}_m$ = Apparent affinity constant, calculated at 50% of the initial B/F value

$$= \frac{\text{B/F (at 50\% of initial B/F value)}}{R_o - \text{B (at 50\% of initial B/F value)}} \qquad nM^{-1}$$

APPENDIX 7 Reaction protocol for the rat adipocyte insulin receptor binding assay. Reagents were added in the order indicated in the table.

Tube no.	Tris Buffer (µl)	Concentration of stock insulin standard (ng/ml)	Volume of unlabelled insulin standard to be added (µl)	Final insulin concentration (ng/ml)	^{125}I-insulin* (µl)	Adipocyte suspension (µl)	Final assay volume (µl)
1 (blank)	225	0	0	0	25	0	250
2 (total binding)	25	0	0	0	25	200	250
3	-	10	25	1	25	200	250
4	-	20	25	2	25	200	250
5	-	50	25	5	25	200	250
6	-	80	25	8	25	200	250
7	-	100	25	10	25	200	250
8	-	5×10^2	25	50	25	200	250
9	-	1×10^3	25	100	25	200	250
10	-	2×10^3	25	200	25	200	260
11	-	2.5×10^3	25	250	25	200	250
12	-	4×10^3	25	400	25	200	250
13	-	5×10^3	25	500	25	200	250
14	-	8×10^3	25	800	25	200	250
15	-	10^4	25	10^3	25	200	250
16 (non specific binding)	-	10^6	25	10^5	25	200	250

* 25 µl contained either 0.1 ng (25,000 cpm) or 0.28 ng (70,000 cpm) of ^{125}I-insulin.

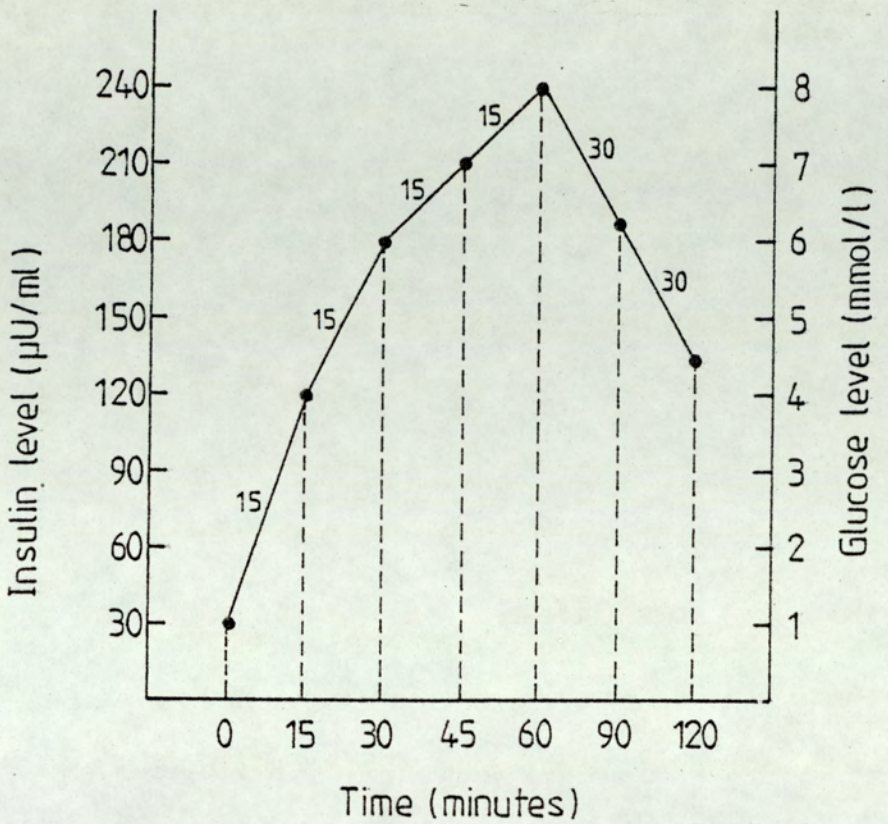
APPENDIX 8 Reaction protocol for the mouse adipocyte insulin receptor binding assay. Reagents were added in the order indicated in the table.

Tube no.	Hepes Buffer (µl)	Concentration of stock insulin standard (ng/ml)	Volume of unlabelled insulin standard to be added (µl)	Final insulin concentration (ng/ml)	^{125}I-insulin* (µl)	Adipocyte suspension (µl)	Final assay volume (µl)
1 (blank)	450	0	0	0	50	0	500
2 (total binding)	50	0	0	0	50	400	500
3	-	10	50	1	50	400	500
4	-	20	50	2	50	400	500
5	-	50	50	5	50	400	500
6	-	80	50	8	50	400	500
7	-	100	50	10	50	400	500
8	-	5×10^2	50	50	50	400	500
9	-	1×10^3	50	100	50	400	500
10	-	2×10^3	50	200	50	400	500
11	-	2.5×10^3	50	250	50	400	500
12	-	4×10^3	50	400	50	400	500
13	-	5×10^3	50	500	50	400	500
14 (non specific binding)	-	10^6	50	10^5	50	400	500

* 50 µl contained either 0.08 ng (20,000 cpm) of ^{125}I-insulin.

APPENDIX 9

Calculation of the area under the OGTT curve or OGTT insulin curve:



Area under the curve for either glucose or insulin was calculated using the following formula:

$$= \frac{bx.0 + bx.15}{2} \times 15 + \frac{bx.15 + bx.30}{2} \times 15 + \frac{bx.30 + bx.45}{2} \times 15$$

$$+ \frac{bx.45 + bx.60}{2} \times 15 + \frac{bx.60 + bx.90}{2} \times 30 + \frac{bx.90 + bx.120}{2} \times 30$$

which reduces to:-

$$7.5\ (bx.0 + 2bx.15 + 2bx.30 + 2bx.45 + bx.60) + 15\ (bx.60 + 2bx.90 + bx.120)$$

Where bx. = the value for either insulin or glucose at each time point.

APPENDIX 10

Protocols for Clinical Studies

Bendrofluazide and nifedipine trial protocol

HOSP. NO.

NAME: _____ D.O.B. _____ |_____|

WEIGHT: _____ kg

HEIGHT: _____ metres SEX _____

PAST DIAGNOSES:

1. _____

2. _____

3. _____

4. _____

5. _____

DRUGS:

1. _____ Dose _____

2. _____ _____

3. _____ _____

4. _____ _____

SMOKES: (per day) _____ ALCOHOL: (per week) _____ gm alcohol

FAMILY HISTORY-DM: FAMILY HISTORY - HYPERTENSION:

ADDRESS:

Nifedipine	Treatment 1st visit	Withdrawal 2nd Visit	Restoration of treatment 3rd visit		
Bendrofluazide	Visit 1	Visit 2 Placebo	Visit 3 1/12 Thiazide	Visit 4 3/12 Thiazide	Visit 5 Thiazide $+K^+$ suppl.
Hb					
WBC					
Platelets					
MCV					
Profile					
Urea					
Na^+					
K^+					
$HCO3^-$					
Cl^-					
Creat.					
BS					
Urate					
Total Prot.					
Alb.					
Ca^{2+}					
Phos.					
Bil.					
Alk. Phos.					
Asp. T.					
GT					
T4					
Free T4					
Total Chol.					
Tg.					
HDL/HDL2					
GTT	Sug. Ins.	Sug. Ins.	Sug. Ins.	Sug. Ins.	Sug. Ins.
0					
15 mins					
30 mins					
45 mins					
1 hr					
1½ hr					
2 hrs					
Fast. insulin					
Receptors					
Urinalysis					
Prot.					
Sugar					
Haem.					
Urine					
Sugar					
Weight					
Pulse					
BP					

Protocol for guarem trial

Name: Number: Height: _____

Date of Birth: / / Age: Duration of NIDDM: _____

Address: Alcohol intake: _____

_____ Smoking habits: _____

_____ Drug treatment: 1) _____

Trial Number: 2) _____

Entry Date: / / 3) _____

	1st baseline 1st visit	2nd baseline 2nd visit	Treatment 3rd visit
Weight			
Blood pressure			
HbA_1			
Glucose: Pre			
30 mins			
60 mins			
90 mins			
120 mins			
Cholesterol			
Triglyceride			
Sodium			
Potassium			
Gamma G.T.			
Asp. A.T.			
Urea			
Uric Acid			

Continued overleaf .....

	1st baseline 1st visit	2nd baseline 2nd visit	Treatment 3rd visit
Creatinine			
Calcium			
Phosphate			
Alk. Phos.			
Bilirubin			
24h urinary Na^+			
24h urinary K^+			
24h urinary Glc			

APPENDIX 11

Normal values for haematology and biochemistry in adult patients (North Birmingham Health District 1976, Clinical Chemistry Department).

Parameter	Value	
Haematology:		
MCV	80-96	fl
Platelets	150-300	$x10^9/l$
Biochemistry (in serum)		
Cholesterol	3-7.3	mmol/l
Triglycerides	1.8	mmol/l
Uric acid	0.16-0.4	mmol/l
Urea	3-7	mmol/l
Calcium	2.2-2.6	mmol/l
Chloride	95-105	mmol/l
Potassium	3.8-5.0	mmol/l
Sodium	136-148	mmol/l
Glycosylated haemoglobin	3-8	%

APPENDIX 12 The sequence for the addition of reagents in the insulin radioimmunoassay

	Tube no.	Initial reactants (50 µl)	Insulin binding reagent (50 µl)	^{125}I-insulin (50 µl)	Washing buffer dilution buffer (500 µl)
Zeros	1-3	Buffer	+	+	+
Insulin standard	4-6	12.5 µU/ml	+	+	+
	7-9	25 µU/ml	+	+	+
	10-12	50 µU/ml	+	+	+
	13-15	100 µU/ml	+	+	+
	16-18	200 µU/ml	+	+	+
Blank	19-21	Buffer	Buffer	+	+
Total count	22-24	-	-	+	-
Sample 1	25-26	+	+	+	+
Sample 2	27-28	+	+	+	+
Sample 3	29-30	+	+	+	+
n					

+ = reagent was added.
- = no reagent was added.

APPENDIX 13

Computer program in BASIC for the analysis of insulin RIA data using either human or mouse standards.

```
  5 REM          INSULIN ASSAY
 10 REM
 15 REM
 20 REM
 25 REM  DATA statements :"Species","units",no. of standards,standard concns.
 30 DATA "Mouse","ng/ml",5,.5,1,2.5,5,10
 35 DATA "Human","uU/ml",5,12.5,25,50,100,200
 40 INPUT "Do you want printout ",PR$
 45 IF ASCII(CVT$$(PR$,32)) = 89 THEN 55
 50 LET PR$ = "N" : GOTO 140
 55 LET PR$ = "Y"
 60 PRINT : INPUT "File name for printout ",FL$ : IF FL$ = "INS" THEN 60
 65 ON ERROR GOTO 85
 70 GENERATE FL$
 75 ON ERROR GOTO 0
 80 ASSIGN #11 = FL$ : OPEN #11 : GOTO 115
 85 IF ERROR >< 2150 THEN DO : ON ERROR GOTO 0 : GOTO 70 : DOEND
 90 PRINT:PRINT"Area already exists - do you want to delete existing data in it"
 95 INPUT "( WARNING - do not delete a program area with this name ) ",AN$
100 IF ASCII(CVT$$(AN$,32)) >< 89 THEN 60
105 ELIMINATE FL$
110 GOTO 70
115 PRINT : PRINT "Experiment title etc. for printout"
120 INPUT TI$ : PRINT #11 TI$
125 REM ********************************************
130 REM Input standard cpm's & compute best line
135 REM ********************************************
140 INPUT "STANDARDS : Mouse or Human or Both ( M or H or B ) ",MHB$
145 LET MHB$ = CVT$$(MHB$,32)
155 IF MHB$ = "H" THEN 185
160 IF MHB$ >< "B" THEN 140
165 GOSUB 235
170 LET INM = IN
175 LET SLM = SL
180 IF MHB$ = "M" THEN 440
185 RESTORE
190 READ R$,R$,RZ
195 FOR JZ = 1 TO RZ
200 READ R
205 NEXT JZ
210 GOSUB 235
215 LET INH = IN
220 LET SLH = SL
225 GOTO 440
230 REM ********************* SUB
235 READ Sp$,Un$
240 PRINT : PRINT Sp$
245 PRINT "Standards","  cpm      (if value not available enter '0')"
250 PRINT Un$
255 IF PR$ = "N" THEN 275
260 PRINT #11 : PRINT #11 Sp$
265 PRINT #11 "Standards","  cpm"
```

```
270 PRINT #11 Un$
275 LET X,Q,Y,S,W,NZ = 0
280 READ StNZ
285 FOR JZ = 1 TO StNZ
290 READ StCo ! Standard concentration
295 PRINT StCo," ";
300 INPUT StCpm
305 IF StCpm >< 0 THEN 320
310 IF PR$ = "N" THEN 365
315 PRINT #11 USING "####.#                -", StCo : GOTO 365
320 IF PR$ = "N" THEN 330
325 PRINT #11 USING "####.#     ##############", StCo,StCpm
330 LET StCo = LOG10(StCo)
335 LET X = X+StCpm
340 LET Q = Q+StCpm^2
345 LET Y = Y+StCo
350 LET S = S+StCo^2
355 LET W = W+StCo*StCpm
360 LET NZ = NZ+1
365 NEXT JZ
370 LET SL = (W-X*Y/NZ)/(Q-X^2/NZ)
375 LET IN = (Y-SL*X)/NZ
380 LET CoCo =(W-X*Y/NZ)/SQR((Q-X^2/NZ)*(S-Y^2/NZ))
385 PRINT : PRINT "Computed best line : Intercept (cpm = 0)      = ";IN
390 PRINT TAB(22),"Slope",TAB(46),"= ";SL
395 PRINT TAB(22),"Correlation coefficient = ";CoCo
400 IF PR$ = "N" THEN 420
405 PRINT #11 : PRINT #11 "Computed best line : Intercept (cpm = 0)      = ";IN
410 PRINT #11,TAB(22),"Slope",TAB(46),"= ";SL
415 PRINT #11,TAB(22),"Correlation coefficient = ";CoCo
420 RETURN
425 REM ************************************************
430 REM               Print headings
435 REM ************************************************
440 PRINT : PRINT "Input sample name , or 'END' to stop program"
445 PRINT TAB(21),"or 'DIL' to change dilution factor"
450 IF PR$ = "N" THEN 460
455 PRINT TAB(21),"or 'SUBH' to insert subheading for printout"
460 PRINT "followed by cpm"
465 PRINT : PRINT "'*' indicates result outside linear region of curve"
470 PRINT "     and will be incorrectly calculated by this program"
475 REM Limits of linearity set in 640,645,690,695
480 IF MHB$ = "B" THEN 545
485 PRINT : PRINT TAB(38),"Insulin concn."
490 PRINT TAB(38),"ref. ";Sp$;" standard"
495 IF PR$ = "N" THEN 510
500 PRINT #11 : PRINT #11 TAB(39),"Insulin concentration"
505 PRINT #11 TAB(27),"Dilution      ref. ";Sp$;" standard"
510 IF MHB$ = "H" THEN 530
515 PRINT "Sample","  cpm","    ng/ml","  uU/ml"
520 IF PR$ = "N" THEN 600
525 PRINT #11 "Sample","  cpm      factor       ng/ml          uU/ml" : GOTO 580
530 PRINT "Sample","  cpm","    uU/ml"
535 IF PR$ = "N" THEN 600
540 PRINT #11 "Sample","  cpm      factor",TAB(45),"uU/ml" : GOTO 580
545 PRINT : PRINT TAB(38),"Insulin concentration     Insulin concn."
550 PRINT TAB(38),"ref. Mouse standard       ref. Human st."
555 PRINT "Sample","cpm","  ng/ml","uU/ml","  uU/ml"
560 IF PR$ = "N" THEN 600
```

```
570 PRINT #11 TAB(26),"Dilution      ref. Mouse standard      ref. Human standard"
575 PRINT #11 "Sample","cpm      factor       ng/ml          uU/ml",TAB(69),"uU/ml"
580 PRINT #11
585 REM ************************************************
590 REM          Calculate sample concentrations
595 REM ************************************************
600 PRINT : INPUT "Dilution factor ",DF : PRINT
605 LET MX$,HX$ = "*"
610 INPUT Sam$
615 IF PR$ = "N" THEN 630
620 IF CVT$$(Sam$,32) >< "SUBH" THEN 630
625 INPUT TI$ : PRINT #11 : PRINT #11 "   ";TI$ : PRINT : GOTO 610
630 IF CVT$$(Sam$,32) = "END" THEN 780
635 IF CVT$$(Sam$,32) = "DIL" THEN 600
640 PRINT " "," ";  : INPUT Cpm : PRINT Sam$,Cpm," ";
645 IF MHB$ = "H" THEN 700
650 LET CoM = INT(EXP((INM+SLM*Cpm)*LOG(10))*10+.5)/10
655 IF CoM < .5 THEN 670
660 IF CoM > 12.8 THEN 670
665 LET MX$ = " "
670 IF MHB$ = "B" THEN 700
675 PRINT USING "!#####.#       !######.#",MX$,DF*CoM,MX$,25*DF*CoM
680 IF PR$ = "N" THEN 695
685 LET FMM$ = "\                \######     ######      !######.#      !#######.#"
690 PRINT #11 USING FMM$,  Sam$,Cpm,DF,MX$,DF*CoM,MX$,25*DF*CoM
695 IF MHB$ = "M" THEN 605
700 LET CoH = INT(EXP((INH+SLH*Cpm)*LOG(10))+.5)
705 IF CoH < 12.5 THEN 720
710 IF CoH > 320 THEN 720
715 LET HX$ = " "
720 IF MHB$ = "B" THEN 745
725 PRINT USING "!######",HX$,DF*CoH
730 IF PR$ = "N" THEN 605
735 LET FMH$ = "\                \######     ######          !########"
740 PRINT #11 USING FMH$,  Sam$,Cpm,DF,HX$,DF*CoH : GOTO 605
745 LET FMBT$ = "!#####.#       !######.#          !######"
750 PRINT USING FMBT$,  MX$,DF*CoM,MX$,25*DF*CoM,HX$,DF*CoH
755 IF PR$ = "N" THEN 605
760 LET FMB1$ = "\               \######     ######      "
765 LET FMB$ = FMB1$+"!######.#      !#######.#          !########"
770 PRINT #11 USING FMB$,  Sam$,Cpm,DF,MX$,DF*CoM,MX$,25*DF*CoM,HX$,DF*CoH
775 GOTO 605
780 IF PR$ = "N" THEN STOP
785 PRINT : PRINT "File for printout : ";FL$
```

****

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****

APPENDIX 14

Computer program in BASIC for the area under the curve.

```
  10CLS:PRINT:PRINT:
  20PRINT CHR$(141);"THIS PROGRAM WAS WRITTEN BY K.HUGHES"
  30PRINT CHR$(141);"THIS PROGRAM WAS WRITTEN BY K.HUGHES"
  40PRINT CHR$(141);TAB(12)"NOVEMBER 1985"
  50PRINT CHR$(141);TAB(12)"NOVEMBER 1985"
  60INPUT TAB(7,20)"PRESS RETURN WHEN READY ";A$:IFA$=INKEY$(-74)  THEN 70 ELSE
10
  70DIM A(100):DIM BX(100)
  80CLS:PROCstimulated:PROCbasal:PROCtest:PROCprint1:
  90INPUT"      DO YOU WANT TO PRINT                    OUT OF THESE RESULTS ";
$
 100IF Y$="YES" OR Y$="Y" THEN PROCprint2 ELSE END
 110PRINT:PRINT:
 120INPUT"DO YOU WANT TO ENTER MORE DATA ";D$:IF D$="YES" OR D$="Y" THEN 80 ELS
 END
 130PRINT:PRINT:END
 140DEF PROCstimulated
 150CLS
 160PRINT:PRINT:
 170INPUT"INPUT NUMBER OF STIMULUS EXPT'S ";N
 180PRINT:PRINT:
 190A=0:B=0
 200FOR I=1 TO N
 210AREA=0
 220PRINT "      TIME (MINS)";TAB(20);"INSULIN (uU/min)"
 230FOR T=31 TO 91 STEP5
 240PRINT T;
 250INPUT TAB(20)"INPUT VALUE ";BX(T)
 260IF T=31 THEN NEXT T
 270AREA=AREA+((BX(T-5)+BX(T)))*2.5
 280NEXT T
 290CLS
 300 PRINT "DATA FOR EXPERIMENT ";I;" NOW BEING STORED!"
 310PRINT:PRINT:
 320LET A(I)=AREA
 330A=A+A(I)
 340B=B+A(I)^2
 350NEXT I
 360C=A/N
 370V=(B-(C*A))/(N-1)
 380D=SQR(V)
 390FORX=1TO1000
 400NEXT X
 410ENDPROC
 420DEF PROCbasal
 430CLS
 440PRINT:PRINT:
 450INPUT"INPUT NUMBER OF BASALS ";N1
 460PRINT:PRINT:
 470A=0:B=0
 480FOR I=1 TO N1
 490AREA=0
 500PRINT "      TIME (MINS)";TAB(20);"INSULIN (uU/min)"
 510FOR T=31 TO 91 STEP5
 520PRINT T;
```

```
530INPUT TAB(20)"INPUT VALUE ";BX(T)
540IF T=31 THEN NEXT T
550AREA=AREA+((BX(T-5)+BX(T)))*2.5
560NEXT T
570CLS
580 PRINT "DATA FOR EXPERIMENT ";I;" NOW BEING STORED!"
590PRINT:PRINT:
600LET A(I)=AREA
610A=A+A(I)
620B=B+A(I)^2
630NEXT I
640C1=A/N1
650V=(B-(C1*A))/(N1-1)
660D1=SQR(V)
670FORX=1TO1000
680NEXT X
690ENDPROC
700DEF PROCtest
710T=(C-C1)/SQR(D^2/N+D1^2/N1)
720ENDPROC
730DEF PROCprint1
740CLS
750PRINT:PRINT:
760PRINT"MEAN FOR STIMULATED  = ";C
770PRINT
780PRINT"S.D. FOR STIMULATED  = ";D
790PRINT
800PRINT"NO. OF STIMULATED    = ";N
810PRINT
820PRINT"MEAN FOR BASAL       = ";C1
830PRINT
840PRINT"S.D. FOR BASAL       = ";D1
850PRINT
860PRINT"NO. OF BASAL         = ";N1
870PRINT
880PRINT"RESULT OF T TEST     = ";T
890PRINT
900PRINT"DEGREES OF FREEDOM   = ";(N+N1)-2
910PRINT:PRINT:
920ENDPROC
930DEF PROCprint2
940CLS
950PRINT:PRINT:
960INPUT"ENTER EXPERIMENTAL TITLE ";EX$
970VDU2
980*FX6
990VDU21
1000PRINT:PRINT
1010PRINT TAB(20);EX$
1020PRINT:PRINT:
1030PRINT TAB(15);"MEAN FOR BASAL     = ";C1
1040PRINT TAB(15);"S.D. FOR BASAL     = ";D1
1050PRINT TAB(15);"NUMBER OF BASAL    = ";N1
1060PRINT:PRINT
1070PRINT TAB(15);"MEAN FOR STIMULUS  = ";C
1080PRINT TAB(15);"S.D. FOR STIMULUS  = ";D
1090PRINT TAB(15);"NUMBER OF STIMULUS = ";N
1100PRINT:PRINT
1110PRINT TAB(15);"RESULT OF T.TEST   = ";T
1120PRINT TAB(15);"DEGREES OF FREEDOM = ";(N+N1)-2
1130VDU6
1140VDU3
1150ENDPROC
```

APPENDIX 15

Publications arising from this thesis.

Possible Role for Insulin Receptors in the Mechanism of Thiazide Induced Glucose Tolerance

Jaswinder S. Gill, Nabeel Al-Hussary*, Terence W. Atkins*, Kenneth G. Taylor† and D. Gareth Beevers

The role of cellular insulin receptors in the mechanism of thiazide induced glucose intolerance was studied in 10 non-diabetics and six diet controlled type II diabetics with mild essential hypertension. Glucose tolerance tests (75 g) were performed at the start of the study, after one month of placebo and after one month on bendrofluazide 5 mg daily. Erythrocyte insulin receptor status was measured on each occasion in the fasting state.

In non-diabetics, low affinity insulin receptor concentration increased after bendrofluazide but high affinity receptor concentration remained unchanged. In the diabetics, there was no change in either high or low affinity insulin receptor concentration. No change in insulin receptor affinity occurred in either group. In the long term, non-diabetics may maintain normal glucose tolerance on thiazide diuretics by increasing insulin receptor numbers. This adjustment did not occur in diabetic patients which may explain the deterioration in glucose tolerance.

Journal of Hypertension 2 (suppl 3): 573-576, 1984

Keywords: Thiazide diuretics, glucose intolerance, insulin receptors.

Introduction

Thiazide diuretics are cheap and effective antihypertensive drugs. They are, however, known to precipitate and aggravate diabetes mellitus [1-4]. When these agents are used as antihypertensive treatment the benefits derived from correcting hypertension, may be offset by the occurrence of diabetes mellitus. Attempts to prevent the diabetogenic effect of thiazide diuretics must be based on an understanding of their effects on insulin secretion and peripheral insulin sensitivity.

The actions of insulin upon intermediary metabolism are mediated through a specific insulin receptor present in the plasma membrane of target cells [5]. This receptor is also present in the membrane of circulating erythrocytes. Alterations in the cellular insulin receptor concentration have been reported in many conditions, including obesity [6], diabetes mellitus [7], steroid therapy [8], thyrotoxicosis [9] and after biguanide treatment [10]. The effect of thiazide diuretic treatment upon insulin receptor status was investigated in an attempt to understand the mechanism of thiazide-induced glucose-intolerance.

Patients and methods

Ten non-diabetic patients (four males) and six diet controlled type II diabetics (four males) with mild essential hypertension were recruited. The mean ages were 51.5 (s.d. 6.5) years and 60.7 (s.d. 6.4) years, respectively. All patients had blood pressures greater than 160/95 mmHg on two separate clinic visits. Pressures were taken seated using a Hawksley random zero sphygmomanometer, using phase V diastolic pressures. Secondary hypertension was excluded.

Patients were prescribed placebo tablets for one month followed by bendrofluazide 5 mg daily for one month in a single-blind study. After a 12 h overnight fast, a 75 g oral glucose tolerance test (OGTT) was performed at recruitment, after placebo and after active bendrofluazide. Glucose was measured in venous blood. Routine haematology (haemoglobin, white cell count, platelets) and biochemistry (urea, electrolytes, creatinine, liver function tests, fasting lipids and thyroid function tests) were performed before each OGTT. Fasting erythrocyte insulin receptor status was measured on each occasion using a modification of the insulin binding assay

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(Ghambir *et al.*) [11]. Analysis of binding was performed using Scatchard analysis [12] with computer assistance to determine the density of insulin binding sites on erythrocytes. The Scatchard plot for erythrocyte insulin binding is curvilinear. It is not established whether this represents two or more classes of receptor with different and fixed affinities or one class of receptors exhibiting negatively cooperative site-site interactions [13]. The present data were analysed using a model which assumes two classes of receptors. Affinity constants for insulin binding to these sites (Ke and Kf) were also calculated [14].

Statistical analysis was by paired t-tests. Ethical committee approval was obtained for the study.

Results

Baseline insulin receptor concentration was higher in the non-diabetic than diabetic patients. There were no changes in total, high or low affinity receptor concentration between baseline and placebo measurements (Fig. 1). Total and low affinity receptor concentration increased in the non-diabetic group after bendrofluazide when compared with the baseline and placebo values. In diabetic patients there was no change in total and low affinity receptor concentration after bendrofluazide. High affinity receptor sites were not changed by bendrofluazide in either group. There were no significant change in receptor site affinities after placebo or bendrofluazide treatment.

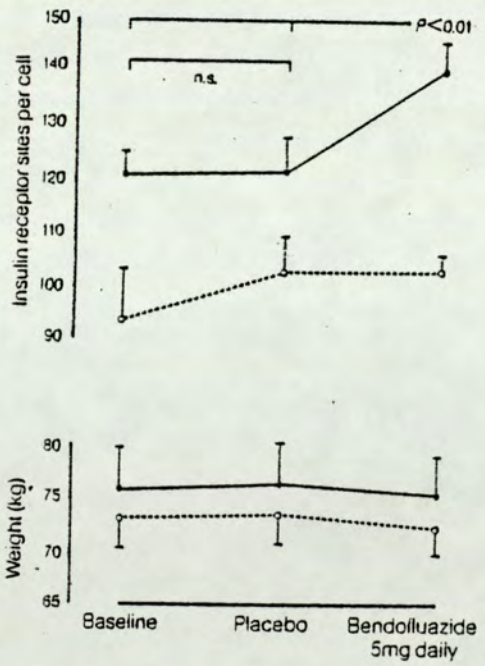

Fig. 1. Changes in low affinity insulin receptor number and weight in the non-diabetic (●) and diabetic (○) groups n.s. = not significant.

No change in glucose tolerance was observed in the non-diabetic or diabetic patients during placebo or bendrofluazide treatment. Blood counts and red cell indices were unaltered during the study. There were no changes in biochemical variables following placebo but there were changes after bendrofluazide as shown in Table 1. There were no changes in the body weight or pulse rate during the study in either group. Blood pressure decreased from 164/98 to 147/94 mmHg in the non-diabetic group and from 170/95 to 153/85 mmHg in the diabetic group after bendrofluazide.

Discussion

The present study was conducted in a single-blind fashion so that the effect of placebo could be examined. A diurnal variation in insulin receptor number has been reported [15] and we were careful to examine the insulin receptor status at the same time of the day on each occasion. Variations in insulin receptor number also occur in association with the menstrual cycle [16]. We recruited only men or post-menopausal females. In the one exception we performed the glucose tolerance test at the same stage of the cycle (luteal phase).

Although some previous studies on glucose intolerance during thiazide administration have demonstrated a decrease in insulin secretion [17], others suggest that thiazides may increase insulin secretion [18]. Furthermore some studies have indicated that glucose intolerance does not occur even with prolonged thiazide administration [19]. Decrease in insulin secretion may be related to hypokalaemia and has been reported in many other conditions in which this occurs including Bartters and Cushings syndromes, primary aldosteronism and oral administration of potassium binding resins [20]. During administration of thiazide diuretics, there is a decrease in the total body potassium even when serum potassium is maintained within normal limits [21]. Potassium seems to be involved in insulin release although magnesium and calcium may be more important.

The increase in insulin receptor number may be a response to decreased insulin secretion and might allow the non-diabetic group to maintain glucose tolerance. Alterations in insulin receptor concentration in response to changes in insulin secretion have been described [22], although the relationship is not entirely clear. In the diabetic group insulin receptors did not increase so that reduced insulin secretion would result in a deterioration in glucose tolerance. Glucose tolerance deteriorates earlier in diabetic and latent diabetic patients [23] and this may be related to the inability to increase insulin receptor number. If insulin secretion is increased then insulin resistance is implied. There were no weight changes in either group indicating that this was not the factor in the change in insulin receptor number. None of the present patients exhibited hypokalaemia during this period of thiazide diuretic therapy.

It is difficult to envisage the mechanism of the change in insulin receptor number in erythrocytes which have

Table 1. Changes in pulse, blood pressure, high affinity receptors and biochemistry after placebo and bendrofluazide. ND = non-diabetics, D = diabetics. Figures in parentheses are standard errors of means. The level of statistical significance (on bendrofluazide compared with placebo and baseline) is indicated by *P<0.05, **P<0.01

		Baseline	Placebo	Bendrofluazide	
Systolic blood pressure (mmHg)	ND	163.6 (6.0)	161.4 (4.6)	147.4 (4.4)*
	D	170.3 (12.4)	168.3 (11.8)	153.7 (9.1)
Diastolic blood pressure (mmHg)	ND	98.4 (4.2)	96.2 (2.3)	93.6 (4.4)
	D	95.3 (5.5)	91.3 (3.0)	85.3 (3.1)
Pulse rate (beats/min)	ND	73.8 (3.5)	75.6 (2.7)	73.4 (3.2)
	D	76.0 (5.2)	70.0 (5.5)	78.0 (5.6)
High affinity receptor sites per erythrocyte	ND	14.3 (1.5)	12.0 (1.3)	13.5 (0.9)
	D	13.5 (1.1)	13.1 (1.1)	13.1 (1.0)
Potassium (mmol/l)	ND	4.6 (0.13)	4.6 (0.11)	4.3 (0.13)
	D	4.5 (0.3)	4.5 (0.2)	4.3 (0.7)
Bicarbonate (mmol/l)	ND	24.6 (0)	26.9 (2.1)	28.3 (0.9)
	D	23.1 (1.4)	25.3 (1.4)	27.8 (0.7)
Urate (mmol/l)	ND	0.33 (0.04)	0.37 (0.04)	0.44 (0.04)**
	D	0.38 (0.04)	0.39 (0.05)	0.39 (0.05)
Cholesterol (mmol/l)	ND	6.0 (0.3)	5.8 (0.3)	6.2 (0.2)
	D	5.9 (0.3)	5.5 (0.3)	5.8 (0.3)
Triglyceride (mmol/l)	ND	1.4 (0.19)	1.4 (0.19)	1.5 (0.2)
	D	1.4 (0.18)	1.3 (0.19)	1.8 (0.3)*
Fasting blood sugar (mmol/l)	ND	4.2 (0.2)	4.4 (0.3)	4.4 (0.2)
	D	8.0 (1.0)	7.4 (1.1)	8.3 (1.0)
2 h OGGT blood sugar (mmol/l)	ND	5.6 (0.6)	5.3 (0.6)	5.8 (0.7)
	D	14.7 (1.3)	13.0 (1.7)	14.1 (1.4)

little capacity for protein biosynthesis. Three mechanisms are proposed for the change in insulin receptor number. First, new cells may be recruited into the circulating pool and these may differ in their insulin receptor number from those already in the circulation. This mechanism alone is unlikely to account for the degree of change in insulin receptor status observed. Second, receptors may be internalized by endocytosis and then recycled to the external plasma membrane by the lysosome [24]. Third, thiazides may cause a conformational change in the insulin receptor rendering it unable to bind insulin. This effect may be mediated by the thiazide diuretic itself or through change in insulin secretion. There is evidence to suggest that changes in receptor affinity may occur following changes in insulin concentration [25]. Furthermore, the number of available insulin receptor sites measured by Scatchard analysis is determined by the prevailing insulin levels, although the number of receptors which can be extracted by detergent remain unchanged [26]. Thus, it is probable that erythrocytes can modulate insulin receptor number and Dons *et al* have been able to correlate changes in erythrocyte insulin receptor number with those in the other target tissues for insulin [27]. In conclusion changes in insulin receptor number were observed in patients receiving thiazide diuretics. These may compensate for changes in insulin secretion or may be a direct effect of the thiazide diuretic. Patients unable to modulate insulin receptor number may be more likely to suffer impairment of glucose tolerance.

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References

1. Murphy MB, Lewis PJ, Kohner E, Schumer B: Glucose intolerance in hypertensive patients with diuretics: a fourteen-year follow-up. Lancet 1982, ii:1293-1295.
2. Report of Medical Research Council Working Party on Mild to Moderate Hypertension: Adverse reaction to bendrofluazide and propranolol for the treatment of mild hypertension. Lancet 1981, ii:539-542.
3. Amery A, Berthoux P, Bulpitt C, Deruyttere M, De Schaepdryver A, Dollery C, Fagard R, Forette F, Hellemans J, Lund-Johansen P, Mutsers A, Tuomilento J: Glucose intolerance during diuretic therapy. Results of trial by the European Working Party on Hypertension in the Elderly. Lancet 1978, i:681-683.
4. Goldner MG, Zarowitz H, Akgun S: Hyperglycaemia and glycosuria due to thiazide derivatives administered in diabetes mellitus. New Engl J Med 1960, 262:403-405.
5. Ginsberg BH: The insulin receptor: Properties and regulation. *In* Biochemical Actions of Hormones edited by Litwack G *et al*. New York: Academic Press, 1977, pp 313-349.
6. Olefsky JM: Insulin resistance and insulin action: an *in vitro* and *in vivo* perspective. Diabetes 1981, 30:148-162.
7. Olefsky JM, Reaven GM: Insulin-binding in diabetes. Relationships with plasma insulin levels and insulin hypersensitivity. Diabetes 1977, 26:680-688.
8. Beck-Neilsen H, DePirro R, Pedersen O: Prednisolone increases the number of insulin receptors on monocytes from normal subjects. J Clin Endocrinol Metab 1980, 50:1-4.
9. Gammage MD, Atkins TW, Grey TA, Fletcher RF, Taylor KG: Effect of hyperthyroidism on erythrocyte insulin receptors in man. Proceedings of the Second Joint Meeting of the British Endocrinol Society, York 1983. Abstract III: p56.
10. Lord JM, White SI, Bailey CJ, Atkins TW, Fletcher RF, Taylor KG: Effect of metformin on insulin receptor binding and glycaemic control in type II diabetes. Br Med J 1983, 286:830-831.
11. Gamonir KK, Archer JA, Bradley CJ: Characteristics of human erythrocyte insulin receptors. Diabetes 1978, 27:701-708.
12. Scatchard G: The attractions of proteins for small molecules and ions. Ann NY Acad Sci 1949, 51:660-672.

13. Bailey CJ, Lord JM, Atkins TW: The insulin receptor and diabetes. *In* Recent Advances in Diabetes edited by Nattrass M, Santiago JV. Edinburgh: Churchill-Livingstone, 1984, 1 pp 27-44.
14. Beck-Nielsen H, Pedersen O, Lindskov HO: Increased insulin sensitivity and cellular insulin binding in obese diabetics following treatment with glibenclamide. Acta Endocrinol (Copenh) 1979, 90:451-462.
15. Beck-Nielsen H, Pedersen O: Diurnal variation in insulin binding to human monocytes. J Clin Endocrinol Metab 1978, 47:385-390.
16. Bertoli A, DePirro R, Fusco A, Greco AV, Magnatta R, Lauro R: Differences in insulin receptors between men and menstruating women and influence of sex hormones on insulin binding during the menstrual cycle. J Clin Endocrinol Metab 1980, 50:246-250.
17. Helderman JH, Elahi D, Andersen DK, Riazes GS, Tobin JD, Shocken D, Andres R: Prevention of glucose intolerance of thiazide diuretics by maintenance of body potassium. Diabetes 1983, 32:106-111.
18. Wise PH, Rihan Z, Dinwiddy G, Whichelow MJ, Butterfield WJH: Clopamide and carbohydrate metabolism. Med J Aust 1969, 2:795-798.
19. Bergfund G, Andersson O: Beta-blockers or diuretics in hypertension? A six-year follow up of blood pressure and metabolic side effects. Lancet 1981, I:744-747.
20. Grunfeld C, Chapell DA: Hypokalaemia and diabetes mellitus (editorial). Amer J Med 1983, 75:553-554.
21. Edmonds CJ, Jasani B: Total body potassium in hypertensive patients during prolonged diuretic therapy. Lancet 1972, II:8-12.
22. Bar RS, Roth J, Kahn CR, DeMeyts P: Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients: effects of starvation, refeeding and dieting. J Clin Invest 1976, 58:1123-1135.
23. Shapiro AP, Benedek TG, Small JL: Effect of thiazides on carbohydrate metabolism in patients with hypertension. N Engl J Med 1961, 265:1028-1033.
24. Gorden P, Carpenter JL, Freychet P, Orci L: Internalisation of polypeptide hormones, mechanism, intracellular localisation and significance. Diabetologia 1980, 8:263-274.
25. DeMeyts P, Bianco AR, Roth J: Site-site interactions among insulin receptors: characterisation of negative co-operativity. J Biol Chem 1976, 281:1877-1888.
26. Krupps M, Lane MD: On the mechanism of ligand-induced down-regulation of insulin receptor level in the liver cell. J Biol Chem 1981, 256:1689-1694.
27. Dons RF, Ryan J, Gordon P, Wachslicht-Rodbard H: Erythrocyte and leucocyte insulin binding in man. A comparative analysis in normal and disease states. Diabetes 1981, 30:896-902.

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27 Insulin receptor status of bendrofluazide treated mild hypertensive patients with and without type II diabetes.

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The role of cellular insulin receptors in the mechanism of thiazide induced glucose intolerance was studied in 10 non-diabetic and 6 diet controlled type II diabetics with mild essential hypertension. A 75 g glucose tolerance test was performed at recruitment, after one month on placebo and after one month on bendrofluazide 5 mg daily. Fasting red blood cell insulin receptor status was measured on each occasion by an insulin binding assay and a computer assisted Scatchard plot. In non-diabetics no change in glucose tolerance or insulin secretion occurred on placebo or bendrofluazide. Low affinity insulin receptor number did not alter from baseline (123±4) to placebo (121.4±6.3) but increased after bendrofluazide (140±4.5, $p<0.01$). High affinity receptors were unchanged. In the diabetics glucose tolerance exhibited a small non-significant deterioration but insulin secretion was unaltered. There was no change in high affinity or low affinity insulin receptors. No change in insulin binding affinity occurred in either group. Therefore in non-diabetics normal glucose tolerance on thiazide diuretics may be maintained by increasing insulin receptor numbers whereas diabetics unable to do this suffer a deterioration in glucose tolerance.

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POSSIBLE ROLE FOR INSULIN RECEPTORS IN THE MECHANISM OF THIAZIDE INDUCED GLUCOSE INTOLERANCE

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Deterioration in glucose tolerance in patients receiving thiazide diuretics has been shown by many studies and there is evidence that this operates through impaired insulin release. We have studied the role of cellular insulin receptors in the mechanism of thiazide induced glucose intolerance.

Eleven patients with mild essential hypertension were studied, 6 were non-diabetic and 5 diet controlled type II diabetics. A 75 g oral glucose tolerance test was performed at recruitment, after one month on placebo and after one month on bendrofluazide 5 mg daily, in a single blind trial. Fasting insulin receptor status was measured on each occasion by an insulin binding assay (modification of the method of Ghambir) and a computer assisted Scatchard plot. Analysis was by paired t-tests.

No significant change in glucose tolerance was observed on placebo or bendrofluazide. There was no significant change in total, high affinity or low affinity receptors between the baseline and placebo measurements in the diabetic and non-diabetic groups. In the non-diabetic group there was a significant increase in red cell insulin receptors between placebo and thiazide phases (total receptors: 131.4±7.5 to 154.9±7.82, high affinity receptors: 11.4±1.89 to 15.6±2.45, low affinity receptors: 120±8.35 to 139.5±2.8 respectively, $p<0.01$). No significant change was found in the diabetics. There was no alteration in insulin binding affinity in either group. In non-diabetics therefore an increase in insulin receptor numbers may maintain normal glucose tolerance in the face of reduced insulin release, whereas diabetics are unable to do this resulting in impaired glucose tolerance.

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Medical and Scientific Section ,
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28 - 30 th March , 1985 .

Insulin receptor binding and insulin action in subcutaneous and abdominal fat of obese mice.

Regional differences in adipose tissue insulin sensitivity have been documented for obese human subjects but not for animal models of obesity. Insulin receptor binding, insulin stimulated glucose oxidation and ^{125}I-insulin degradation have been determined in isolated adipocytes and fat segments from the lower abdominal (Ab) and upper femoral region (Sc) of obese mice. ^{14}C-glucose oxidation was continuously monitored with an electrometer and ionisation chamber. The total insulin receptor concentration was significantly higher in Sc adipocytes than Ab (25.7 ± 2.04 and 18.7 ± 1.07 per μm^2 respectively, $P < 0.01$). The apparent insulin receptor affinity was significantly higher in Ab adipocytes than Sc (0.071± 0.005 and 0.044 ± 0.004 nM^{-1} respectively, $P<0.01$). There was no significant difference in either the rate of insulin stimulated glucose oxidation or cell diameter between Sc and Ab adipocytes and no significant difference in ^{125}I-insulin degradation between Sc and Ab sites whether using isolated adipocytes or fat segments. Differences between subcutaneous and abdominal fat of obese mice appear to be restricted to the level of insulin receptor binding with little evidence of differences in postreceptor activity.

British Diabetic Association ,
Medical and Scientific Section ,
Belfast , Abstract No . 27 ,
5 - 7 th September , 1985 .

Failure of potassium supplementation to prevent the effects of bendrofluazide on glucose tolerance, insulin and insulin receptors

Nine non-diabetic (ND) patients (4 males mean age 51.7±7.0 years) and 9 diet-controlled diabetic (D) patients (8 males mean age 64.3±4.6 years) with mild hypertension were studied. A 75 g of oral glucose tolerance test (OGTT) was performed at recruitment, after 1 month placebo treatment, after 1 month bendrofluazide (BF) 5 mg daily, a further 3 months BF and 1 month after potassium supplementation (20 mmol thrice daily).

Comparing placebo values to those after 4 months treatment with BF glucose tolerance deteriorated (area under OGTT curve ND 781±186 to 848±192; D 1440±118 to 151±143 mmol/l/min) and insulin secretion decreased. (area under OGTT curve ND25975±17963 to 24272±13968; D 14316±12950 to 13337±13967 uU/ml/min).

Fasting erythrocyte insulin receptors increased in the ND group (135±19 to 154±25 sites/cell) but were unchanged in the D group (107±18 to 96±20 sites/cell). Serum potassium decreased from 4.5±4 to 4.3±0.4 mmol/l.

Following potassium supplementation, serum insulin fell further (ND 18291±10361; D 9534±1101 uU/ml/min) but there were no other significant changes.

We conclude that thiazide induced glucose tolerance is consequent to decreased insulin secretion. Oral potassium supplementation does not prevent deterioration of these changes.

REFERENCES

1. Roth, J.: Peptide hormone binding to receptors: a review of direct studies in vitro. Metabolism, 22: 1059-1073, 1973.

2. Roth, J., Kahn, C.R., Lesniak, M.A., Gorden, P., De Meyts, P., Megyeski, K., Neville, D.M. Jr., Gavin, J.R. III., Soll, A.H., Freychet, P., Goldfine, I.D., Bar, R.S. & Archer, J.A.: Receptors for insulin, NSILA-S, and growth hormone: applications to disease states in man. Recent Prog. Horm. Res., 31: 95-139, 1975.

3. Kahn, C.R., Baird, K.L., Flier, J.S., Grunfeld, C., Harmon, J.T., Harrison, L.C., Karlsson, F.A., Kasuga, M., King, G.L., Lang, U.C., Podskalny, J.M. & Van Obberghen, E.: Insulin receptors, receptor antibodies, and the mechanism of insulin action. Recent Prog. Horm. Res., 37: 477-538, 1981.

4. Drury, P.L.: Diabetes and arterial hypertension. Diabetologia, 24: 1-9, 1983.

5. Wilhelmsen, L. & Svärdsudd, K.: Hypertension risk indicators. In: Mild Hypertension: Recent Advances. Gross, F. & Strasser, T. (eds.), Raven Press, New York, 1983, pp 123-130.

6. AHA Committee Report: Recommendations for human blood pressure determination by sphygmomanometers. Hypertension, 3: 510A-519A, 1981.

7. Korner, P.I.: Pathophysiological mechanisms in hypertension. In: Hypertension: Mechanisms and Management. Kincaid-Smith, P.S. & Whitworth, J.A. (eds.), ADIS Health Science Press, New York, 1982, pp 1-11.

8. Gavras, H., Brunner, H.R., Vaughan, E.D. Jnr. & Laragh, J.H.: Angiotensin - sodium interactions in blood pressure maintenance of renal hypertensive and normotensive rats. Science, 180: 1369-1372, 1973.

9. McGiff, J.C. & Nasjletti, A.J.: Kinnins, renal function and blood pressure regulation. Fed. Proc. Am. Soc. Exper. Biol., 35: 172-206, 1975.

10. Freeman, R.H., Davis, J.O. & Vatkins, B.E.: Development of chronic perinephritis hypertension in dogs without volume expansion. Am. J. Physiol., 233: F278-F281, 1977.

11. Doyle, A.E.: Vascular reactivity in hypertension. In: Hypertension: Mechanims and Management. Kincaid-Smith, P.S. & Whitworth, J.A. (eds.), ADIS Health Science Press, New York, 1982, pp 12-17.

12. Folkow, B.: The haemodynamic consequences of adaptive structural changes of the resistance vessels in hypertension. Clin. Sci., 41: 1-12, 1971.

13. Moulds, R.F.W.: Vascular smooth muscle in hypertension. In: Hypertension: Mechanisms and Management. Kincaid-Smith, P.S. & Whitworth, J.A. (eds.), ADIS Health Science Press, New York, 1982, pp 18-24.

14. Sannerstedt, T.: Hemodynamic findings at rest and during exercise in mild arterial hypertension. Am. J. Med. Sci., 258: 70-79, 1969.

15. Jarrett, R.J., Keen, H., McCartney, M., Fuller, J.M., Hamilton, P.J.S., Reid, D.D. & Rose, G.: Glucose tolerance and blood pressure in two population samples: their relation to diabetes mellitus and hypertension. Int. J. Epidemiol., 7: 15-24, 1978.

16. McGrath, B.: Catecholamines in hypertension. In: Hypertension: Mechanisms and Management. Kincaid-Smith, P.S. & Whitworth, J.A. (eds.), ADIS Health Science Press, New York, 1982, pp 25-35.

17. Goldstein, D.S.: Plasma norepinephrine in essential hypertension: a steady of the studies. Hypertension, 3: 48-52, 1981.

18. Christlieb, A.R., Kaldany, A. & D'Elia, J.A.: Plasma renin activity and hypertension in diabetes mellitus. Diabetes, 25: 969-974, 1976.

19. Brown, J.J., Fraser, R., Leckie, B., Lever, A.F., Morton, J.J., Padfield, P.L., Semple, P.F. & Robertson, J.I.S.: Significance of renin and angiotensin in hypertension. Cardiovascular Clinics, 9: 55-89, 1978.

20. Yamori, Y.: The genetics of essential hypertension. In: Mild Hypertension, Recent Advances. Gross, F. & Strasser, T. (eds.), Raven Press, New York, 1983, pp 187-196.

21. Scherstèn , B. & Bitzèn, P-O.: The diagnosis of diabetes mellitus. Acta Endocrinol. (Copenh.), Suppl. 262: 11-17, 1984.

22. Soeldner, J.S.: The etiology of diabetes. In: Clinical Diabetes Mellitus. Kozak, G.P. (ed.), W.B. Saunders Company, London, 1982, pp 21-31.

23. Beck-Nielsen, H. & Sørensen, N.S.: Natural history of non-insulin dependent diabetes mellitus. Acta Endocrinol. (Copenh.), Suppl. 262: 7-9, 1984.

24. Fajans, S.S., Cloutier, M.C. & Crowther, R.L.: Clinical and etiologic heterogeneity of idiopathic diabetes mellitus. Diabetes, 27: 1112-1125, 1978.

25. Tolbert, B.M., Kirk, M. & Baker, E.M.: Continuous $^{14}CO_2$ and CO_2 excretion studies in experimental animals. Am. J. Physiol., 185: 269-274, 1956.

26. Schwabe, A.D., Bennett, L.R. & Bowman, L.P.: Octanoic acid absorption and oxidation in humans. J. Appl. Physiol., 19: 335-337, 1964.

27. De Fronzo, R.A., Simonson, D. & Ferrannini, E.: Hepatic and peripheral insulin resistance: A common feature of Type II and Type I diabetes mellitus. Diabetologia, 23: 313-319, 1982.

28. Seltzer, H.S., Allen, W.W., Herron, A.L. & Brennan, M.T.: Insulin secretion in response to glycemic stimulus: relation of delayed initial release to carbohydrate intolerance in mild diabetes mellitus. J. Clin. Invest., 46: 323-335, 1967.

29. Beck-Nielsen, H., Pedersen, O. & Sørensen, N.S.: Effects of dietary changes on cellular insulin binding and in vivo sensitivity. Metabolism, 29: 482-487, 1980.

30. Olefsky, J.M.: Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. J. Clin. Invest., 57: 1165-1172, 1976.

31. Olefsky, J.M.: The insulin receptor: its role in insulin resistance of obesity and diabetics. Diabetes, 25: 1154-1162, 1976.

32. Tuck, M.L., Somers, J. & Dornfeld, O.: The effect of weight reduction on blood pressure, plasma renin activity and plasma aldosterone level in obese patients. N. Engl. J. Med., 304: 930-933, 1981.

33. Maxwell, M.H., Waks, A.V., Schroth, P.C., Karam, M. & Dornfeld, L.P.: Error in blood pressure measurement due to incorrect cuff size in obese patients. Lancet, 2: 33-35, 1982.

34. Major, S.G.: Blood pressure in diabetes mellitus: a statistical study. Arch. Intern. Med., 44: 797-812, 1929.

35. Adams, S.: A study of the blood pressure of patients with diabetes mellitus. Am. J. Med. Sci., 177: 195-201, 1929.

36. Freedman, P., Moulton, R. & Spencer, A.G.: Hypertension and diabetes mellitus. Q. J. Med., 27: 293-305, 1958.

37. Pell, S. & D'Alonzo, C.A.: Some aspects of hypertension in diabetes mellitus. J.A.M.A., 20: 104-110, 1967.

38. Dawber, T.R.: Diabetes and Cardiovascular Disease: The Framingham Study. Harvard University Press, Cambridge, 1980, pp 190-201.

39. Barrett-Conner, E., Criqui, M.H., Klauber, M.R. & Holdbook, M.: Diabetes and hypertension in a community of older adults. Am. J. Epidemiol., 113: 276-284, 1981.

40. Fagerberg, S-E.: Diabetes and arterial hypertension. Acta. Endocrinol. (Copenh.), Suppl. 262: 79-82, 1984.

41. Siperstein, M.D.: Pathogenesis of Diabetes Mellitus. Nobel Symposium 13. Cerasi, E. & Luft, R. (eds.), Uppsala, Almqvist and Wiksell, 1970.

42. De Fronzo, R.A.: The effect of insulin on renal sodium metabolism. A review with clinical implications. Diabetologia, 21: 165-171, 1981.

43. Weidmann, P., Beretta-Piccoli, C., Keusch, G., Gluck, Z., Mujagic, M., Grimm, M., Meier, A. & Ziegler, W.H.: Sodium - volume factor, cardiovascular reactivity and hyptensive mechanism of diuretic therapy in mild hypertension associated with diabetes mellitus. Am. J. Med., 67: 779-784, 1979.

44. Burden, A.C. & Thurston, H.: Plasma renin activity in diabetes mellitus. Clin. Sci., 56: 255-259, 1979.

45. Christlieb, A.R.: Vascular reactivity to angiotensin II and to norepinephrine in diabetic subjects. Diabetes, 25: 268-274, 1976.

46. Moulds, R.F.W.: The pharmacological basis of drug therapy. In: Hypertension: Mechanisms and Management. Kincaid-Smith, P.S. & Whitworth, J.A. (eds.), ADIS Health Science Press, New York, 1982, pp 68-77.

47. Dustan, H.P., Page, I.H., Tarazi, R.C. & Frahlich, E.D.: Arterial pressure response to discontinuing antihypertensive drugs. Circulation, 37: 370-379, 1968.

48. Doyle, A.E.: When to begin treatment in hypertension. In: Hypertension: Mechanisms and Management. Kincaid-Smith, P.S. & Whitworth, J.A. (eds.), ADIS Health Science Press, New York, 1982, pp 61-63.

49. Oliver, M.F.: Risks of correcting the risks of coronary disease and stroke with drugs. N. Engl. J. Med., 6: 297-298, 1982.

50. Finnerty, F.A.: Step-down therapy in hypertension. Importance in long-term management. J. Am. Med. Assoc., 246: 2593-2596, 1981.

51. Björntorp, P.: Hypertension and excercise. Hypertension, 4 (Suppl. 111): 111-56-111-59, 1982.

52. Björntorp, P.: Hypertension and obesity. Acta Med. Scand., 24: 241-242, 1982.

53. De Fronzo, R.A., Goldberg, M. & Agus, Z.: The effect of glucose and insulin on renal electrolyte transport. J. Clin. Invest., 58: 83-90, 1976.

54. Sacks, F.M., Castelli, W.P., Donner, A. & Kass, E.H.: Plasma lipids and lipoproteins in vegetarians and controls. N.Engl. J. Med., 292: 1148-1151, 1975.

55. Trowell, H.C.: Hypertension, obesity, diabetes mellitus and coronary heart disease. In: Western Diseases: Their Emergence and Prevention. Trowell, H.C. & Burkitt, D.P. (eds.), Edward Arnold, London, 1981, pp 3-32.

56. Anderson, J.W.: Plant fibre and blood pressure. Ann. Intern. Med., 98: 842-846, 1983.

57. Dodson, P.M. & Humphreys, D.: Hypertension and angina. In: Western Disease: Their Emergence and Prevention. Trowell, H.C. & Burkitt, D.P. (eds.), Edward Arnold, London, 1981, pp 411-420.

58. Anderson, J.W. & Chen, W-J.L.: Plant fibre: carbohydrate and lipid metabolism. Am. J. Clin. Nutr., 32: 346-363, 1979.

59. Anderson, J.W.: Plant fibre treatment for metabolic diseases. Spe. Top. Endocrinol. Metab., 2: 1-42, 1981.

60. De Fronzo, R.A., Cooke, C.R., Andres, R., Faloona, G.R. & David, P.J.: The effect of insulin on renal handling of sodium, potassium, calcium and phosphate in man. J. Clin. Invest., 55: 845-855, 1975.

61. Jenkins, D.J.A., Leeds, A.R., Gassull, M.A., Cochet, B. & Alberti, K.G.M.M.: Decreased in postprandial insulin and glucose concentrations by guar and pectin. Ann. Intern. Med., 86: 20-23, 1977.

62. Björntorp, P. Holm, G., Jacobsson, B., Schiller - de Joungey, K., Lundberg, P.A., Björström, L., Smith, U. & Sullivan, L.: Physical training in human hyperplastic obesity. IV. Effects on the hormonal status. Metabolism, 26: 319-328, 1977.

63. Krotkiewski, M., Mandroukas, K., Sjöstrom, L., Sullivan, L., Wetterqvist, H. & Björntorp, P.: Effects of long term physical training on body fat metabolism and blood pressure in obesity. Metabolism, 28: 650-658, 1979.

64. MacGregor, G.A., Markandu, N., Best, F., Elder, D., Cam, J., Sagnella, G.A. & Squires, M.: Double blind randomised crossover trial of moderate sodium restriction in essential hypertension. Lancet, i: 351-355, 1982.

65. Murphy, M.B., Lewis, P.J., Kohner, E., Schumer, B. & Dollery, C.T.: Glucose intolerance in hypertensive patients treated with diuretics: a fourteen-year follow-up. Lancet, ii: 1293-1295, 1982.

66. Pacy, P.J., Dodson, P.M., Kubicki, A.J., Fletcher, R.F. & Taylor, K.G.: Comparison of the hypertensive and metabolic effects of bendrofluazide therapy and a high fibre, low fat, low sodium diet in diabetic subjects with mild hypertension. J. Hypertens., 2: 215-220, 1984.

67. Nutrition Sub-Committee of the British Diabetic Association. Dietary recommendations for diabetic for the 1980's - a policy statement. British Diabetic Association: London, 1983.

68. Kannel, W.B. & McGee, D.L.: Diabetes and cardiovascular risk factors; the Framingham study. Circulation, 59: 8-13, 1979.

69. Puska, P., Iacono, J.M., Nissinen, A., Korhonen, H.J., Vartiainen, E., Pietinen, P., Dougherty, R., Leino, U., Mutanen, M., Moisio, S. & Huttunen, J.: Controlled, randomised trial of the effect of dietary fat on blood pressure. Lancet, i: 1-5, 1983.

70. MacGregor, G.A., Smith, S.J., Markandu, N.D., Banks, R.A. & Sagnella, G.A.: Moderate potassium supplementation in essential hypertension. Lancet, ii: 567-570, 1982.

71. Beard, T.C., Cooke, H.M., Gray, W.R. & Barge, R.: Randomised controlled trial of a no-added-sodium diet for mild hypertension. Lancet, ii: 455-458, 1982.

72. Dodson, P.M., Pacy, P.J., Bal, P., Fletcher, R.F. & Taylor, K.G.: The hypotensive effect of a low sodium, low fat and high fibre diet in the hypertensive diabetic patient. Diabetologia, 25: 151, 1983. Abstract.

73. Dodson, P.M., Stocks, J., Holdsworth, G. & Galton, D.J.: High fibre and low fat diets in diabetes mellitus. Br. J. Nutr., 46: 289-294, 1981.

74. Smith, S.J., Markandu, N.D., Sagnella, G.A. & MacGregor, G.A.: Does dietary potassium supplementation plus moderate sodium restriction have an additive blood pressure lowering effect in essential hypertension? A double blind placebo controlled trial. Clin. Sci. Mol. Med., 66: 36 , 1984. Abstract.

75. Swales, J.D.: Clinical Hypertension. Chapman and Hall, London, 1979, pp 163-167.

76. Gross, F.: Advances in pharmacotherapy with special reference to mild hypertension. In: Mild Hypertension: Recent Advance. Gross, F. & Strasser, T. (eds.), Raven Press, New York, 1983, pp 341-346.

77. Editorial: Diuretic or beta blocker? Lancet, i: 763, 1981.

78. Amery, A., Berthoux, P., Bulpitt, C., Deruyttère, M., DeSchaepdryver, A., Dollery, C., Fagard, R., Forette, F., Hellemans, J., Lund-Johansen, P., Mutsers, A. & Tuomilehto, J.: Glucose tolerance during diuretic therapy. Results of trial by the European working party on hypertension in the elderly. Lancet, i: 681-683, 1978.

79. Edmonds, C.J. & Jasani, B.: Total body potassium in hypertensive patients during prolonged diuretic therapy. Lancet, ii: 8-12, 1972.

80. Lewis, P.J., Kohner, E.M., Petrie, A. & Dollery, C.T.: Deterioration of glucose tolerance in hypertensive patients on prolonged diuretic treatment. Lancet, i: 564-566, 1976.

81. Grimm, R.H., Leon, A.S., Hunninghake, D.B., Lenz, K., Hannan, P. & Blackburn, H.: Effect of thiazide diuretics on plasma lipids and lipoproteins in mildly hypertensive patients. A double blind controlled trial. Ann. Int. Med., 94: 7-11, 1981.

82. Goldman, A.I., Steele, B.W., Schnaper, H.W., Fitz, A.E., Frohlich, E.D. & Perry, H.M.Jr.: Serum lipoprotein levels during chlorthalidone therapy. J.A.M.A., 244: 1691-1695, 1980.

83. Bulpitt, C.J.: Serum uric acid in hypertensive patients. Br. Heart J., 12: 1210-1215, 1975.

84. Berglund, G., Andersson, O., Larsson, O. & Wilhelmsen, L.: Antihypertensive effect and side-effects of bendrofluazide and propranolol. Acta. Med. Scand., 199: 499-506, 1976.

85. Berglund, G. & Andersson, O.: Beta blockers or diuretics in hypertension? A six year follow-up of blood pressure and metabolic side effects. lancet, i: 744-747, 1981.

86. Gorden, P.: Glucose intolerance with hypokalaemia. Diabetes, 22: 544-551, 1973.

87. Helderman, J.H., Elahi, D., Andersen, D.K., Raizes, G.S., Tobin, J.D. & Shocken, D.: Prevention of the glucose intolerance of thiazide diuretics by maintenance of body potassium. Diabetes, 32: 106-111, 1983.

88. Bauer, G.F. & Hunyor, S.N.: Mild hypertension: is treatment worthwhile? Drugs, 15: 80-86, 1978.

89. Waal-Manning, H.J.: Can B-blockers be used in diabetic patients? Drugs, 17: 157-160, 1979.

90. Wright, A.D., Barber, S.G., Kendall, M.J. & Poole, P.H.: Beta adrenoreceptor-blocking drugs and blood sugar control in diabetes mellitus. Br. Med. J., 1: 159-161, 1979.

91. Freis, E.D.: Should mild hypertension be treated? N. Engl. J. Med., 307: 306-309, 1982.

92. Australian National Blood Pressure Study Management Committee.: The Australian therapeutic trial in mild hypertension. Lancet, i: 1261-1267, 1980.

93. Whitworth, J.A. & Kincaid-Smith, P.: Diuretics or B-blockers first for hypertension? Drugs, 23: 394-402, 1982.

94. Hulthen, U.L., Bolli, P. & Bühler, F.R.: Calcium influex blockers in the treatment of essential hypertension. Acta Med. Scand., Suppl. 681: 101-108, 1984

95. Krebs, R., Graefe, K.H. & Ziegler, R.: Effects of calcium entry antagonists in hypertension. Clin. Exp. Hypertens., A4: 271-284, 1982.

96. Sharma, B.K., Singh, S. & Mehta, R.: Hyperglycaemic effect of nifedipine. Br. Med. J., 283: 19-20, 1981.

97. Greenwood, R.H.: Hyperglycaemic effect of nifedipine. Br. Med. J., 284: 50, 1982.

98. Giugliano, D., Torella, R., Cacciapuoti, F., Gentile, S., Verza, M. & Varricchio, M.: Impairment of insulin secretion in man by nifedipine. Eur. J. Clin. Pharmacol., 18: 395-398, 1980.

99. Report of the Management Committee of the Australian Therapeutic Trial in the Mangement of Hypertension.: Untreated mild hypertension. lancet, i: 185-191, 1982.

100. Kahn, C.R. & Roth, J.: Cell membrane receptors for polypeptide hormones. Am. J. Clin. Pathol., 63: 656-668, 1975.

101. Staidie, W.C., Haugaard, N. & Vaughn, M.: The quantitative relation between insulin and its biological activity. J. Biol. Chem., 200: 745-751, 1953.

102. Newerly, K. & Berson, S.A.: Lack of specificity of insulin ^{131}I-binding by isolated rat diaphragm (23075). Proc. Soc. Exper. Med., 94: 751-755, 1957.

103. Freychet, P. Roth, J. & Neville, D.M.: Insulin receptors in the liver: specific binding of ^{125}I-insulin to the plasma membrane and its relation to insulin bioactivity. Proc. Natl. Acad. Sci. U.S.A., 68: 1833-1837, 1971.

104. Cuatrecasas, P.: Insulin-receptor interactions in adipose tissue cells: direct measurement and properties. Proc. Natl. Acad. Sci. U.S.A., 68: 1264-1268, 1971.

105. Ginsberg, B.H.: The insulin receptor: properties and regulation. In: Biochemical Actions of Hormones. Litwack, G. (ed.), Academic Press, New York, 1977, pp 313-349.

106. Posner, B.I., Bergeron, J.J.M., Josefsberg, Z., Kahn, M.N., Kahn, R.J., Patel, B.A., Sikstrom, R.A. & Verma, A.K.: Polypeptide hormones: intracellular receptors and internalization. Recent Prog. Horm. Res., 37: 539-582, 1981.

107. Bailey, C.J., Lord, J.M. & Atkins, T.W.: The insulin receptor and diabetes. In: Recent advances in diabetes. Nattrass, M. & Santiago, J.V. (eds.), Churchill Livingstone, Edinburgh, 1984, Part 1, pp 27-44.

108. Grunberger, C., Taylor,, S.I., Dons, R.F. & Gorden, P.: Insulin receptors in normal and disease states. Clinic in Endocrinol. Metabol., 12: 191-219, 1983.

109. Flier, J.S.: Insulin receptors and insulin resistance. Ann. Rev. Med., 34: 145-160, 1983.

110. Blecher, M. & Bar, R.S.: Receptors and Human Disease. Williams and Wilkins, Baltimore, 1981.

111. Pastan, I., Roth, J. & Macchia, V.: Binding of hormone to tissue: The first step in polypeptide hormone action. Proc. Natl. Acad. Sci. U.S.A., 56: 1802-1809, 1966.

112. Kono, R.: Destruction of insulin effector system of adipose tissue cells by proteolytic enzymes. J. Biol. Chem., 244: 1772-1778, 1969.

113. Roth, J. & Grunfeld, C.: Endocrine systems: mechanisms of disease, target cells and receptors. In: Textbook of Endocrinology. Williams, R.H. (ed.), Ed. 6. W.B. Saunders, Philadelphia, 1982, pp 15-72.

114. Cech, J.M., Freeman, R.B. Jr., Caro, J.F. & Amatruda, J.M.: Insulin action and binding in isolated hepatocytes from fasted, streptozotocin-diabetic, and other older, spontaneously obese rats. Biochem. J., 188: 839-845, 1980.

115. Le Marchand-Brustel, Y. & Freychet, P.: Effect of fasting and streptozotocin diabetes and insulin binding and action in the isolated mouse soleus muscle. J. Clin. Invest., 64: 1505-1515, 1979.

116. Harrison, L.C., Billington, T., Clark, S., Nichols, R., East, I. & Martin, F.I.R.: Decreased binding of insulin by receptors on placental membranes from diabetic mothers. J. Clin. Endocrinol. Metab., 44: 206-209, 1977.

117. Beck-Nielsen, H., Pedersen, O., Kragballe, K. & Schwartz-Sørensen, N.: The monocyte as a model for the study of insulin receptors in man. Diabetologia, 13: 563-569, 1977.

118. Gavin, J.R. III., Gorden, P., Roth, J., Archer, J. & Buell, D.N.: Characteristics of the human lymphocyte insulin receptor. J. Biol. Chem., 248: 2202-2207, 1973.

119. Fussganger, R.D., Kahn, C.R., Roth, J. & De Meyts, P.: Binding and degradation of insulin by human peripheral granulocytes. J. Biol. Chem., 251: 2761-2769, 1976.

120. Rechler, M.M. & Podskalny, J.M.: Insulin receptors in cultured fibroblasts. Diabetes, 25: 250-255, 1976.

121. Gambhir, K.K., Archer, J.A. & Bradley, C.J.: Characteristics of human erythrocyte insulin receptors. Diabetes, 27: 701-708, 1978.

122. Goldfine, I.D. & Smith, G.J.: Binding of insulin to isolated nuclei. Proc. Natl. Acad. Sci. U.S.A., 73: 1427-1431, 1976.

123. Bergeron, J.J.M., Posner, B.I., Josefsberg, Z. & Sickstrom, R.: Intracellular polypeptide hormone receptors: the demonstration of specific binding sites for insulin and human growth hormone in Golgi fractions isolated from the liver of female rats. J. Biol. Chem., 253: 4058-4066, 1978.

124. Forgue, M-E. & Freychet, P.: Insulin receptors in the heart muscle: demonstration of specific binding sites and impaired of insulin binding in the plasma membrane of the obese hyperglycaemic mouse. Diabetes, 24: 715-723, 1975.

125. Bolinder, J., Engfeldt, P., Ostman, J. & Arner, P.: Site differences in insulin receptor binding and insulin action in subcutaneous fat of obese females. J. Clin. Endocrinol. Metab., 57: 455-461, 1983.

126. Kaplan, S.A.: The insulin receptor. J. Pediatr., 104: 327-336, 1984.

127. Bhaumick, B., Bara, R.M. & Hollenberg, M.D.: Somatomedin receptor of human placenta: solubilization, photolabeling, partial purification, and comparison with insulin receptor. Proc. Natl. Acad, Sci. U.S.A., 78: 4279-4283, 1981.

128. Bar, R.A., Harrison, L.C., Muggeo, M., Gorden, P., Kahn, C.R. & Roth, J.: Regulation of insulin receptors in normal and abnormal physiology in humans. Advanc. Int. Med., 24: 23-52, 1979.

129. Pedersen, O. & Beck-Nielsen, H.: A study of insulin receptors in human mononuclear leucocytes. Acta Endocrinol., 83: 556-564, 1976.

130. Pedersen, O., Hjøllund, E., Beck-Nielsen, H., Lindskov, H.O., Sonne, O. & Glieman, J.: Insulin receptor binding and receptor-mediated insulin degradation in human adipocytes. Diabetologia, 20: 636-641, 1981.

131. Waelbroeck, M., Van Obberghen, E. & De Meyts, P.: Thermo-dynamics of the interaction of insulin with its receptor. J. Biol. Chem., 254: 7736-7740, 1979.

132. Kahn, C.R., Freychet, P., Roth, J. & Neville, D.M. Jr.: Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J. Biol. Chem., 249: 2249-2257, 1974.

133. Terris, S. & Steiner, D.F.: Binding and degradation of ^{125}I-insulin by rat hepatocytes. J. Biol. Chem., 250: 8389-8398, 1975.

134. Gorden, P., Carpentier, J.L., Freychet, P. & Orci, L.: Internalisation of polypeptide hormone mechanism, intracellular localisation and significance. Diabetologia, 8: 263-274, 1980.

135. Gammeltoft, S. & Gliemann, J.: Binding and degradation of ^{125}I-labelled insulin by isolated rat fat cells. Biochim. Biophys. Acta, 320: 16-32, 1973.

136. Draznin, B., Solomons, C.C., Emler, C.A., Schalch, D.S. & Sussmann, K.E.: Decreased insulin binding and degradation associated with depressed intracellular ATP content. Diabetes, 29: 221-226, 1980.

137. Thomopoulos, P., Kosmakos, F.C., Pastan, I. & Lovelace, R.: Cyclic AMP increases the concentration of insulin receptors in cultured fibroblasts and lymphocytes. Biochem. Biophys. Res. Commun., 75: 246-252, 1977.

138. Gavin, J.R. III., Roth, J., Neville, D.M. Jr., De Meyts, P. & Buell, D.N.: Insulin-dependent regulation of insulin receptor concentrations: a direct demonstration in cell culture. Proc. Natl. Acad. Sci. U.S.A., 71: 84-88, 1974.

139. Jacobs, S., Hazum, E., Shechter, Y. & Cuatrecasas, P.: Insulin receptor: Covalent labeling and identification of subunits. Proc. Natl. Acad. Sci. U.S.A., 10: 4918-4921, 1979.

140. Massague, J., Pilch, P.F. & Czech, M.P.: Electrophoretic resolution of three major insulin receptor structures with unique subunit stoichiometries. Proc. Natl. Acad. Sci. U.S.A., 77: 7137-7141, 1980.

141. Jacobs, S., Hazum, E. & Cuatrecasas, P.: Digestion of insulin receptors with proteolytic and glycosidic enzymes - effects on purified and membrane-associated receptor subunits. Biochem. Biophys. Res. Commun., 94: 1066-1073, 1980.

142. Cherqui, G., Caron, M., Capeau, J. & Picard, J.: Further characterization of the insulin receptor glycosidic moiety in rat adipocytes. Mol. Cell. Endocrinol., 23: 297-310, 1981.

143. Ginsberg, B.H., Kahn, C.R., Roth, J. & De Meyts, P.: Insulin-induced dissociation of its receptor into subunits: possible molecular concomitant of negative cooperativity. Biochem. Biophys. Res. Commun., 73: 1068-1074, 1976.

144. Krupp, M.N. & Livingstone, J.N.: Insulin binding to solubilized material from fat cell membranes: evidence for two binding species. Proc. Natl. Acad. Sci. U.S.A., 75: 2579-2597, 1978.

145. Baron, M.D., Wisher, M.H., Thamm, P.M., Saunders, D.J., Brandenburg, D. & Sonksen, P.H.: Hydrodynamic characterization of the photoaffinity-labeled insulin receptor solubilized in Triton X-100. Biochemistry, 20: 4156-4161, 1981.

146. Czech, M.P., Massague J. & Pilch, P.P.: The insulin receptor: structural features. Trends in Biochemical Sciences, 6: 222-225, 1981.

147. Jacobs, S., Hazum, E. & Cuatrecasas, P.: The subunit structure of rat liver insulin receptor. Antibodies directed against the insulin-binding subunit. J. Biol. Chem., 255: 6937-6940, 1980.

148. Pottick, L.A., Moxley, R.T. III. & Livingstone, J.N.: Tissue differences in insulin receptors. Acute changes in insulin binding characteristics induced by wheat germ agglutinin. Diabetes, 30: 196-202, 1981.

149. Cahill, G.F.: Physiology of insulin in man. Diabetes, 20: 785-799, 1971.

150. Czech, M.P.: Molecular basis of insulin action. Ann. Rev. Biochem., 46: 359-384, 1977.

151. Susuki, K. & Kono, T.: Evidence that insulin causes translocation of glucose transport acitivity to the plasma membrane from an intracellular storage site. Proc. Natl. Acad. Sci. U.S.A., 77: 2542-2545, 1980.

152. Wardzala, L.J., Cushman, S.W. & Salans, L.: Mechanism of insulin action on glucose transport in the isolated rat adipose cell. J. Biol. Chem., 253: 8002-8005, 1978.

153. Czech, M.P.: Insulin action. Am. J. Med., 70: 142-150, 1981.

154. Rodbell, M., Scow, R.O. & Chernick, S.S.: Removal and metabolism of triglycerides by perfused liver. J. Biol. Chem., 239: 385-391, 1964.

155. Kahn, C.R., Baird, K., Flier, J.S. & Jarrett, D.B.: Effects of autoantibodies to the insulin receptor on isolated adipocytes. Studies on insulin binding and insulin action. J. Clin. Invest., 60: 1094-1106, 1977.

156. Gliemann, J. & Whitesell, R.R.: Kinetic aspects of 3-O-methylglucose transport in insulin treated rat adipocytes. Diabetologia, 13: 196, 1977. Abstract.

157. Rechler, M.M., Podskalny, J.M., Goldfine, I.D. & Wells, C.A.: DNA synthesis in human fibroblasts: stimulation by insulin and by insulin-like activity (NS/LA-S). J. Clin. Endocrinol. Metab., 39: 512-521, 1974.

158. Purrello, F., Burnham, D.B. & Goldfine, I.D.: Insulin receptor antiserum and plant lectins mimic the direct effects of insulin on nuclear envelope phosphorylation. Science, 221: 462-464, 1983.

159. Roth, J., Lesniak, M.A., Bar, R.S., Muggeo, M., Megyesi, K., Harrison, L.C., Flier, J.S., Wachslicht-Rodbard, H. & Gordon, P.: An introduction to receptors and receptor disorders. Proc. Soc. Exp. Biol. Med., 162: 3-12, 1979.

160. Denton, R.M., Brownsey, R.W. & Belsham, G.J.: A partial view of the mechanism of insulin action. Diabetologia, 21: 347-362, 1981.

161. Larner, J.: Insulin and glycogen synthase. Diabetes, 21 (Suppl. 2): 428-438, 1972.

162. Larner, J., Galasko, G., Cheng, K., De Paoli-Roach, A.A., Huangh Daggy, P. & Kellogy, J.: Generation by insulin of a chemical mediator that controls protein phosphorylation and dephosphorylation. Science, 206: 1408-1410, 1979.

163. Jarrett, L. & Seals, J.R.: Pyruvate dehydrogenase activation in adipocytes mitochondria by an insulin-generated mediator from muscle. Science, 206: 1406-1408, 1979.

164. Kiechle, F.L., Jarett, L., Popp, D.A. & Kotagal, N.: Isolation from rat adipocytes of a chemical mediator for insulin activation of pyruvate dehydrogenase. Diabetes, 29: 852-855, 1980.

165. Cheng, K., Galasko, G., Huang, L., Kellogy, J. & Larner, J.: Studies on the insulin mediator: separation of two antagonistic biologically active materials from fraction II. Diabetes, 29: 659-661, 1980.

166. Larner, J., Cheng, K., Schwartz, C., Kikuchi, K., Tamura, S., Creacy, S., Dubler, R., Galasko, G., Pullin, C. & Katz, M.: A proteolytic mechanism for the action of insulin via oligopeptide mediator formation. Fed. Proc., 41: 2724-2729, 1982.

167. Larner, J.: Insulin mediator - fact or fancy? J. Cyclic Nucleotide Res., 8: 289-296, 1982.

168. Jarett, L., Kiechle, F.L. & Parker, J.C.: Chemical mediator or mediators of insulin action: response to insulin and mode of action. Fed. Proc., 41: 2736-2741, 1982.

169. Karnieli, E., Zarnowski, M.J., Hissin, P.J., Simpson, I.A., Salans, L.B. & Cushman, S.W.: Insulin stimulated translocation of glucose transport systems in the isolated rat adipose cell. J. Biol. Chem., 256: 4772-4777, 1981.

170. Levin, R.: Insulin: The effects and mode of action of the hormone. Vitams. Horm., 39: 145-173, 1982.

171. Czech, M.P.: Insulin action and the regulation of hexose transport. Diabetes, 29: 399-409, 1980.

172. Seals, J.R. & Jarett, L.: Activation of pyruvate dehydrogenase by direct addition of insulin to an isolated plasma membrane-mitochondrial mixture: evidence for generation of insulin's second messenger in a subcellular system. Proc. Natl. Acad. Sci. U.S.A., 77: 77-81, 1981.

173. Seals, J.R. & Czech, M.P.: Evidence that insulin activates an intrinsic plasma membrane protease in generation of a secondary chemical mediator. J. Biol. Chem., 255: 6529-6531, 1980.

174. Seals, J.R. & Czech, M.P.: Production by plasma membranes of chemical mediator of insulin action. Fed. Proc., 41: 2730-2735, 1982.

175. Kasuga, M., Karlsson, F.A. & Khan, C.R.: Insulin stimulates the phosphorylation of the 95,000-Dalton subunit of its own receptor. Science, 215: 185-187, 1982.

176. Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M. & Kahn, C.R.: Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell free system. Nature, 298: 667-669, 1982.

177. Roth, R.A. & Cassell, D.J.: Insulin receptor: evidence that it is a protein kinase. Science, 219: 299-301, 1983.

178. Shia, M.A.& Pilch, P.F.: The B subunit of the insulin receptor is an insulin-activated protein kinase. Biochemistry, 22: 717-721, 1983.

179. Fain, J.N.: Insulin secretion and action. Metabolism, 33: 672-679, 1984.

180. Kahn, C.R.: Insulin resistance, insulin insensitivity and insulin unresponsiveness: a necessary distinction. Metabolism, 27 (Suppl. 2): 1893-1902, 1978.

181. Orci, L., Rufener, C., Malaisse-Lagae, F., Blondel, B., Amherdt, M., Bataille, D., Freychet, P. & Perrelet, A.: A morphological approach to surface receptors in islet and liver cells. Isr. J. Med. Sci., 11: 639-655, 1975.

182. Carpentier, J.L., Van Obberghen, E., Gorden, P. & Orci, L.: Surface redistribution of ^{125}I-insulin in cultured human lymphocytes. Diabetologia, 19: 263, 1980. Abstract.

183. Whittacker, J., Hammond, V.A. & Al-Attas, O.: Insulin interactions with hepatocytes: evidence for ligand stimulated acceleration of pinocytes and receptor recycling. Diabetologia, 21: 343, 1981. Abstract.

184. Krupp, M.N. & Lane, M.D.: Evidence to different pathways for the degradation of insulin and insulin receptors in the chick liver cell. J. Biol. Chem., 257: 1372-1377, 1982.

185. Sonne, O., Linde, S. & Cliemann, J.: Receptor-mediated degradation of insulin in isolated rat adipocytes: evidence for a degradation product of molecular size similar to insulin. Diabetologia, 23: 201, 1982. Abstract.

186. Poole, G.P., O'Conner, K.J., Lazarus, N.R. & Pogson, C.I.: ^{125}I-labelled insulin degradation by isolated rat hepatocytes: the roles of glutathion-insulin transhydrogenase and insulin specific protease. Diabetologia, 23: 49-53, 1982.

187. Sonne, O. & Gliemann, J.: Insulin receptors of cultured human lymphocytes (IM-9). Lack of receptor-mediated degradation. J. Biol. Chem., 255: 7449-7454, 1980.

188. Brush, J.S. & Nascimento, C.G.: Studies on the properties of the insulin protease of rat liver. Biochim. Biophys. Acta, 704: 398-402, 1982.

189. Yokono, K., Imamura, Y., Shii, K., Sakai, H. & Baba, S.: Purification and characterisation of insulin-degrading enzyme from pig skeletal muscle. Endocrinology, 108: 1527-1532, 1981.

190. Bellomo, G., Bellazzi, R., Nicotera, P.L., Francesconi, R. & Fratino, P.: Binding and degradation of ^{125}I-insulin in human erythrocytes. Comparative studies with hemolysate and membranes. J. Endocrinol. Invest., 5: 287-291, 1982.

191. Neal, G.W. & Kitabchi, A.E.: Insulin degradation by human skeletal muscle. Biochim. Biophys. Acta, 719: 259-266, 1982.

192. Beck-Nielsen, H. & Pedersen, O.: Insulin binding, insulin degradation and glucose metabolism in human monocytes. Diabetologia, 17: 77-84, 1979.

193. Hedo, J.A., Kahn, C.R., Hayashi, M. & Yasuda, K.M.: Biosynthesis and glycosylation of the insulin receptor-evidence for a single polypeptide precursor of the 2 major subunits. J. Biol. Chem., 258: 20-26, 1983.

194. Eckel, J. & Reinauer, H.: Involvement of microtubules in insulin-receptor turnover in isolated cardiocytes. Diabetologia, 23: 165, 1982. Abstract.

195. Carpentier, J-L., Fehlmann, M., Van Obberghen, E., Gorden, P. & Orci, L.: Morphological aspects and physiological relevance of insulin receptor internalization and recycling. In: Endocrinology. Labrie, F. & Proulx, L. (eds.), Elsevier Science Publishers, B.V., 1984, pp 349-353.

196. Bar, R.S., Gorden, P., Roth, J., Kahn, C.R. & De Meyts, P.: Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients. J. Clin. Invest., 58: 1123-1135, 1976.

197. Robinson, J.R. & Chefurka, W.: Continuous measurment of $C^{14}O_2$ respired by insects. An ionization chamber method. Anal. Biochem., 9: 197-203, 1964.

198. Krupp, M. & Lane, M.D.: On the mechanism of ligand-induced down regulation of insulin receptor level in the liver cell. J. Biol. Chem., 256: 1689-1694, 1981.

199. Marshall, S. & Olefsky, J.M.: Characterisation of insulin-induced receptor loss and evidence for internalization of the insulin receptor. Diabetes, 30: 746-753, 1981.

200. Caro, J.F. & Amatruda, J.M.: Insulin receptors in hepatocytes: postreceptor events mediate down regulation. Science, 210: 1029-1030, 1980.

201. Kono, T. & Barham, F.W.: The relationship between the insulin binding capacity of fat cells and the cellular response to insulin. Studies with intact and trypsin-treated fat cells. J. Biol. Chem., 246: 6210-6216, 1971.

202. Olefsky, J.M.: Effect of dexamethasone on insulin binding, glucose transport, and glucose oxidation of isolated rat adipocytes. J. Clin. Invest., 56: 1499-1508, 1975.

203. Gliemann, J., Gammeltoft, S. & Vinten, J.: Time course of insulin-receptor binding and insulin-induced lipogenesis in isolated rat fat cells. J. Biol. Chem., 250: 3368-3374, 1975.

204. Frank, H.J.L., Davidson, M.B. & Serbin, P.A.: Insulin binding and action in isolated rat hepatocytes: evidence for spare receptors. Metabolism, 30: 1159-1164, 1981.

205. Soll, A.H., Goldfine, I.D., Roth, J., Kahn, C.R. & Neville, D.M.: Thymic lymphocytes in obese (ob/ob) mice: a mirror of the insulin receptor defect in liver and fat. J. Biol. Chem., 249: 4127-4131, 1974.

206. Olefsky, J.M., Johnson, J., Lin, F., Jen, P. & Reaven, G.M.: The effects of acute and chronic dexamethasone administration on insulin binding to isolated rat hepatocytes and adipocytes. Metabolism, 24: 517-527, 1975.

207. Freychet, P.: Interactions of polypeptide hormones with cell membrane specific receptors: studies with insulin and glucagon. Diabetologia, 12: 83-100, 1976.

208. Le Marchand-Brustel, Y., Jeanrenaud, B. & Freychet, P.: Insulin binding and effects in isolated soleus muscle of lean and obese mice. Am. J. Physiol., 234: E348-E358, 1978.

209. Schwartz, R.H., Bianco, A.R., Handwerger, B.S. & Kahn, C.R.: Demonstration that monocytes rather than lymphocytes are the insulin-binding cells in preparations of human peripheral blood mononuclear leukocytes: implications for studies of insulin-resistance states in man. Proc. Natl. Acad. Sci. U.S.A., 72: 474-478, 1975.

210. Gambhir, K.K., Archer, J.A. & Carter, L.: Insulin radioreceptor assay for human erythrocytes. Clin. Chem., 23: 1590-1595, 1977.

211. Dons, R.F., Ryan, J., Gorden, P. & Wachslicht-Rodbard, N.: Erythrocyte and monocyte insulin binding in man. A comparative analysis in normal and disease states. Diabetes, 30: 896-902, 1981.

212. Lippe, B.M., Kaplan, S.A., Golden, M.P., Hendricks, S.A. & Scott, M.L.: Carbohydrate tolerance and insulin receptor binding in children with hypopituitarism: responses after acute and chronic growth hormone administration. J. Clin. Endocrinol. Metab., 53: 507-513, 1981.

213. Eng, J., Lee, L. & Yalow, R.S.: Influence of the age of erythrocytes on their insulin receptors. Diabetes, 29: 164-166, 1980.

214. Kosmakos, F.C., Nagulesparan, M. & Bennett, P.H.: Insulin binding to erythrocytes: a negative correlation with red cell age. J. Clin. Endocrinol. Metab., 51: 46-50, 1980.

215. Dons, R.F., Corash, L.M. & Gorden, P.: The insulin receptor is an age-dependent integral component of the human erythrocyte membrane. J. Biol. Chem., 256: 2982-2987,1981.

216. Pedersen, O., Hjøllund, E. & Lindskov, H.O. Insulin binding and action in fat cells from young healthy females and males. Am. J. Physiol., 243: E158-E167, 1982.

217. Pedersen, O., Beck-Nielsen, H. & Heding, L.: Increased insulin receptors after exercise in patients with insulin dependent diabetes mellitus. N. Engl. J. Med., 302: 886-892, 1980.

218. Yalow, R.S. & Berson, S.A.: Immunoassay of endogenous plasma insulin in man. J. Clin. Invest., 39: 1157-1175, 1960.

219. Olefsky, J.M., Jen, P. & Reaven, G.M.: Insulin binding to isolated human adipocytes. Diabetes, 23: 565-571, 1974.

220. De Meyts, P.: Insulin and growth hormone receptors in human cultured lymphocytes and peripheral blood monocytes. In: Methods in Receptor Research. Blecher, M. (ed.), Marcel Dekker, New York, Part 1, 1976, pp 301-383.

221. Kolterman, O.G., Gray, R.S., Griffin, J., Burstein, P., Insel, J., Scarlett, J.A. & Olefsky, J.M.: Receptor and postreceptor defects contribute to the insulin resistance in non-insulin dependent diabetes mellitus. J. Clin. Invest., 68: 957-969, 1981.

222. Hollenberg, M.D. & Cuatrecasas, P.: Methods for the biochemical identification of insulin receptors. In: Methods in Receptor Research. Blecher, M. (ed.), Marcel Dekker, New York, Part 2, 1976, pp 429-477.

223. Henquin, J.C., Charles, S., Henquin, M., Mathot, F. & Tamagawa, T.: Diazoxide and D600 inhibition of insulin release. Diabetes, 31: 776-783, 1982.

224. Guldberg, C.M. & Wagge, P.: The Law of Mass Action. A centenary volume: 1864-1964, Det Norske Videnskans, Akademi I Oslow, 1964,pp 7.

225. Munson, P.J. & Rodbard, D.: Number of receptor sites from Scatchard and Klotz graphs: a constructive critique. Science, 220: 979-981, 1983.

226. Scatchard, G., Coleman, J.S. & Shen, A.L.: Physical chemistry of protein solutions - VII The binding of some small anions to serum albumin. J. Am. Chem. Soc., 79: 12-20, 1957.

227. Weder, H.G., Schildknecht, J., Lutz, R.A. & Kesselring, P.: Determination of binding parameters from Scatchard plots. Theoretical and practical considerations. Eur. J. Biochem., 42: 475-481, 1974.

228. Pollet, R.J., Standaert,M.L. & Haase, B.A.: Insulin binding to the human lymphocyte receptor. J. Biol. Chem., 252: 5828-5834, 1977.

229. De Meyts, P., Bianco, A.R. & Roth, J.: Site-site interactions among insulin receptors. Characterisation of negative cooperativity. J. Biol. Chem., 251: 1877-1888, 1976.

230. De Meyts, P. & Roth, J.: Cooperativity in ligand binding: a new graphic analysis. Biochem, Biophys. Res. Commun., 66: 1118-1126, 1975.

231. De Meyts, P. Van Obberghen, E., Roth, J., Wollmer, A. & Brandenburg, D.: Mapping of the residues in the receptors binding region of insulin responsible for negative cooperativity. Nature, 273: 504-508, 1978.

232. Herzberg, V., Boughter, J.M., Carlisle, S. & Hill, D.E.: Evidence for two receptor populations on human erythrocytes. Nature, 286: 279-281, 1980.

233. Donner, D.B.: Regulation of insulin binding to isolated hepatocytes: correction for bound hormone fragments linearizes Scatchard plots. Proc. Natl. Acad. Sci. U.S.A., 77: 3176-3180, 1980.

234. Levitzki, A.: Negative cooperativity in clustered receptors as a possible basis for membrane action. J. Theor. Biol., 44: 367-372, 1974.

235. Levitzki, A.: Negative cooperativity at the insulin receptor. Nature, 289: 442-443, 1981.

236. Jacobs, S. & Cuatrecasas, P.: The mobile receptor hypothesis and "cooperativity" of hormone binding application to insulin. Biochim. Biophys. Acta., 433: 482-495, 1976.

237. Kahn, C.R.: Membrane receptors for hormones and neurotransmitters. J. Cell. Biol., 70: 261-286, 1976.

238. Jarrett, L. & Smith, R.M.: Ultrastructural localization of insulin receptors on adipocytes. Proc. Natl. Acad. Sci. U.S.A., 72: 3526-3530, 1975.

239. Beck-Nielsen, H. & Pedersen, O.: Diurnal variation in insulin binding to human monocytes. J. Clin. Endocrinol. Metab., 47: 385-390, 1978.

240. Misbin, R.I.: Dietary regulation of insulin receptors in obesity. J. Nutr., 111: 475-479, 1981.

241. Grundleger, M.L. & Thenen, S.W.: Decreased insulin binding, glucose transport, and glucose metabolism in soleus muscle of rats fed a high fat diet. Diabetes, 31: 232-237, 1982.

242. Koivisto, V., Soman, V., Conrad, P., Hendler, R., Nadel, E. & Felig, P.: Insulin binding to monocytes in trained athletes: changes in the resting state and after exercise. J. Clin. Invest., 64: 1011-1015, 1979.

243. Hendricks, S.A., Lippe, B.M., Kaplan, S.A., Landaw, E.M., Hertz, D. & Scott, M.: Insulin binding to erythrocytes of normal infants, children and adults: variation with age and sex. J. Clin. Endocrinol. Metab., 52: 969-974, 1981.

244. Puavilai, G., Drobny, E.C., Comont, L.A. & Baumann, G.: Insulin receptors and insulin resistance in human pregnancy: evidence for a postreceptor defect in insulin action. J. Clin. Endocrinol. Metab., 54: 247-253, 1982.

245. Bertoli, K., De Pirro, R., Fusco, A., Greco, A.V., Magnatta, R. & Lauro, R.: Differences in insulin receptors between men and menstruating women and influence of sex hormones on insulin binding during the menstrual cycle. J. Clin. Endocrinol. Metab., 50: 246-250, 1980.

246. Arner, P., Engfeldt, P. & Lithell, H.: Site differences in the basal metabolism of subcutaneous fat in obese women. J. Clin. Endocrinol. Metab., 53: 948-952, 1981.

247. Bolinder, J., Kager, L., Östman, J. & Arner, P.: Differences at the receptor and postreceptor levels between human omental and subcutaneous adipose tissue in the action of insulin on lipolysis. Diabetes, 32: 117-123, 1983.

248. Ginsberg, H., Kimmerling, G., Olefsky, J.M. & Reaven, G.M.: Demonstration of insulin resistance in untreated adult onset diabetic subjects with fasting hyperinsulinaemia. J. Clin. Invest., 55: 454-461, 1975.

249. Reaven, G.M., Berstein, R., Davis, B. & Olefsky, J.M.: Nonketotic diabetes mellitus: insulin deficiency or insulin resistance? Am. J. Med., 60: 80-88, 1976.

250. Olefsky, J.M. & Reaven, G.M.: Insulin binding in diabetes. Relationships with plasma insulin levels and insulin sensitivity. Diabetes, 26: 680-688, 1977.

251. Kolterman, O.G., Gray, R.S., Griffin, J., Burnstein, P., Insel, J., Scarlett, J.A. & Olefsky, J.M.: Receptor and postreceptor defects contribute to the insulin resistance in non-insulin dependent diabetes mellitus. J. Clin. Invest., 68: 957-969, 1981.

252. Grundleger, M.L., Godbole, V.Y. & Thenen, S.W.: Age-dependent development of insulin resistance of soleus-muscle in genetically obese (ob/ob) mice. Am. J. Physiol., 239: E363-E371, 1980.

253. Baxter, D. & Lazarus, N.R.: The control of insulin receptors in the New Zealand obese mouse. Diabetologia, 11: 261-267, 1975.

254. Kern, P., Picard, J., Caron, M. & Veissiere, D.: Decreased binding of insulin to liver plasma membrane receptors in hereditary diabetic mice. Biochim. Biophys. Acta., 389: 281-289, 1975.

255. Broer, Y., Freychet, P. & Rosselin, G.: Insulin and glucagon-receptor interactions in the genetically obese Zucker rat: studies of hormone binding and glucagon stimulated cyclic AMP levels in isolated hepatocytes. Endocrinology, 101: 236-249, 1977.

256. Olczak, S.A., Greenwood, R.H. & Hales, C.N.: Post-receptor insulin resistance after diazoxide in non-insulin-dependent diabetes. Horm. Metab. Res., In press.

257. Pedersen, O.: Studies of insulin receptor binding and insulin action in humans. Danish Med. Bull., 31: 1-32, 1984.

258. Gliemann, J., Laursen, A.L., Foley, J.E. & Sonne, O.: The insulin receptors: looking at the present. In: Current Views on Insulin Receptors. Andreani, D., De Pirro, R., Lauro, R., Olefsky, J.M. & Roth, J. (eds.), Academic Press, 1981, pp 4-12.

259. Chesworth, J.M.: Radioimmunoassays of ovine LH and ovine prolactin using polymerized second antisera. Anal. Biochem., 80: 31-40, 1977.

260. Covelli, I. & Wolfe, J.: Iodohistidine formation in ribonuclease A. J. Biol. Chem., 241: 4444-4451, 1966.

261. Koshland, M.E., Englberger, F.M. & Gaddone, S.M.: Identification of tyrosine at the active site of anti-p-azobenzenearsonic acid antibody. J. Biol. Chem., 238: 1349-1352, 1963.

262. Jovanovic, V., Nemoda, D. & Lwin, K.: An effective electrolytic iodination of human serum albumin at alkaline pH. Int. J. Appl. Rad. Isotop., 23: 242-244, 1972.

263. Samols, E. & Williams, H.S.: Trace-labelling of insulin with iodine. Nature, 109: 1211-1212, 1961.

264. Redshaw, M.R. & Lynch, S.S.: An improved method for the preparation of iodinated antigens for radio-immunoassay. J. Endrocrinol., 60: 527-528, 1974.

265. Pennisi, F. & Rosa, U.: Preparation for radioiodinated insulin by constant current electrolysis. J. Nucl. Biol. Med., 13: 64-70, 1969.

266. Marchalonis, J.J.: An enzymatic method for the trace iodination of immunoglobulins and other proteins. Biochem. J., 113: 299-305, 1969.

267. Scatchard, G.: The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci., 51: 660-672, 1949.

268. Gliemann, J., Sonne, O., Linde, S. & Hansen, B.: Biological potency and binding affinity of monoiodinsulin with iodine in tyrosine A14 or tyrosine A19. Biochem. Biophys. Res. Commun., 87: 1183-1190, 1979.

269. Linde, S., Hansen, B., Sonne, O., Holst, J.J. & Gliemann, J.: Tyrosine A14 ^{125}I monoiodoinsulin: preparation, biological properties and long term stability. Diabetes, 30: 1-8, 1981.

270. Jorgensen, K.H. & Larsen, U.D.: Homogeneous mono-^{125}I-insulins. Preparation and characterization of mono-^{125}I-(tyr A14)- and mono-^{125}I-(tyr A19)-insulin. Diabetologia, 19: 546-554, 1980.

271. Sodoyez, J.C., Sodoyez-Goffaux, F., Goff, M.M., Zimmerman, A.E. & Arquilla, E.R.: ^{127}I or carrier-free ^{125}I monoiodoinsulin preparation: physiological, immunological and biological propoerties, and susceptibility to "insulinase" degradation. J. Biol. Chem., 250: 4268-4277, 1975.

272. Seon, B.K., Roholt, O.A. & Pressman, D.: Environments of individual tyrosines in protein. Differences in rates of mono- and diiodination. Biochim. Biophys. Acta., 221: 114-118, 1970.

273. Freedlender, A. & Cathou, R.E.: Iodination. In: Radioimmunoassay Methods. Kirkham, K.E. & Hunter, M.W. (eds.), Churchill Livingstone, Edinburgh, 1971, pp 94.

274. Freychet, P. Roth, J. & Neville, D.M.Jr.: Monoiodoinsulin: demonstration of its biological activity and binding to fat cells and liver membranes. Biochem. Biophys. Res. Comm., 43: 400-408, 1971.

275. Pullen, R.A., Lindsay, D.G., Woods, P. Tickle, I.J., Blundell, T.L., Wollmer, A., Kiail, G., Brandenberg, D., Zahn, H., Gliemann, J. & Gammeltoft, S.: Receptor-binding region of insulin. Nature, 259: 369-373, 1976.

276. Podlecki, D.A., Frank, N., Kao, M., Horikoshi, G., Freidenberg, G., Marshall, S., Ciaraldi, T. & Olefsky, J.: Characterization of the receptor binding properties of monoiodoinsulin isomers and the identification of different insulin receptor specificities in hepatic and extra-hepatic tissues. Diabetes, 32: 697-704, 1983.

277. Stentz, F.B., Wright, F.B., Wright, R.K. & Kitabchi, A.E.: A rapid means of separation A14 ^{125}I-insulin from heterogeneously labelled insulin molecules for biological studies. Diabetes, 31: 1128-1131, 1982.

278. Hansen, B., Linde, S. & Lernmark, A.: Preparation of iodinated peptide hormone tracers by polyacrylamide gel electrophoresis. In: Current Views on Insulin Receptors. Andreani, D., De Pirro, R., Lauro, R., Olefsky, J.M. & Roth, J. (eds.), Academic Press, New York. 1981, pp 115-121.

279. Hunter, W.M. & Greenwood, F.C.: Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature, 194: 495-496, 1962.

280. Thompson, E.E., Freychet, P. & Roth, J.: Monoiodo-oxytocin: demonstration of its biological activity and specific binding to isolated fat cells. Endocrinology, 91: 1199-1205, 1972.

281. Freychet, P., Kahn, R., Roth, J. & Neville, D.M.Jr.: Insulin interactions with liver plasma membranes. Independence of binding of the hormone and its degradation. J. Biol. Chem., 247: 3953-3961, 1972.

282. Hammond, J.M., Jarett, L., Mariz, I.K. & Daughaday, W.H.: Heterogenity of insulin receptors on fat cell membranes. Biochem. Biophys. Res. Commun., 49: 1122-1128, 1972.

283. Lesniak, M.A., Roth, J., Gorden, P. & Gavin, J.R. III: Human growth hormone radioreceptor study using cultured human lymphocytes. Nature, 241: 20-22, 1973.

284. Hunter, W.M.: Preparation and assessment of radioactive tracers. Br. Med. Bulletin, 30: 18-23, 1974.

285. Greenwood, F.C. & Hunter, W.M.: Preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J., 89: 114-123, 1963.

286. Ganguli, P.C. & Hunter, W.M.: Iodination of gastrin. In: Radioimmunoassay Method. Kirkham, K.M. & Hunter, W.M. (eds.), Churchill Livingstone, Edinburgh, 1971, pp 54.

287. Yalow, R.S.: Radioimmunologic methods to problems in insulin antigenicity and hormonal assay. Acta Med. Scan. Suppl., 601:149-196, 1976.

288. Izzo, J.L., Roncone, A., Izzo, M.J. & Bale, W.F.: Relationship between degree of iodination of insulin and its biological electrophoretic and immunological properties. J. Biol. Chem., 239: 3749-3754, 1964.

289. Garratt, C.J., Harrison, D.M. & Wicks, M.: The effect of iodination of particular tyrosine residues on the hormonal activity of insulin. Biochem. J., 126: 123-131, 1972.

290. Brunfeld, K., Hanson, B.A. & Jorgensen, K.R.: The immunological reactivity and biological activity of iodinated insulin. Acta Endocrinol. (Copenh.), 57: 307-329, 1968.

291. Lee, C.Y. & Ryan, R.J.: The uptake of human luteinizing hormone (hLH) by slices of luteinized rat ovaries. Endocrinology, 89: 1515-1523, 1971.

292. Low, A.G., Rainbird, A.L. & Gurr, M.I.: Animal models for studying the effect of fibre on gastrointestinal function. J. Plant Foods, 4: 29-32, 1982.

293. Sonksen, P.H. & Refetoft, S.: Iodination. In: Radioimmunoassay Methods. Kirkham, K.E. & Hunter, W.M. (eds.), Churchill Livingstone, Edinburgh, 1971, pp 89.

294. Gavin, J.R., Roth, J., Jen, P. & Freychet, P.: Insulin receptors in human circulating cells and fibroblasts. Proc. Natl. Acad. Sci. U.S.A., 69: 747-751, 1972.

295. Berson, S.A. & Yalow, R.S.: Radioimmunoassay. In: Methods in Investigative and Diagnostic Endocrinology. Vol. 2a. Peptide Hormones, Part 1, General Methodology. Berson, S.A. & Yalow, R.S. (eds.), North Holland Publishing Company, Amsterdam and London, 1973, pp 84-120.

296. Martinnen, A., Koivula, T., Jokela, H., Lehtinen, M. & Pasternack, A.: Quality control of insulin radioreceptor assay for human erythrocytes. Effect of ageing of mono-^{125}I-Tyr-A14-insulin preparation. Scand. J. Clin. Lab. Invest., 44: 401-408, 1984.

297. Steck, T.L.: The organisation of proteins in human red cell membrane. J. Cell. Biol., 62: 1-19, 1974.

298. Boyum, A.: Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Invest., 21: (Suppl. 97): 77-89, 1968.

299. Moore, G.E., Ito, E., Ulrich, K. & Sanderberg, A.A.: Culture of human leukemia cells. Cancer, 19: 713-723, 1966.

300. Pedersen, O., Beck-Nielsen, H., Sørensen, N.S. & Svejgaard, A.: Heterogeneity of insulin receptors in patients with untreated insulin-dependent diabetes mellitus. J. Clin. Endocrinol. Metab., 55: 30-39, 1982.

301. Anderson, O., Gliemann, J. & Gammeltoft, S.: Receptor binding and biological effect of insulin in human adipocytes. Diabetologia, 13: 589-593, 1977.

302. Olefsky, J.M. & Reaven, G.M.: Effects of age and obesity on insulin binding to isolated adipocytes. Endocrinology, 96: 1486-1496, 1975.

303. Rodbell, M.: Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J. Biol. Chem., 239: 375-380, 1964.

304. Gliemann, J. & Sonne, O.: Binding and receptor mediated degradation of insulin in adipocytes. J. Biol. Chem., 253: 7857-7863, 1978.

305. Lorch, E. & Rentsch, G.: A simple method for staining and counting isolated adipose tissue fat cells. Diabetologia, 5: 356-357, 1969.

306. Ginsberg, B.H.: The insulin receptor: properties and regulation. In: Biochemical Actions of Hormones. Litwack, G. (ed.), Academic Press, New York, Vol. 4, 1977, pp 313-349.

307. Insel, J.R., Kolterman, O.G., Saekow, M. & Olefsky, J.M.: Short-term regulation of insulin receptor affinity in man. Diabetes, 29: 132-139, 1980.

308. Dawson, R.M.C., Elliott, D.C., Elliott, W.H. & Jones, K.M.: Data for Biochemical Research. Oxford University Press, 1959, pp 208.

309. Olefsky, J.M., Marshall, S., Berhanu, P., Saekow, M., Heidenreich, K. & Green, A.: Internalization and intracelluar processing of insulin and insulin receptors in adipocytes. Metabolism, 31: 670-690, 1982.

310. Gliemann, J., Qsterlind, K., Vinten, J. & Gammeltoft, S.: A procedure for measurement of distribution spaces in isolated fat cells. Biochim. Biophys. Acta., 286: 1-9, 1972.

311. Klotz, I.M. & Hunston, D.L.: Properties of graphical representations of multiple classes of binding sites. Biochemistry, 10: 3065-3069, 1971.

312. De Meyts, P., Roth, J., Neville, D.M., Gavin, J.R. & Lesniak, M.A.: Insulin interactions with receptor: experimental evidence for negative cooperativity. Biochem. Biophys. Res. Commun., 54: 154-161, 1973.

313. Schwartz, S.E. & Levine, G.D.: Effects of dietary fibre on intestinal glucose absorption and glucose tolerance in rats. Gastroenterology, 79: 833-836, 1980.

314. Gu, J.L. & De Meyts, P.: Negative cooperativity induced during the slow phase of ^{125}I-insulin degradation is identical to that induced at time zero of dissociation. Diabetologia, 23: 171, 1982. Abstract.

315. Rodbard, D.: Mathematics of hormone-receptor interaction. I. Basic principles. In: Receptors for Reproductive Hormones. O'Malley, B.W. & Means, A.R. (eds.), Plenum Press, New York, London, 1973, pp 289-326.

316. Berson, S.A. & Yallow, R.s.: Ethanol fractionation of plasma and electrophoretic identification of insulin binding antibody. J. Clin. Invest., 36: 642-647, 1957.

317. Hales, C.N. & Randle, P.J.: Immunoassay of insulin with insulin antibody precipitate. Biochem. J., 88: 137-146, 1963.

318. Stevens, J.F.: Determination of glucose by an automatic analyser. Clin. Chem. Acta., 32: 199-201, 1971.

319. Vallee, B.L. & Thiers, R.E.: Flame Photometry in Treatise on Analytical Chemistry. Vol. 6. Kolthoff, I.M. & Elving, P.J. (eds.), Interscience, New York, 1965, pp 3463.

320. Gochman, N. & Schmitz, J.M.: Automated determination of uric acid, with use of a uricase-peroxidase system. Clin. Chem., 17: 1154-1159, 1971.

321. Wybenga. D.R., Di Giorgio, J. & Pileggi, V.J.: Manual and automated methods for urea nitrogen measurement in whole serum. Clin. Chem., 17: 891-895, 1971.

322. Deeg, R. & Ziegenhorn, J.: Kinetic enzymatic method for automated determination of serum total cholesterol [CH]. Clin. Chem., 28: 1574, 1982. Abstract.

323. Esders, T.W. & Michrina, C.A.: Purification and properties of L-x-Glycerophosphate oxidase from streptococcus faecium ATCC 12755. J. Biol. Chem., 254: 2710-2713, 1979.

324. Mallia, A.K., Hermanson, G.T., Krohn, R.I., Fujimoto, E.K. & Smith, P.K.: Preparation of a boronic acid affinity support for separation and quantitation of glycosylated hemoglobins. Anal. Lett., 14: 649-661, 1981.

325. Dacie, J.V. & Lewis, S.M.: Practical Haemotology. Churchill Livingstone, Edinburgh, London, Maelbourne and New York, 1985, pp 22-49.

326. Anderson, J.W. & Herman, R.H.: Effect of fasting, caloric restriction and refeeding on glucose tolerance of normal man. Am. J. Clin. Nutr., 25: 41-52, 1972.

327. Muggeo, M., Bar, R.S. & Roth, J.: Change in affinity of insulin receptors following oral glucose in normal adults. J. Clin. Endocrinol. Metab., 44: 1206-1209, 1977.

328. Bhathena, S.J., Voyles, N.R., Joshi, B., Engel, W.K., Smith, S.S. & Recant, L.: Acute changes in human erythrocyte insulin receptors. Horm. Metab. Res., 13: 179-180, 1981.

329. McElduff, A. & Eastman, C.J.: The erythrocyte insulin receptor response to either feeding or a short fast. Horm. Metab. Res., 16: 275-278, 1984.

330. Pedersen, O., Hjøllund, E. & Sørensen, N.S.: Insulin receptor binding and insulin action in human fat cells: effects of obesity and fasting. Metabolism, 31: 884-895, 1982.

331. Spanheimer, R.G., Bar, R.S., Ginsberg, B.H., Peacock, M.L. & Martino, I.: Comparison of insulin binding to cells of fed and fasted obese patients: results in erythrocytes and monocytes. J. Clin. Endocrinol. Metab., 54: 40-47, 1982.

332. De Fronzo, R.A., Soman, V., Sherwin, R.S., Hendler, R. & Felig, P.: Insulin binding to monocytes and insulin action in human obesity, starvation, and refeeding. J. Clin. Invest., 62: 204-213, 1978.

333. Reckless, J.P.D.: Fibre in the diet of the diabetic and its effects on blood lipids. In: Dietary Fibre in the Management of the Diabetic: Proceedings of the British Diabetic Association Symposium held in London, Medical Education Services Ltd., Oxford, 1984, pp 15-20.

334. Kolterman, O.G., Saekow, M. & Olefsky, M.M.: The effectsof acute and chronic starvation on insulin binding to isolated human adipocytes. J. Clin. Endocrinol. Metab., 48: 836-842, 1979.

335. Olefsky, J.M. & Kobayashi, M.: Mechanisms of the fasting-induced increase in insulin binding to rat adipocytes. J. Clin. Invest., 61: 329-338,1978.

336. Almira, E.C. & Reddy, W.J.: Effect of fasting on insulin binding to hepatocytes and liver plasma membranes from rats. Endocrinology, 104: 205-211, 1979.

337. Freychet, P., Laudat, M.H., Laudat, P., Rosselin, G., Kah, C.R., Gorden, P. & Roth, J.: Impairment of insulin binding to fat cell plasma membrane in the obese hyperglycaemic mouse. F.E.B.S. Letts., 25: 339-342, 1972.

338. Olefsky, J.M.: Effect of fasting on insulin binding, glucose transport, and glucose oxidation in ioslated rat adipocytes. Relationships between insulin receptors and insulin action. J. Clin. Invest., 58: 1450-1460, 1976.

339. Soll, A.H., Kahn, R., Neville, D.M.Jr. & Roth, J.: Insulin receptor deficiency in genetic and acquired obesity. J. Clin. Invest., 56: 769-780, 1975.

340. Kappy, M.S. & Plotnick, L.: Studies of insulin binding in children using human erythrocytes in small amounts of blood. Diabetes, 28: 1001-1005, 1979.

341. Parijs, J., Joossens, J.V., Van Der Linden, L., Verstreken, C. & Amery, A.K.P.C.: Moderate sodium restriction and diuretics in the treatment of hypertension. Am. Heart J., 85: 22-34, 1973.

342. Bengtsson, C., Johnsson, G., Sannerstedt, R. & Werkö, L.: Effect of different doses of chlorthalidone on blood pressure, serum potassium and serum urate. Br. Med. J., 1: 197-199, 1975.

343. Kincaid-Smith, P.S.: The selection of drugs in the treatment of hypertension. In: Hypertension: Mechanisms and Management. Kincaid-Smith, P.S. & Whitworth, J.A. (eds.), ADIS Health Science Press, New York, 1982, pp 78-87.

344. Healy, J.J., McKenna, T.J., Canning, B., Brienst, J., Duffy, G.J. & Muldowney, F.P.: Body composition changes in hyper-tensive subjects on long-term oral diuretic therapy. Br. Med. J., 1: 716-719, 1970.

345. Begnbol, B., Dorph, S. & Marner, T.: The effect of different diuretics on elevated blood pressure and serum potassium. Acta. Med. Scand., 193: 407-410, 1973.

346. Pressel, S., Langford, H.G., Blanfox, M.D. & Oberman, A.: Two types of response to thiazide type diuretics - lowering potassium and elevating creatinine. Clin. Res., 31: 844A, 1983. Abstract.

347. Report of Medical Research Council Working Party on Mild to Moderate Hypertension: Adverse reactions to bendrofluazide and propranolol for the treatment of mild hypertension. Lancet, ii: 539-543, 1981.

348. Kohner, E.M., Dollery, C.t., Lowy, C. & Schumer, B.: Effect of diuretic therapy on glucose tolerance in hypertensive patients. Lancet, i: 986-990, 1971.

349. Multicentre Study Group: Hydrochlorothiazide and bendroflumethiazide in low doses - a comparative trial. Acta. Pharmacol, Toxicol., 54 (Suppl. 1): 47-51, 1984.

350. Breckenridge, A., Welborn, T.A., Dollery, C.T. & Fraser, R.: Glucose tolerance in hypertensive patients on long term diuretic therapy. Lancet, i: 61-64, 1967.

351. Grunfeld, C. & David, A.C.: Hypokalaemia and diabetes mellitus. Am. J. Med., 75: 553-554, 1983.

352. Goldner, M.G., Zarowitz, H. & Akgun, S.: Hyperglycemia and glucosuria due to thiazide derivatives administered in diabetes mellitus. N. Engl. J. Med., 262: 403-405, 1960.

353. Fajans, S.S., Floyd, J.C., Knopf, R.F., Bull, J., Gunstsche, E.M. & Conn, J.W.: Benzothiadiazine suppression of insulin release from normal and abnormal islet cell tissue in man. J. Clin. Invest., 45: 481-493, 1966.

354. Weller, J.N. & Borondy, P.E.: Effects of benzothiadiazine drugs on carbohydrate metabolism. Metabolism, 14: 708-714, 1965.

355. Shapiro, A.P., Benedek, T.G. & Small, J.L.: Effect of thiazides on carbohydrate metabolism in patients with hypertension. N. Engl. J. Med., 265: 1028-1033, 1961.

356. Gorden, P.: Glucose intolerance with hypokalaemia. Diabetes, 22: 544-551, 1973.

357. Hart, J.T.: Hypertension. Churchill Livingstone, Edinburgh, 1980, pp 181-188.

358. Newland, H.: Hyperuricaemia in coronary, cerebral and peripheral arterial disease: an explanation. Med. Hypoth., 1: 152-155, 1975.

359. Gordon, T. & Kannel, W.B.: Predisposition to atherosclerosis in the head, heart and legs. The Framingham study. J.A.M.A., 221: 661-666, 1972.

360. Ames, R.P. & Hill, P.: Elevation of serum lipid levels during diuretic therapy of hypertension. Am. J. Med., 61: 748-757, 1976.

361. Helgeland, A., Hjermann, I., Holme, I. & Laren, P.: Serum triglycerides and serum uric acid in untreated and thiazide-treated patients with mild hypertension. Am. J. Med., 64: 34-38, 1978.

362. Bengtsson, C.: Impairment of glucose metabolism during treatment with antihypertensive drugs. Acta. Med. Scand., Suppl. 628: 63-67, 1979.

363. The Pharmaceutical Codex, Incorporating the British Pharmaceutical Codex, The Pharmaceutical Press, London, 11th Ed., 1979, pp 81-82.

364. Runyan, J. W.: Influence of thiazide diuretics on carbohydrate metabolism in patents with mild diabetes. N. Eng. J. Med., 267: 541-543, 1962.

365. Gorden, P., Sherman, B.M. & Simopoulos, A.P.: Glucose intolerance with hypokalaemia: an increased proportion of circulating proinsulin-like component. J. Clin. Endocrinol. Metab., 34: 235-240, 1972.

366. Cox, M., Sterns, R.H. & Singer, I.: The defense against hyperkalaemia: the roles of insulin and aldosterone. N. Engl. J. Med., 299: 525-532, 1978.

367. Gitelman, H.J.: An improved automated procedure for the determination of calcium in biochemical specimen. Anal. Biochem., 18: 521-531, 1967.

368. Wise, P.H., Rihan, Z., Dinwiddy, G., Whichelow, M.J. & Butterfield, W.J.H.: Clopamide and carbohydrate metabolism. Med. J. Aust., 2: 795-798, 1969.

369. Rowe, J.W., Tobin, J.D., Rosa, R.M. & Andres, R.: Effects of experimental potassium deficiency on glucose and insulin metabolism. Metabolism, 29: 498-502, 1980.

370. Wigand, J.P. & Blackard, W.G.: Downregulation of insulin receptors in obese man. Diabetes, 28: 287-291, 1979.

371. Berglund, G., Andersson, O. & Wilhelmsen, L.: Prevalence of primary and secondary hypertension: studies in a random population. Br. Med. J., 2: 554-556, 1976.

372. Jarrett, R.J., Keen, H., Fuller, J.H. & McCartney, M.: Treatment of borderline diabetes: controlled trial using carbohydrate restriction and phenformine. Br. Med. J., 2: 861-865, 1977.

373. Edmonds, C.J. & Wilson, G.M.: The action of hydroflumethiazide in relation to adrenal steroids and potassium loss. Lancet, i: 505-509, 1960.

374. Talso, P.J. & Carballo, A.J.: Effects of benzothiadiazines on serum and total body electrolytes. Ann. N.Y. Acad. Sci., 88: 822-840, 1960.

375. Malaisse, W.J.: Insulin secretion: multifactorial regulation for a single process of release. Diabetologia, 9: 167-173, 1973.

376. Odlind, B.: Site and mechanism of the action of diuretics. Acta Pharmacol. Toxicol., 54 (suppl. 1): 1-15, 1984.

377. Wollheim, C.B. & Sharp, G.W.G.: The regulation of insulin release by calcium. Physiol. Rev., 61: 914-973, 1981.

378. Andersson, O.: The use of diuretics in modern antihypertensive therapy. Acta Pharmacol. Toxicol., 54 (suppl. 1): 79-85, 1984.

379. Bryant, J.M., Yu, T.F., Berger, L., Schvartz, N., Torosdag, S., Fletcher, L.Jr., Fertig, H., Schwartz, M.S. & Quan, R.B.: Hyperuricaemia induced by the administration of chlorthalidone and other sulfonamide diuretics. Am. J. Med., 33: 408-420, 1962.

380. Ames, R.P. & Hill, P.: Raised serum lipid concentrations during diuretic treatment of hypertension: a study of predictive indexes. Clin. Sci. Mol. Med., Suppl. 55: 311S-314S, 1978.

381. Kannel, W.B. & Castelli, W.P.: Is the serum total cholesterol an anachronism. Lancet, ii: 950-951, 1979.

382. Hipsley, E.H.: Dietary fibre and pregnancy toxaemia. Br. Med. J., 2: 420-422, 1953.

383. Trowell, H.C.: Ischemic heart disease and dietary fibre. Am. J. Clin. Nutr., 25: 926-932, 1972.

384. Trowell, H.C.: Dietary fibre hypothesis of the etiology of diabetes mellitus. Diabetes, 24: 762-765, 1975.

385. McCance, R.A. & Lawrence, R.D.: The Carbohydrate Content of Foods. Medical Research Council Special Report Series. 135, HMSO, London, 1929.

386. Southgate, D.A.T.: The chemistry of dietary fibre. In: Fibre in Human Nutrition. Spiller, G.A. and Amens, R.J. (eds.), Plenum Press, New York, 1976, pp 31-72.

387. Bhan, S. & Prasad, R.: Guar has many uses. Indian Farm, 17: 17-19, 1967.

388. Blackburn, N.A., Redfern, J.S., Jarjis, H., Holgate, A.M., Hanning, I., Scarpello, J.H.B., Johnson, I.T. & Read, N.W.: The mechanism of action of guar gum in improving glucose tolerance in man. Clin. Sci., 66: 329-336, 1984.

389. Holt, S., Heading, R.C. & Clements, J.: Effects of dietary fibre (Correspondence). Gastroenterology, 80: 1611-1612, 1981.

390. Holt, S., Heading, R.C., Carter, D.C., Prescott, L.F. & Tothill, P.: Effect of gel fibre on gastric emptying and absorption of glucose and paracetamol. Lancet, i: 636-639, 1979.

391. Forman, L.P. & Schneeman, B.O.: Dietary pectins' effect on starch utilization in rats. J. Nutr., 112: 528-533, 1982.

392. Rainbird, A.L., Low, A.G. & Sambrook, I.E.: Lack of effect of guar gum on gastric emptying in pigs. Proc. Nutr. Soc., 42: 24A, 1982. Abstract.

393. Blackburn, N.A. & Johnson, I.T.: The effect of guar gum on the viscosity of the gastrointestinal contents and on glucose uptake from the perfused jejunum in the rat. Br. J. Nutr., 46: 239-246, 1981.

394. Goulder, T.J., Morgan, L., Marks, V., Smythe, P. & Hinks, L.: Effects of guar on the metabolic and hormonal response to meals in normal and diabetic subjects. Diabetologia, 15: 235, 1978. Abstract.

395. Johnson, I.T. & Gee, J.M.: Effect of gel-forming gums on the intestinal unstirred layer and sugar transport in vitro. Gut, 22: 398-403, 1981.

396. Gassull, M.A., Goff, D.V., Haisman, P., Hockaday, T.D.R., Jenkins, D.J.A., Jones, K., Leeds, A.R. & Wolever, T.M.S.: The effect of unavailable carbohydrate gelling agents in reducing the postprandial glycaemia in normal volunteers and diabetics. J. Physiol., 259: 52P-53P, 1976.

397. Brown, J.C., Dryburg , J.R., Ross, S.A. & Dupre, J.: Identification and actions of gastric inhibitory polypeptide. Recent Prog. Horm. Res., 31: 487-532, 1975.

398. Morgan, L.M., Goulder, T.J., Tsiolakis, D., Marks, V. & Alberti, K.G.M.M.: The effect of unabsorbable carbohydrate on gut hormones. Diabetologia, 17: 85-89, 1979.

399. Jenkins, D.J.A., Leeds, A.R., Gassull, M.A., Wolever, T.M.S., Goff, D.V., Alberti, K.G.M.M. & Hockaday, T.D.R.: Unabsorbable carbohydrates and diabetes: decreased post-prandial hyperglycaemia. Lancet, ii: 172-174, 1976.

400. Kiehm, T.G., Anderson, J.W. & Ward, K.: Beneficial effects of a high carbohydrate high fibre diet on hyperglycemic diabetic man. Am. J. Clin. Nutr., 29: 895-899, 1976.

401. Trowell, H.: Definition of dietary fibre and hypotheses that it is a protective factor in certain diseases. Am. J. Clin. Nutr., 29: 417-427, 1976.

402. Jenkins, D.J.A., Wolever, T.M.S., Leeds, A.R., Gassull, M.A., Haisman, P., Dilawari, J., Goff, D.V., Metz, G.K. & Alberti, K.G.M.M.: Dietary fibres, fibre analogues and glucose tolerance: importance of viscosity. Br. Med. J., 1: 1392-1394, 1978.

403. Smith, U.: Potential alleviation of insulin resistance in diabetes by fibre treatment. In: Dietary Fibre in the Management of the Diabetic: Proceedings of the British Diabetic Association Symposium held in London, Medical Education Services Ltd, Oxford, 1984, pp 27-31.

404. Anderson, J.W. & Ward, K.: High carbohydrate, high fibre diets for insulin-treated men with diabetes mellitus. Am. J. Clin. Nutr., 32: 2312-2321, 1979.

405. Karlström, B., Vessby, B., Asp, N-G., Boberg, M., Gustafsson, I-B., Lithell, H. & Werner, I.: Effects of an increased content of cereal fibre in the diet of type 2 (non-insulin-dependent) diabetic patients. Diabetologia, 26: 272-277, 1984.

406. Hjøllund, E., Pedersen, O., Richelsen, B., Beck-Nielsen, H. & Sørensen, N.S.: Increased insulin binding to adipocytes and monocytes and increased insulin sensitivity of glucose transport and metabolism in adipocytes from non-insulin-dependent diabetics after a low-fat/high-starch/high-fibre diet. Metabolism, 32: 1067-1075, 1983.

407. Aro, A., Uusitupa, M., Voutilainen, E., Hersio, K., Korhonen, T. & Siitonen, O.: Improved diabetic control and hypocholesterolaemic effect induced by long-term dietary supplementation with guar gum in type 2 (insulin-independent) diabetes. Diabetologia, 21: 29-33, 1981.

408. Jenkins, D.J.A., Wolever, R.M.S., Nineham, R., Taylor, R., Metz, G.L., Bacon, S. & Hockaday, T.D.R.: Guar crispbread in the diabetic diet. Br. Med. J., 2: 1744-1746, 1978.

409. Jenkins, D.J.A., Wolever, T.M.S., Taylor, R.H., Reynolds, D., Nineham, R. & Hockaday, T.D.R.: Diabetic glucose control, lipids and trace elements on long-term guar. Br. Med. J., 1: 1353-1354, 1980.

410. Genuth, S.M.: Plasma insulin and glucose profiles in normal , obese and diabetic persons. Ann. Intern. Med., 79: 812-822, 1973.

411. Smith, U. & Holm, G.: Effect of a modified guar gum preparation on glucose and lipid levels in diabetics and healthy volunteers. Atherosclerosis, 45: 1-10, 1982.

412. Krotkiewski, M.: Effect of guar gum on body weight, metabolism and hunger ratings in obese subjects. Br. J. Nutr., 52: 97-105, 1984.

413. Tuomilehto, J., Voutilainen, E., Huttunen, J., Vinni, S. & Homan, K.: Effect of guar gum on body weight and serum lipids in hypercholesterolemic females. Acta Med. Scand., 208: 45-48, 1980.

414. Tuomilehto, J., Uuscitupa, M. & Karttunen, P.: Long term effects of granulated guar gum on metabolic control, serum lipids and blood pressure level in type 2 diabetic patients. Acta Endocrinol. (Copenh.), Suppl. 257: 72, 1983. Abstract.

415. Chen, W-J.L. & Anderson, J.W.: Effects of guar gum and wheat bran on lipid metabolism of rats. J. Nutr., 109: 1028-1034, 1979.

416. Peterson, D.: Guar in the management of non-insulin dependent diabetic patients. In: Dietary Fibre in the Management of the Diabetic: Proceedings of the British Diabetic Association Symposium held in London, Medical Educational Services Ltd., Oxford, 1984, pp 33-38.

417. Jenkins, D.J.A., Leeds, A.R., Cummings, J.H. & Newton, C.: Effect of pectin, guar gum and wheat fibre on serum-cholesterol. lancet, i: 1116-1117, 1975.

418. Jenkins, D.J.A., Leeds, A.R., Slavin, B., Mann, J. & Jepson, E.M.: Dietary fibre and blood lipids: reduction of serum chlosterol in type II hyperlipidaemia by guar gum. Am. J. Clin. Nutr., 32: 16-18, 1979.

419. Anderson, J.W., Chen, W.L. & Sicling, B.: Hypolipidemic effects of high carbohydrate, high fibre diets. Metabolism, 29: 551-558, 1980.

420. Riccardi, B.A. & Fahrenbach, M.J.: Effects of guar gum and pectin N.F. on serum and liver lipids of cholesterol fed rats. Proc. Soc. Exp. Biol. Med., 124: 749-752, 1967.

421. Boyer, P.D., Lardy, H.A. & Phillips, P.H.: Further studies on the role of potassium and other ions in the phosphorylation of the adenylic system. J. Biol. Chem., 149: 529-541, 1943.

422. Pedersen, O., Hjøllund, E., Lindskov, H.O. & Sørensen, N.S.: Increased insulin receptors on monocytes from insulin-dependent diabetes after a high-starch, high-fibre diet. Diabetologia, 19: 306, 1980. Abstract.

423. Pedersen, O., Hjøllund, E., Lindskov, H.O., Helms, P., Sørensen, N.S. & Ditzel, J.: Increased insulin receptor binding to monocytes from insulin-dependent diabetics after a low fat, high starch, high fibre diet. Diabetes Care, 5: 284-291, 1982.

424. Tagliaferro, V., Bozzo, C., Bruno, A., Lombardi, A., Cassader, M. & Cravero, L.: Metabolic effects of diet guar gum supplementation on glucose production, metabolic clearance rate of glucose, insulin binding, and lipid profile in type 2 (non-insulin-dependent) diabetic patients. Diabetologia, 25: 197, 1983. Abstract.

425. Jarjis, H.A., Blackburn, N.A., Redfern, J.S. & Read, N.W.: The effect of isphagula (Fybogel) and guar gum on glucose tolerance in man. Br. J. Nutr., 51: 371-378, 1984.

426. Wolever, T.M.S., Jenkins, D.J.A., Nineham, R. & Alberti, k.G.M.M.: Guar gum and reduction of postprandial glycaemia: effect of incorporation into solid food, liquid food, and both. Br. J. Nutr., 41: 505-510, 1979.

427. Kobayashi, M. & Olefsky, J.M.: Effects of streptozotocin induced diabetes on insulin binding, glucose transport, and intracellular glucose metabolism in isolated rat adipocytes. Diabetes, 28: 87-95, 1979.

428. Busick, E.J.Jr.: Natural history and diagnosis. In: Clinical Diabetes Mellitus. Kozak, G.P. (ed.). W.B. Saunders Company, London, 1982, pp 32-42.

429. Totterman, K., Groop, L. & Groop, P-H.: Metabolic effects of dietary fibres (guar gum) on plasma glucose, serum insulin and serum c-peptide in obese healthy subjects. Acta Endocrinol. (Copenh.), Suppl. 257: 74, 1983. Abstract.

430. Hagander, B., Schersten, B., Asp, N-G., Sartor, G., Agardh, C-D., Schrezenmeir, J., Kasper, H., Ahren, B. & Lundquist, I.: Effect of dietary fibre on blood glucose, plasma immunoreactive insulin, c-peptide and GIP responses in non-insulin dependent (type 2) diabetics and controls. Acta Med. Scand., 215: 205-213, 1984.

431. Ebert, R. & Creutzfeldt, W.: Aspects of GIP pathology. In: Gut Hormones. Bloom, S.R. (ed.), Churchill-Livingstone, Edinburgh, London, New York, 1978, pp 294-300.

432. Elsenbans, B., Sufke, U., Blume, R. & Caspary, W.F.: The influence of carbohydrate gelling agents on rat intestinal transport of monosaccharides and neutral amino acids in vitro. Clin. Sci., 59: 373-380, 1980.

433. Greenwood, M.R.C. & Hirsch, J.: Postnatal development of adipocyte cellularity in the normal rat. J. Lipid. Res., 15: 474-483, 1974.

434. Olefsky, J.M. & Chang, R.: Insulin binding to adipocytes. Evidence for functionally distinct receptors. Diabetes, 37: 946-958, 1978.

435. Blackard, W.G. & Nelson, N.C.: Portal and peripheral vein immunoreactive insulin concentrations before and after glucose infusion. Diabetes, 19: 302-306, 1970.

436. Kosmakos, F.C. & Roth, J.: Insulin-induced loss of the insulin receptor in IM-9 lymphocytes. J. Biol. Chem., 255: 9860-9869, 1980.

437. Greenwood, M.R.C., Johnson, P.R. & Hirsch, J.: Relationship of age and cellularity to metabolic activity in C57B mice. Proc. Soc. Exp. Biol. Med., 133: 944-947, 1970.

438. Di Girolamo, M. & Rudman, D.: Variations in glucose metabolism and sensitivity to insulin of the rat's adipose tissue, in relation to age and body weight. Endocrinology, 82: 1133-1141, 1968.

439. Westman, S.: Development of the obese hyperglycaemic syndrome in mice. Diabetologia, 14: 141-149, 1968.

440. Johnson, P.R. & Hirsch, J.: Cellularity of adipose depots in six strains of genetically obese mice. J. Lipid Res., 13: 2-11, 1972.

441. Genuth, S.M., Przybylski, R.J. & Rosenberg, D.M.: Insulin resistance in genetically obese, hyperglycemic mice. Endocrinology, 88: 1230-1238, 1971.

442. Salans, L.B., Knittle, J.L. & Hirsch, J.: The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity. J. Clin. Invest., 47: 153-165, 1968.

443. Kahn, C.R., Neville, D.M.Jr., Gorden, P., Freychet,P. & Roth, J.: Insulin receptor defect in insulin resistance: studies in the obese-hyperglycemic mouse. Biochem. Biophys. Res. Commmun., 48: 135-142, 1972.

444. Mahler, R.J., Mobley, P.W. & Dubuc, P.U.: Increased gluconeogenesis in the ob/ob mouse - a previously undefined parameter of insulin resistance. Endocrinology,94:236, 1974. Abstract.

445. Freychet, I. & Forgue, E.: Insulin receptors in the heart muscle: impairment of insulin binding to the plasma membrane in the obese hyperglycaemic mouse. Diabetes, 23 (Suppl. 1): 354, 1974. Abstract.

446. Becker, S.G., Smolin, P.S., Richardson, D.K. & Czech, M.P.: Insulin resistance in epitrochlearis muscles in the spontaneously obese rat. Horm. Metab. Res., 10: 204-208, 1978.

447. Olefsky, J.M.: The effects of spontaneous obesity on insulin binding, glucose transport and glucose oxidation of isolated rat adipocytes. J. Clin. Invest., 57: 842-851, 1976.

448. Salans, L.B. & Dougherty, J.W.: The effect of insulin upon glucose metabolism by adipose cells of different size: influence of cell lipid and protein content, age, and nutritional state. J. Clin. Invest., 50: 1399-1410, 1971.

449. Salans, L.B., Bray, G.A., Cushman, S.W., Danforth, E., Clennon, J.A.Jr., Horton, E.S. & Sims, E.A.H.: Glucose metabolism and the response to insulin by human adipose tissue in spontaneous and experimental obesity effects of dietary composition and adipose cell size. J. Clin. Invest., 53: 848-856, 1974.

450. Livingston, J.N. & Lockwood, D.H.: Direct measurements of sugar uptake in small and large adipocytes from young and adult rats. Biochem. Biophys. Res. Commun., 61: 989-996, 1974.

451. Vague, J. & Fenasse, R.: Comparative anatomy of adipose tissue. In: Handbook of Physiology. Section 5. Adipose tissue. Renold, A.E. & Cahill, G.F.Jr. (eds.), American Physiological Society, Washington D.C., 1965, pp 25.

452. Sjöström, L., Smith, U., Björntorp, P. & Krotkievski, M.: Cellularity in different regions of adipose tissue in young men and women. Metabolism, 21: 1143-1153, 1972.

453. Östman, J., Arner, P. Engfeldt, P. & Kager, L.: Regional differences in the control of lipolysis in human adipose tissue. Metabolism, 28: 1198-1205, 1979.

454. Bolinder, J. Arner, P. & Östman, J.: Post-receptor defects causing insulin resistance in normotensive non-inulin-dependent diabetes mellitus. Diabetes, 31: 911-916, 1982.

455. Soll, A.H., Kahn, C.H. & Neville, D.M.Jr.: The decreased insulin binding to liver plasma membranes in the obese hyperglycaemic mouse (ob/ob): demonstration of a decreased number of functionally normal receptors. J. Biol. Chem., 250: 4702-4707, 1975.

456. Smith, U.: Effect of cell size on lipid synthesis by human adipose tissue in vitro. J. Lipid. Res., 12: 65-70, 1971.

457. Storstein, L., Larsen, A., Midtbø, K. & Soevareid, L.: Pharmacokinetics of calcium blockers in patients with renal insufficiency and in geriatric patients. Acta Med. Scand., Suppl. 681: 25-30, 1984.

458. Manery, J.F.: Effects of Ca ions on membranes. Fed. Proc., 25: 1804-1810, 1966.

459. Kneer, N.M., Wagner, M.J. & Lardy, H.A.: Regulation by calcium of hormonal effects on gluconeogenesis. J. Biol. Chem., 254: 12160-12168, 1979.

460. Anderson, K-E. & Högestätt, E.D.: On the mechanism of action of calcium antagonists. Acta Med. Scand., Suppl. 681: 11-24, 1984.

461. Charles, S., Ketelslegers, J.M., Buysschaert, M. & Lambert, A.E.: Hyperglycaemic effect of nifedipine. Br. Med. J., 283: 19-20, 1981.

462. Johansson, B.: Processes involved in vascular smooth muscle contraction and relaxation. Circul. Res., 43 (Suppl. 1): 1-14-20, 1978.

463. Röjdmark, S. & Andersson, D.E.H.: Influence of verapamil on glucose tolerance. Acta Med. Scand., Suppl. 681: 37-42, 1984.

464. Brauman, A., Golik, A., Gilboa, Y. & Hertzianu, I.: Effect of nifedipine on metabolism of glucose and secretion of insulin in diabetic patients. Diabetologia, 27: 260A, 1984. Abstract.

465. Donnelly, T. & Harrower, A.D.B: Effect of nifedipine on glucose tolerance and insulin secretion in diabetic and non-diabetic patients. Curr. Med. Res. Opin., 6: 690-693, 1980.

466. Pedersen, O. & Hjøllund, E.; Insulin receptor binding to fat and blood cells and insulin action in fat cells from insulin dependent diabetics. Diabetes, 31: 706-715, 1982.

467. Dormandy, T.L.: The mechanism of insulin action. The effect of insulin on allosteric properties of intracellular haemoglobin. J. Physiol., 183: 378-406, 1966.

468. Dormandy, T.L.: The mechanism of insulin action: The effect of insulin on phosphate turnover in red-cell systems. J. Physiol., 180: 708-721, 1965.

469. Dormandy, T.L. & Zarday, Z.: The mechanism of insulin action: The immediate electrochemical effects of insulin on red cell systems. J. Physiol., 180: 684-707, 1965.

470. Collins, W.C.J., Cranny, A., Cullen, M.J. & Feely, J.: Calcium channel blocker therapy and glucose tolerance in non-insulin-dependent diabetics. Abstract 28, British Diabetic Association Meeting, Belfast, September 1985.

471. Czech, M.P., Richardson, D.K. & Smith, C.J.: Biochemical basis of fat cell insulin resistance in obese rodents and man. Metabolism, 26: 1057-1077, 1977.

472. Cahill, G.F.: Human evolution and insulin dependent (IDD) and non-insulin dependent diabetes (NIDD). Metabolism, 28: 389-393, 1979.

473. Olefsky, J.M.: Mechanisms of decreased insulin responsiveness of large adipocytes. Endocrinology, 100: 1169-1177, 1977.

474. Kolterman, O.G., Insel, J., Saekow, M. & Olefsky, J.M.: Mechanisms of insulin resistance in human obesity: evidence for receptor and postreceptor defects. J. Clin. Invest., 65: 1272-1284, 1980.

475. Ginsberg, H. & Rayfield, E.J.: Effect of insulin therapy on insulin resistance type 2 diabetic subjects: evidence for heterogeneity. Diabetes, 30: 739-745, 1981.

476. Hastings, A.B. & Buchanan, J.M.: The role of intracellular cations on liver glycogen formation in vitro. Proc. Natl. Acad. Sci. U.S.A., 28: 478-482, 1942.

477. Schlessinger, J., Schechter, Y., Willingham, M.C. & Pastan, I.: Direct visualisation of binding, aggregation and internalization of insulin and epidermal growth factor on living fibroblastic cells. Proc. Natl. Acad. Sci. U.S.A., 75: 2659-2663, 1978.

478. Czech, M.P., Richardson, D.K., Becker, S.G. & Walters, G.V.: Insulin response in skeltal muscle and fat cells of the genetically obese Zucker rat. Metabolism, 27: 1967-1981, 1978.

479. Van Obberghen, E., Gazzano, H., Ballotti, R., Fehlmann, M. Rossi, B., Gammeltoft, S., Sadoul, J.L. & Le Marchand-Brustel, Y.: The insulin receptor kinase. In: Endocrinology. Labrie, F. & Proulx, L. (eds.), Elsevier Science Publishers, B.V., 1984, pp 344-348.

480. Curry, D.L., Bennett, L.L. & Grodsky, G.M.: Requirement for calcium ion in insulin secretion by the perfused rat pancreas. Am. J. Physiol., 214: 174-178, 1968.

481. Posner, P., Miller, B.L. & Lambert, C.R.: The effect of verapamil on potassium fluxes in canine cardiac Purkinje fibers. Eur. J. Pharmacol., 34: 369-372, 1975.

482. Koch, G.: Plasma renine activity, epinephrine and norepinephrine at rest and during exercise in young adults and boys. Scand. J. Clin. Lab. Invest., 37 (Suppl. 147): 107, 1977. Abstract.

483. Jenkins, D.J.A., Nineham, R., Craddock, C., Craig-McFeely, P., Donaldson, K., Leigh, T. & Snook, J.: Fibre and diabetes. Lancet, i: 434-435, 1979.

484. Lord, J.M., White, S.I., Bailey, C.J., Atkins, T.W., Fletcher, R.F. & Taylor, K.G.: The effect of metformin on insulin receptor binding and glycaemic control in type 2 diabetes. Br. Med. J., 286: 830-831, 1983.

485. Grofford, O.B., Rogers, N.L. & Russell, W.G.: The effect of insulin on fat cells, an insulin degrading system extracted from plasma membranes of insulin responsive cells. Diabetes, 21 (Suppl. 2): 403-413, 1972.

486. Taylor, R.: Insulin receptor assays - clinical application and limitations. Diabetic Med., 1: 181-188, 1985.