THE EFFECT OF ORAL-HYPOGLYCAEMIC DRUGS AND OBESITY ON INSULIN RECEPTOR BINDING.

by

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A thesis submitted for the degree of

Doctor of Philosophy

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SUMMARY

This thesis is concerned with the regulation of insulin receptors, with respect to receptor number, affinity and certain postreceptor effects of insulin action.

The Aston obese hyperglycaemic (ob/ob) mouse was used as an animal model of obesity and type 2, NID diabetes mellitus. Hepatocyte insulin receptor number and soleus muscle insulin binding were reduced in these mice compared to lean littermates. The rate of insulin stimulated glycogen synthesis was reduced in the obese mouse between 5 and 40 weeks of age. This defect preceeded the appearance of reduced insulin receptor binding at 10 weeks and a defect beyond the insulin receptor is therefore proposed as a primary aetiological factor in the development of insulin resistance in the obese mouse.

The effect of the oral hypoglycaemic agents, metformin and glibenclamide on insulin receptor status has also been investigated. These drugs are used in the treatment of type 2, NID diabetes in man and both produced an increase in hepatocyte insulin receptor number in normal mice. Metformin was also administered to obese mice, but a supraclinical dose was required to produce an increase in receptor number. The increase in receptor number produced by metformin was rapid, appearing within 24 hours in lean mice. Metformin and glibenclamide were also administered to streptozotocin diabetic mice, but did not further increase the already raised hepatocyte insulin receptor number. However, insulin-induced hypoglycaemia was increased in the drug treated mice. Furthermore, metformin produced an increase in insulin stimulated glycogen synthesis in soleus muscle from streptozotocin diabetic mice, with no increase in insulin binding. These data provide evidence that metformin and glibenclamide mediate their therapeutic effects via modulation of the postreceptor actions of insulin. Metformin also produced an increase in erythrocyte insulin binding in obese type 2 diabetic patients, accompanied by an improvement in glycaemic control.

Key words: diabetes. insulin receptor. obesity. oral hypoglycaemic. postreceptor.

This thesis is dedicated to the memory of my father

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Terminology and Abbreviations

Terminology

1. Insulin bioactivity = The degree of response of a sensitive cell to a set amount of insulin.

2. Insulin receptor status refers to insulin receptor number, and the affinity of the receptor for the insulin molecule.

3. Maximum biological activity = The maximum response that can be elicited in a responsive cell or tissue by insulin.

4. Postreceptor effects/ mechanisms / actions = Those biological responses resulting from the binding of insulin to its receptor, that are involved in mediating its action in responsive cells and that culminate in a biological effect.

Abbreviations

NID = non-insulin dependent

ID = insulin dependent.

IGT = impaired glucose tolerance

5. DIABETES MELLITUS:

TYPE 1 = Juvenile onset, insulin dependent, ketosis prone diabetes.

TYPE 2 = Maturity onset, non-insulin dependent diabetes (frequently accompanied by obesity).

CHAPTER ONE

INTRODUCTION

Introduction

Insulin is a polypeptide hormone synthesised and released from the β cells of the pancreatic Islets of Langerhans, it plays a central role in intermediary metabolism regulating the metabolism of carbohydrates, fats and proteins. Insulin interacts with a specific receptor in the plasma membrane of target cells. This marks the first link in a chain of cellular events which culminate in a biological action. In the early nineteen seventies, improved techniques for the radiolabelling of peptide hormones gave a new impetus to receptor research (Cuatrecasas, 1971; Freychet et al., 1971a). The subsequent advances in this field of study have been admirably charted in a succession of excellent reviews (Roth, 1973; Cuatrecasas, 1974; Roth et al., 1975; Freychet, 1976a; Freychet, 1976b; Kahn, 1976; Olefsky, 1976a; Bar and Roth, 1977; Kahn et al., 1977; Bar et al., 1979; Kahn, 1980; Blecher and Bar, 1981; Kahn et al., 1981; Posner et al., 1981). A resume of our present understanding of insulin receptor structure and function is presented herein as a basis for considering insulin receptor status in diabetes mellitus, its association with insulin resistance and the possible modulatory effect of age and oral hypoglycaemic agents (with special reference to metformin).

Insulin action

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 (Randle et al., 1966; Cahill, 1971). Insulin promptly stimulates the membranal transport of glucose, and modulates the cellular pathways of glucose and lipid metabolism by enzyme activation and

inhibition (Pilkis and Park, 1974; Czech, 1977; Denton et al., 1981). Within minutes insulin alters protein synthesis, probably at the level of translation (Manchester, 1979; Sato et al., 1981). This alteration in protein synthesis is associated with the induction and repression of enzymes, and the stimulation of amino acid transport across the plasma membrane of target cells. All of these actions are dependent upon the binding of insulin with its receptor in the plasma membrane, and seemingly they do not require the insulin molecule to enter the target cell (Kahn et al., 1981).

Although a major factor in the regulation of the bioactivity of insulin in vivo must be the alteration of circulating concentrations of insulin, modulation of the biological action of any given concentration of insulin may also occur at the level of the insulin receptor.

A general expression for the bioeffect (E) of insulin, usually termed the occupancy theory, is given below. It is thought to be a function of the concentration of the occupied receptor [HR].

$$E = F[HR] \qquad (1)$$

The exact nature of F is not yet clear, but it is likely to be complex as it must account for several of insulin's binding properties, including the influence of negative cooperativity and the role of spare receptors (see page 20). However it is possible to approximate the binding of insulin to its receptor by the law of mass action, thus;

$$[H]+[R] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [HR] \qquad (2)$$

$$K_a = \frac{k_1}{k_{-1}} = \frac{[HR]}{[H][R]} \qquad (3)$$

KEY: [H] = free hormone concentration

[R] = free receptor concentration

[HR] = hormone-receptor concentration

K_a = association constant

From equation (1-3) an expression for the bioeffect of insulin can be derived (4) indicating that the bioactivity of a given amount of insulin will depend upon the receptor concentration and affinity of the receptor for the insulin molecule.

$$E = F(K_a [H][R]) \qquad (4)$$

Therefore, conditions that reflect altered insulin action, such as obesity (Bailey, 1978) and aging (Davidson, 1979) may result from changes in the status of the insulin receptor (receptor number or affinity). This theory has been elaborated upon in the following review of the present literature, and the experimental work in this project has attempted to add to our present knowledge of the development of insulin resistance in both obese and aging animals. Furthermore, the basis for the therapeutic actions of oral hypoglycaemic agents (with special reference to metformin) is investigated, to establish whether their reduction of insulin resistance in type 2 diabetics is connected with an effect on insulin receptor concentration or affinity.

In addition to its rapid and potent effects on intermediary metabolism, insulin exerts a mild long term stimulatory effect on cell growth, division and differentiation (Kahn et al., 1981). This appears to be mediated via receptors which are distinctly separate from the receptors that mediate the effects on intermediary metabolism.

Receptors responsible for growth related effects show a high degree of specificity for a group of polypeptides known as insulin-like growth factors (IGFS) or somatomedins (King et al., 1980; Kasuga et al., 1981). Sufficient structural similarities exist between insulin and IGFS to allow insulin to interact weakly with IGF receptors, and to allow IGFS to interact weakly with the insulin receptor (Rinderknecht and Humbel, 1978; Zapf et al., 1978; King et al., 1980).

In this thesis the insulin receptor represents that receptor which shows a very high degree of specificity for insulin, and which mediates the effects of insulin on intermediary metabolism.

Insulin receptor structure

The insulin receptor has been shown to be a large glycoprotein component of the plasma membrane. The receptor binds insulin at the extracellular surface of the membrane, shows a high degree of affinity for insulin, and reacts in a specific, rapid, saturable and reversible manner (Cuatrecasas, 1974; Roth et al., 1975). Recent advances in affinity labelling and affinity purification techniques have contributed immensely to the identification of the insulin receptor, and to an understanding of its subunit composition (Heinrich et al., 1980; Yip et al., 1980; Czech et al., 1981). In addition, the immunoprecipitation of insulin receptors using anti-insulin receptor antibodies has been used to probe the molecular structure of the insulin receptor (Harrison and Kahn, 1980; Jacobs et al., 1980a). The insulin receptor is comprised of a minimum of four glycoprotein subunits (Figure 1). These subunits form a large globular complex with a molecular radius of about

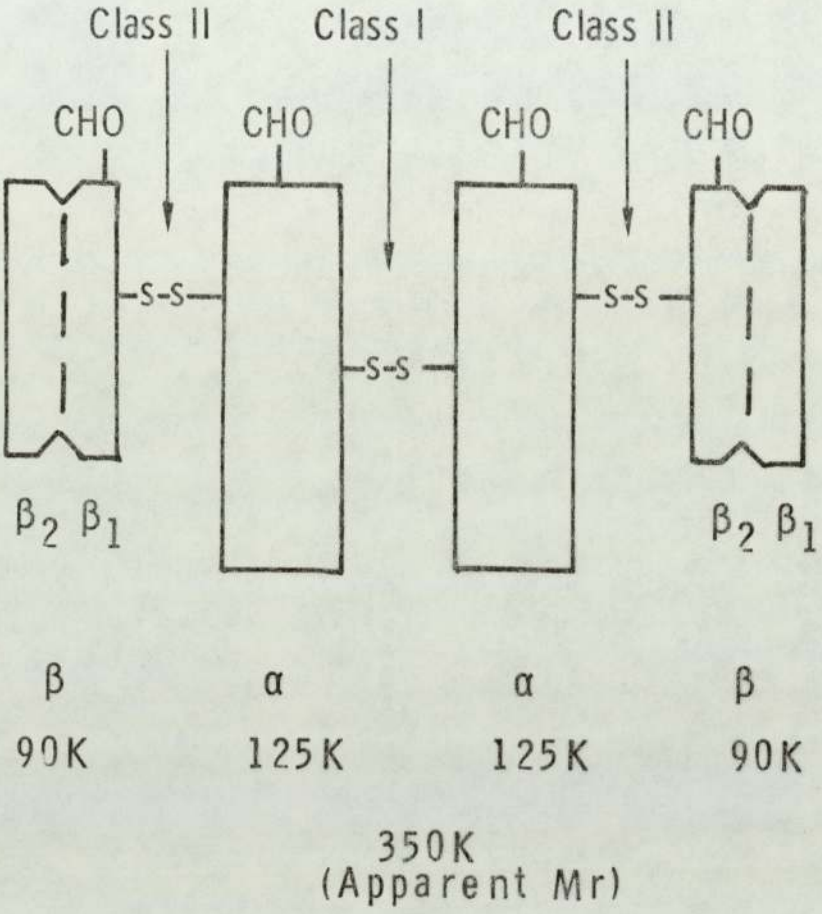


Figure 1. Representation of the subunit organisation of the insulin receptor as described by Czech et al. (1981). The two larger subunits (α) are linked together by one or more disulphide bonds (class I S-S bonds), and the two smaller subunits (β) are disulphide linked (class II S-S bonds) to the α-subunits. The apparent Mr of the receptor complex assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is lower than expected from the subunit composition. This may result partly because interchain disulphide bonds maintain a compact structure during SDS-PAGE purification.

7nm, and an apparent M_r of 300-350K (Jacobs et al., 1979; Massague et al., 1980). There are two larger subunits designated α , which are linked together by one or more disulphide bonds (class I S-S bonds), and two smaller subunits designated β which are disulphide linked (class II S-S bonds) to the α subunits. This subunit composition is called the $(\alpha\beta)_2$ complex. There may also be further subunits which are not covalently linked to the complex and different arrangements of the various subunits. Oligosaccharide side chains appear to be attached to both the α and β subunits (Jacobs et al., 1980b). Indeed, a sialylated glycosidic moiety has been shown to participate in the interaction with insulin, involving D-galactose, N-acetyl-D-glucosamine and D-mannose residues (Cherqui et al., 1981). The exact location of the insulin binding site on the receptor complex, however, has not yet been established.

Several investigators have identified an active receptor moiety with a molecular radius of about 4nm, suggesting that the high molecular weight receptor complex (M_r 300-350 K) may comprise two active components (Ginsberg et al., 1976 ; Krupp and Livingston, 1978; Baron et al., 1981). 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 (Czech et al., 1981). Proteolytic fragmentation of the β subunits 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 (Massague et al., 1980). 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 $(\alpha\beta_1)_2$ (Czech et al.,

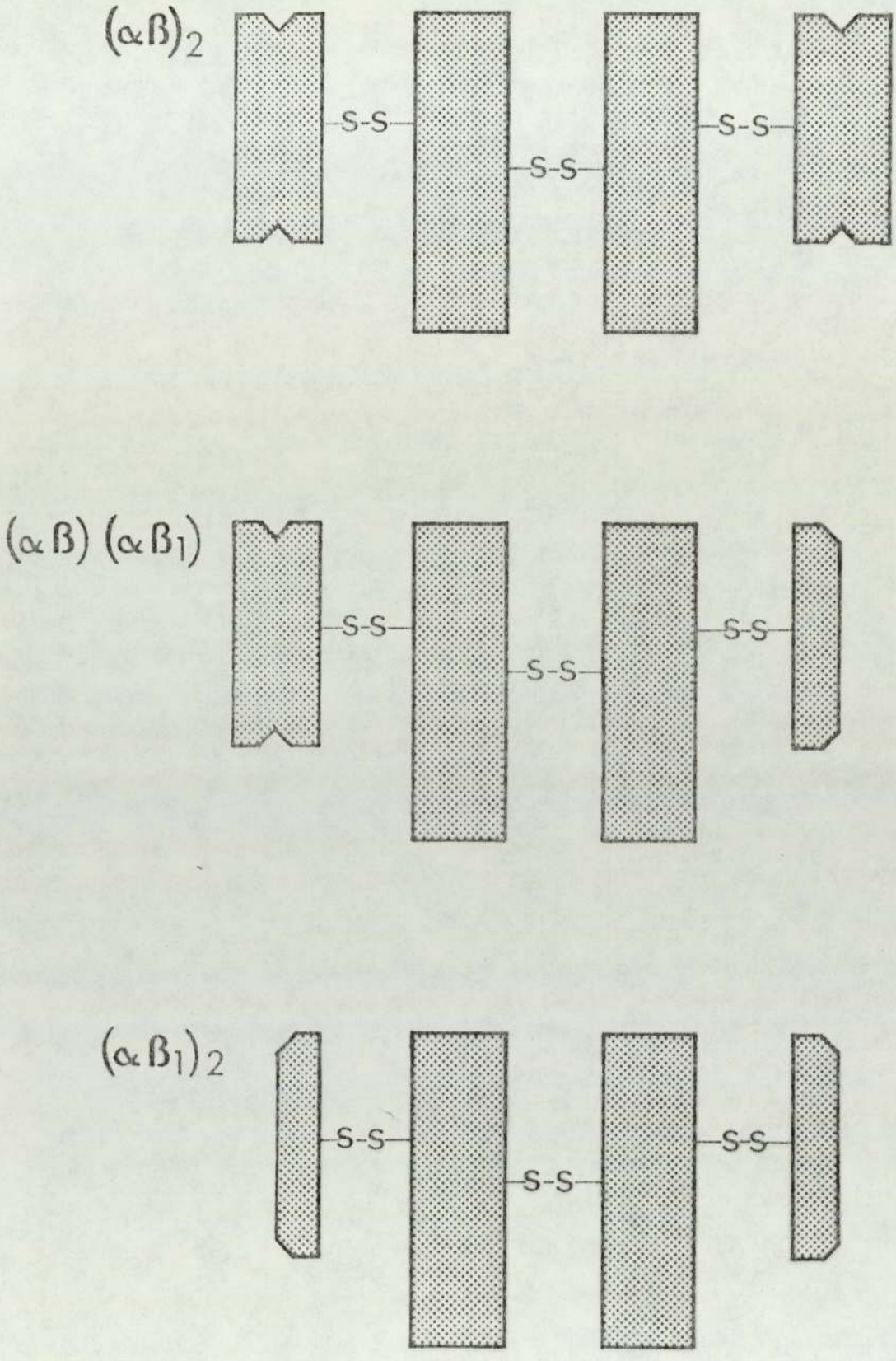


Figure 2. Proteolytic fragmentation of the $(\alpha\beta)_2$ insulin receptor complex into two additional forms of the insulin receptor, $(\alpha\beta)(\alpha\beta_1)$ and $(\alpha\beta_1)_2$, as described by Massague et al. (1980).

1981). Recent evidence suggests that different cell types may exhibit variations in insulin receptor structure (Pottick et al., 1981). Estimates of the number of insulin receptor sites suggest considerable variation between cell types. For example, expressed per μm^2 of plasma membrane, estimates range from fourteen sites on human erythrocytes, to twenty four on cultured human lymphocytes, and sixty three in rat hepatocytes (Ginsberg, 1977b).

Internalisation , recycling and biosynthesis of insulin receptors

When a target cell is exposed to insulin there follows an aggregation of occupied receptors (and possibly unoccupied receptors - see page 20) into 'coated pits' on the plasma membrane. Aggregates of receptors are then taken into the cell ('internalisation') by endocytosis (Gorden et al., 1980; Posner et al., 1981). In a steady state binding situation, as much as 40 per cent of the insulin associated with a target cell may be localised within the cell. The process of internalisation appears to provide a pathway for the cellular degradation of insulin, and for the recycling and degradation of receptors (Figure 3). Endocytotic vesicles containing the internalised receptors combine with other vesicular elements associated with the Golgi fraction. The involvement of the various Golgi fractions and biochemically defined lysosomes in the process remains to be defined. Enzymatic degradation of insulin and receptors is believed to occur within lysosomes, and some receptors appear to be recycled via Golgi elements (Goldstein and Livingston, 1981). Binding of insulin to plasma membrane receptors not only accelerates the process of internalisation (down regulation - discussed on page 20),

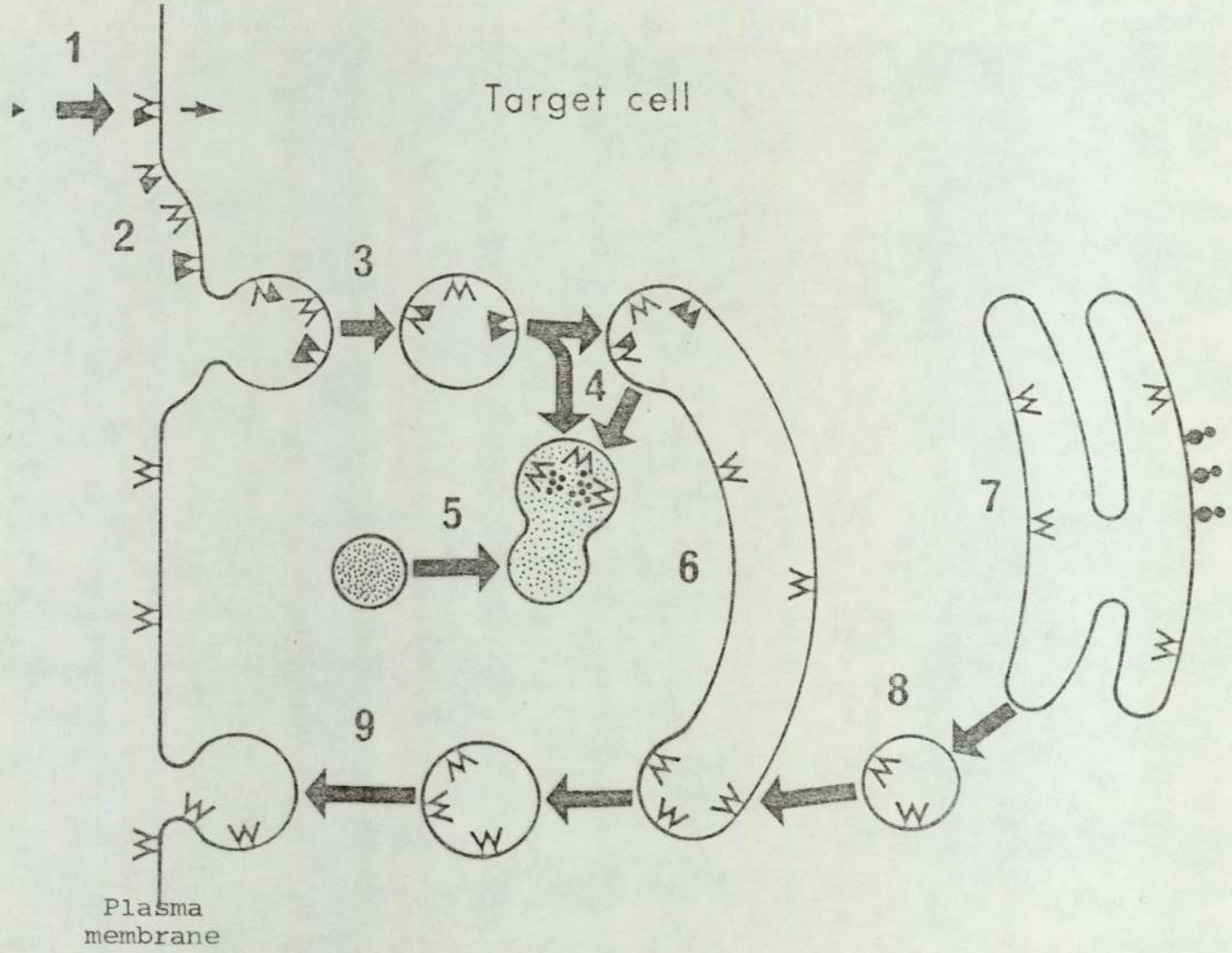


Figure 3. Schematic representation of insulin receptor binding, 'internalisation', insulin degradation and insulin receptor turnover. 1. Insulin binds with a plasma membrane receptor site. 2. Receptors aggregate. 3. Receptors are internalised by endocytosis. 4. Endocytotic vesicles merge with Golgi and lysosomal elements. 5. Insulin (and receptors?) is enzymatically degraded. 6. Some receptors are recycled via the Golgi. 7. Insulin receptors are synthesised at the rough endoplasmic reticulum. 8. Newly synthesised receptors are transferred to the Golgi. 9. Newly synthesised and recycled receptors pass to the plasma membrane by exocytosis. Based on Kahn et al. (1981) and Posner et al. (1981)

but also stimulates the production of new receptors (Posner et al., 1981). Biosynthesis of insulin receptors takes place at the rough endoplasmic reticulum; receptors concentrate in the Golgi region, and are transferred to the plasma membrane by exocytosis of Golgi vesicles. Recycled receptors are probably transferred to the plasma membrane in the same manner.

Postreceptor events

As noted previously, the interaction of insulin with its plasma membrane receptor initiates a series of cellular events which leads to a biological action. These events comprise the postreceptor pathways. It is envisaged that postreceptor pathways are activated by a conformational change in the insulin receptor due to insulin binding (Pilch and Czech, 1980; Harmon et al., 1981). One theory of insulin action suggests that an early postreceptor event is the activation, possibly by a change in phosphorylation state, of a protein kinase associated with the plasma membrane. The activated kinase controls phosphorylation of intracellular proteins and enzymes responsible for the biological actions of insulin (Czech, 1981). Very recent work by Kasuga has demonstrated that the insulin receptor itself is phosphorylated upon binding of insulin and may in fact be a tyrosine protein kinase itself, activated by phosphorylation (Kasuga et al., 1982). Alternative evidence suggests that the postreceptor sequence comprises one or more peptide like factors which are released from the membrane into the cytoplasm. These factors then control protein and enzyme phosphorylation (Cheng et al., 1980; Seals and Czech, 1981). Whether insulin stimulates membranal glucose transport by a mechanism operating

entirely within the confines of the membrane, or via a cytoplasmic postreceptor sequence is unresolved.

Insulin receptor binding

Assessment of insulin receptor binding using a radio-receptor assay involves methods similar to those used for hormone radioimmunoassay. A high specific activity tracer, ^{125}I- monoiodoinsulin, is incubated with a homogenous preparation of isolated cells or plasma membranes in the presence of varying amounts of unlabelled insulin. At steady state the bound and free hormone fractions are separated by filtration or centrifugation, and the radio-activity associated with the bound fraction is counted. Correction is made for non-specific binding, and data can be analysed by a number of methods which will be discussed later in the text. It is appreciated that changes in receptor function may occur during the incubation period required for steady state binding in the receptor assay procedure; insulin receptor binding is highly dependent upon temperature, and sub-physiological temperatures are often used to achieve steady state binding during receptor assays. Thus the observed binding data of the receptors *in vitro* may not reflect precisely their properties *in vivo*.

Estimation of the concentration and affinity of insulin receptor sites is most commonly based on the method of Scatchard (1949), in which the ratio of the moles of bound: free labelled insulin (B/F) is plotted against the moles of bound insulin (B). This method of analysis of ligand binding by receptors, although widely used, is not without its flaws and it is therefore employed with an awareness of its limitations. In the simplest situation, namely with an homogenous population of receptors, the Scatchard plot is a

reliable device for measuring the binding constant. In this case the Scatchard plot is linear and the intercept on the abcissa is the total number of receptor sites. eg. Human growth hormone interaction with its receptors on cultured lymphocytes (Lesniak et al., 1973). However, in many biological systems, including insulin binding to its receptor, the Scatchard plot is not linear and raises the possibility of the presence of more than one class of receptor binding site. Klotz (1982) has criticised the use of the Scatchard plots for the estimation of the number of receptor binding sites in cases where the existence of a single class of receptors cannot be confirmed. Despite suggesting a variety of alternative methods for the analysis of binding data, Klotz does admit that more reliable information could be derived " if the experimenter is willing to make, and the system is amenable to the collection of, the necessary extensive measurements ". In an already time consuming technique, the acquisition of further measurements in the insulin radioreceptor assay may be of limited advantage. As the Scatchard plot for insulin is curvilinear with upward concavity (Figure 4), the intercept on the abcissa is gained by extrapolation of the plot. A more accurate intercept would therefore be gained from points falling on the latter portion of the plot, these are gained from the incubations of receptor bearing cells at high insulin concentrations. In practice the B/F ratio changes only slightly with increasing insulin concentration at the higher insulin concentrations (> 10mM). Further measurements would therefore not produce a more accurate extrapolation, therefore until further methodological advances are made, the estimates of receptor site

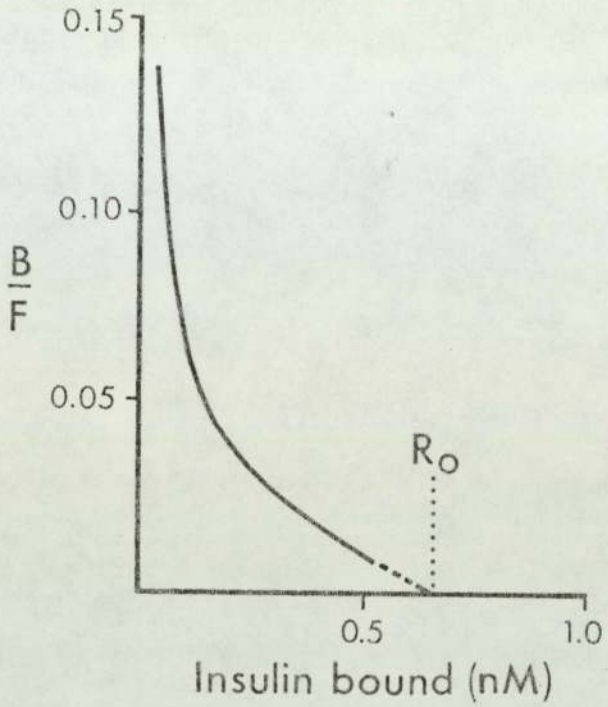


Figure 4. Typical Scatchard plot for the binding of insulin to mouse hepatocytes at 20°C. The slope of the curve indicates receptor affinity (see text). Total binding capacity (R_o) is estimated by extrapolating the line to the abscissa. The total number of receptor sites per cell is calculated as

$$\frac{R_o \text{ (molar)} \times 6.02 \times 10^{23} \text{ (Avogadro's number)}}{\text{Cells per litre}}$$

concentration using the Scatchard plot will remain as 'approximations' of the true value.

As has been stated, the Scatchard plot for insulin receptor binding is curvilinear with upward concavity (Figure 4). This was originally interpreted as evidence for two or more classes of insulin receptors with different but fixed affinity constants (Gavin et al., 1973). Subsequently it was argued, however, that the profile reflects a single population of receptors showing negatively cooperative site-site interactions (De Meyts et al., 1976). This implies that there exists a single group of insulin receptors whose affinity is variable, and decreases as the occupancy of the receptors increases. Consistent with this interpretation De Meyts and Roth (1975) proposed the use of an average affinity profile to analyse steady state binding data (Figure 5). The profile indicates two interconvertible sites: a high affinity site with a binding constant $\bar{K}_e$ and a low fractional occupancy $\bar{Y}_e$: and a low affinity site with a binding constant $\bar{K}_f$ and a high fractional occupancy $\bar{Y}_f$. Therefore receptors may 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 the 'negative cooperativity' theory of insulin receptor binding, notably De Meyts and coworkers (1973) incubated cultured lymphocytes with [^{125}I]- labelled insulin to steady state, followed by infinite dilution (100x) of the insulin receptor complexes to prevent rebinding of labelled hormone. The dissociation rate of ^{125}I-insulin was then measured in either the presence or absence of unlabelled insulin. The dissociation rate of insulin from independent

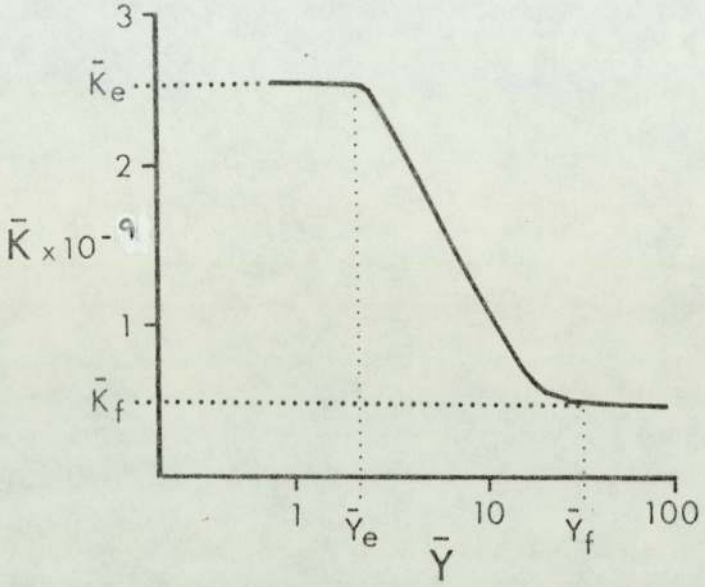


Figure 5. Typical average affinity profile for the binding of insulin to mouse hepatocytes at 20°C. The profile is obtained by plotting the average affinity ($\bar{K}$) against the fractional occupancy ($\bar{Y}$). Both functions are derived from the Scatchard plot: $\bar{K} = (B/F)/(R_o - B)$; $\bar{Y} = B/R_o$

receptors should not be affected by insulin concentration (De Meyts et al., 1973) but this study showed an increased rate of dissociation in the presence of insulin. Thus, the occupation of receptors by unlabelled insulin increased the rate of dissociation of labelled insulin from other receptor sites, i.e. there were site-site interactions.

The other main body of evidence has been derived from studies involving insulin analogs eg. desoctapeptide and desalanine-desasparagine insulin (De Meyts et al., 1976; Pullen et al., 1976; De Meyts et al., 1978). This work has indicated the presence of two discrete areas for insulin binding to the receptor. One site is necessary for the biological postreceptor actions of insulin and involves residues 12, 16 and 24-26 on the B chain and residues 1, 4 19 and 21 of the A chain, the other site is represented by the last 8 residues of the B chain and is thought to be important for the induction of cooperative interactions.

The concept of negative cooperativity has not found universal acceptance. Herzberg and coworkers (1980a) have demonstrated a complete block of high affinity sites by concanavalin A without any effect upon low affinity sites. However, this work was carried out using erythrocytes, which have been shown to exhibit heterogeneity of receptor concentration with age (Kosmakos et al., 1980). It is therefore possible that the 'high' and 'low' affinity receptors, on the erythrocytes, reside in separate cell populations, only one of which was affected by concanavalin A. Furthermore, intact hepatocytes have been shown to degrade [^{125}I]-insulin into fragments which show a reduced binding capacity (Donner, 1980; Levitzki, 1981) and if this degradation is taken into account a linear Scatchard plot

results. This effect may however be limited to the hepatocyte, with its high content of endogenous proteases, since Sonne and Gliemann (1980) have failed to confirm insulin fragmentation using cultured lymphocytes.

Insulin receptor models

One mechanistic explanation for the cooperative model proposes interactions between (or conformational changes in) the receptor subunits induced by increasing insulin concentrations (Levitzki, 1974; De Meyts et al., 1976). The altered subunit structure reduces the affinity of the receptor and induces a fast dissociating state. De Meyts et al. (1976) have drawn an analogy with the oxygen dissociation curve for haemoglobin. At low oxygen tension the haemoglobin molecule has a low affinity for oxygen, and oxygen dissociates rapidly from the haemoglobin molecule. At high oxygen tension the affinity of haemoglobin for oxygen 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 a 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 concentrations, 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 (Jacobs and Cuatrecasas, 1976; Kahn, 1976). Aggregation (or 'clustering') of insulin receptors in the presence of rising concentrations of insulin has been

demonstrated experimentally (Jarrett and Smith, 1975; Orci et al., 1975). Levitzki (1974) proposed that insulin 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 (Figure 6). It has also been suggested that insulin binding enables the newly occupied receptors to aggregate unoccupied receptors. A requirement for the movement of receptors might explain why negative cooperativity is not observed in cultured lymphocytes in the presence of concanavalin A (De Meyts et al., 1974).

Spare receptors

It is well established that target cells with a normal sensitivity to insulin show a maximum biological response to insulin when only a small percentage (10-30 per cent) of the receptor population is occupied. The unoccupied receptors are known as 'spare' receptors (Kono and Barham, 1971). It has been argued that all receptors are potentially available for binding and the 10-30 per cent 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.

Down regulation

Insulin, in addition to the induction of changes in receptor affinity, also alters the number of insulin receptors, although the latter is a more chronic effect (Gavin et al., 1974; Bar et al., 1976). In general, raised concentrations of insulin are associated with a reduced number of insulin receptors. This insulin-induced receptor

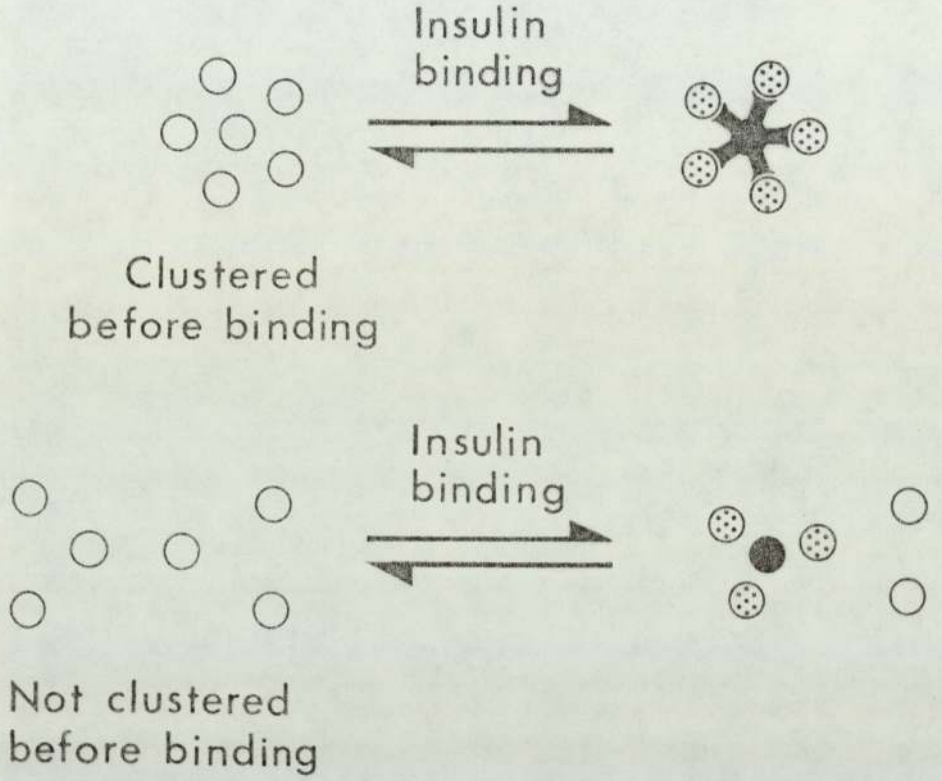


Figure 6. Speculative mechanisms to account for the organisation of insulin receptors and a change in receptive state after interaction with insulin. Unoccupied receptors exist in a more receptive state. Insulin binding renders the occupied receptors less receptive and might be envisaged to compromise the receptive state of adjacent receptors.

loss is termed 'down-regulation'. At high insulin concentrations there is no apparent alteration in the rate of receptor synthesis or degradation (Krupp and Lane, 1981), 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 (1981) 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 activity which act distally to the receptor, such as spermine and Vitamin K_5 , Caro and Amatruda (1980a) have also demonstrated receptor loss. This indicates the importance of postreceptor events in the regulation of insulin receptors.

Cell types used to study the human insulin receptor

Radioreceptor binding assays have been performed using a variety of cell and membrane preparations, from a range of species, including lymphocytes (Gavin et al., 1973), erythrocytes (Gambhir et al., 1978), hepatocytes (Kahn et al., 1974), adipocytes (Cuatrecasas, 1971) and placenta (Haour and Bertrand, 1974). In addition to these studies the binding properties of solubilised insulin receptors, from erythrocytes, have also been examined (Ginsberg et al., 1976). From these experiments it is clear that despite either the tissue or species of origin, the receptor binding properties are very similar; regulation of receptor binding activity may however vary between different tissues.

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 the clinical situation should examine each of these cell types, but practical and ethical limitations exist. Animal studies have indicated a general agreement between the regulation of insulin receptors in adipocytes, hepatocytes and muscle cells (Soll et al., 1974; Olefsky et al., 1975; Le Marchand-Brustel et al., 1978), but comparative studies in man are still awaited. For convenience, assessments of insulin receptor activity in man have relied mostly on the use of circulating monocytes (Schwartz et al., 1975; Bar et al., 1976; Beck-Nielsen and Pedersen, 1979) and erythrocytes (Gambhir et al., 1977; Gambhir et al., 1978; Gambhir et al., 1981). The responses of monocyte insulin receptors have been shown to mirror closely the responses of adipocyte insulin receptors in a number of experimental and pathological conditions (Bar et al., 1976; Olefsky, 1976b; Beck-Nielsen et al., 1977; Beck-Nielsen and Pedersen, 1979).

Erythrocytes offer the advantage that only a relatively small sample of blood is required (Gambhir et al., 1977; Gambhir et al., 1978). The insulin receptors of erythrocytes respond in a similar manner to those of monocytes, although changes in erythrocytes may occur more slowly (Gambhir et al., 1978; Dons et al., 1981a; Lippe et al., 1981). This may reflect the incapacity of erythrocytes to undertake 'de novo' protein synthesis, which is anticipated to compromise receptor turnover, with 'up regulation' being especially vulnerable and may depend to some extent on the recruitment

of new cells into the erythrocyte pool. Furthermore, it is known that within erythrocyte preparations, 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 (Eng et al., 1980; Kosmakos et al., 1980; Dons et al., 1981b). Thus, unless patients included in clinical studies have served as their own controls, groups with haematological disturbances likely to alter the proportion or mean age of circulating erythrocytes have not been studied.

At present, cultured lymphocytes (Krug et al., 1972; Pollet et al., 1977; Helderman and Strom, 1978) and fibroblasts (Gavin et al., 1972; Thomopoulos et al., 1976; Mott et al., 1979; Prince and Olefsky, 1980) are proving useful for studies on human cells. In the future it is likely that cultured adipocytes (Blackard et al., 1978) will receive more extensive use. However, despite the general similarities between insulin receptor activity in different cell types, sufficient differences have been observed (Olefsky et al., 1975; Dons et al., 1981a) to invite further investigation. Hence the extrapolation of receptor data beyond the cell type studied should be undertaken with only extreme caution.

Conditions of altered insulin receptor binding

The foregoing sections have emphasised that the insulin receptor is not an inert structure: its activity may vary between tissues, and it is regulated by the circulating concentration of insulin. Indeed, both the number of insulin receptor sites and their affinity fluctuate in response to many acute and chronic influences. Acute influences include meal patterns (Muggeo et al., 1977; De Pirro et al., 1979) and physical activity (Koivisto et al., 1979; Pedersen et

al., 1980). These are superimposed upon longer term modulation by dietary factors (Beck-Nielsen et al., 1978; Wigand et al., 1979), age (Thorsson and Hintz, 1977; Kappy and Plotnick, 1979; Herzberg et al., 1980b; Pagano et al., 1981) and sex (Bertoli et al., 1980; Hendricks et al., 1981). Adjustments in receptor status may also occur during the menstrual cycle, pregnancy and oral contraceptive usage (Beck-Nielsen et al., 1979a; Bertoli et al., 1980; Tsibris et al., 1980; De Pirro et al., 1981; Moore et al., 1981), and may accompany changes in the circulating concentrations of growth hormone (Muggeo et al., 1979; Lippe et al., 1981) and glucocorticoids (Cigolini and Smith, 1979; Beck-Nielsen et al., 1980; De Pirro et al.,1980a). Pathological conditions which affect insulin receptor function have been reviewed recently (Harrison and Kahn, 1980; Blecher and Bar, 1981) and the following section will be confined to a consideration of defects occurring in obesity and diabetes mellitus. At this point, it may be helpful to classify certain terms used in the text with reference to diabetes and obesity, that may otherwise be misleading.

Insulin resistance

An impaired biological action of insulin is described by the term insulin resistance. This is a general term which for practical purposes is identified by an inability of insulin to lower blood glucose. Insulin resistance may be caused by elevated concentrations of circulating insulin antagonists (eg. counter regulatory hormones), or by defects in target tissues.

Insulin antagonists

Insulin antagonists are blood borne agents which oppose or prevent the biological action of insulin. Again, the

definition is based on antagonism of the blood glucose lowering action of insulin.

Insulin antagonists include counter regulatory hormones such as glucagon, catecholamines, growth hormone and glucocorticoids (Felig et al., 1979). These agents can raise plasma glucose concentrations independently of an effect on insulin receptor binding, although both growth hormone and glucocorticoids are known to modify insulin receptor binding. Other antagonists which interfere or compete with insulin for binding to its' receptor include insulin antibodies (Blecher and Bar, 1981), insulin receptor antibodies (Harrison and Kahn, 1980), abnormal insulins (Tager et al., 1979), proinsulin (Freychet, 1974), insulin-like growth factors (King et al., 1980) and possibly insulin degrading enzymes (Goldstein and Livingstone, 1981).

Insulin insensitivity and insulin unresponsiveness

Target tissue defects which may contribute to insulin resistance reside either at the level of the insulin receptor or at some point in the postreceptor pathways. Techniques used to investigate the structure of the insulin receptor and the postreceptor signals have yet to be applied to human diabetes. However, if the levels of insulin antagonists are not abnormally raised, it is possible to distinguish defects located at the insulin receptor from those located at a postreceptor site. This is based on the interpretation of the dose response curve for a biological action of insulin (Figure 7). A maximum biological effect of insulin is generated when only a small proportion (normally 10-30%) of the insulin receptor sites are occupied (see page 20). Thus, a defect which resides predominantly at the insulin receptor should impair the subsequent

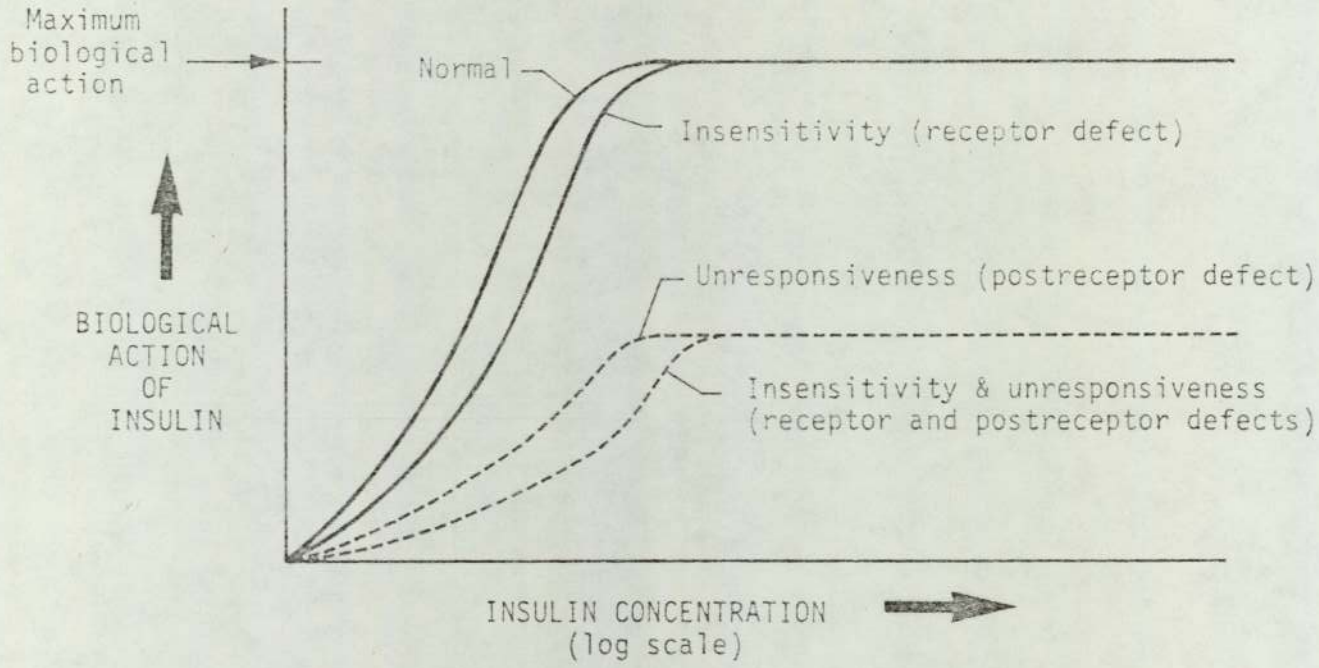


Figure 7. Theoretical dose-response curves for a biological action of insulin. Based on Kahn (1978) and Olefsky (1981a)

biological action of insulin at submaximally effective insulin concentrations, but a normal biological action should be achieved at maximally effective insulin concentrations. This assumes that an adequate concentration of patent receptor sites (10-30% of the normal complement) is available for binding and that the postreceptor defect is not rate limiting. Accepting these assumptions, the dose response curve is displaced to the right and described as insulin insensitivity (Figure 7).

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 maximal biological action, even at inordinately raised insulin concentrations. Thus, the dose response curve is proportionally reduced at all insulin concentrations, and this is designated insulin unresponsiveness (Figure 7).

Complete elimination of interference with insulin is difficult in diabetic subjects (Reaven and Olefsky, 1978), although a preliminary study has suggested that glucagon does not affect insulin receptor binding (Gill and Hart, 1981). However, a possible alteration by glucagon of the rate of insulin degradation, and increased proinsulin concentrations (Duckworth et al., 1972a)cannot be disregarded.

Insulin receptor binding in obesity

Obesity is a common condition in which hyperinsulinaemia and insulin resistance (Bailey, 1978; Olefsky, 1981a) are symptomatic and in which insulin receptor activity has been examined extensively. As reviewed elsewhere (Olefsky, 1976a; Olefsky, 1981a;Freychet, 1976a;Blecher and Bar, 1981) obese humans and animals show a decrease in the total specific

insulin binding (see Terminology page 1) in a wide range of cell types. This is due to a decrease in the concentration of their insulin receptors. More recently it has been emphasised that among obese subjects there exists a spectrum of defects in insulin action. Obese subjects with mild insulin resistance have only a reduced concentration of insulin receptors, whereas subjects with more severe resistance exhibit a reduced concentration of receptors together with one or more postreceptor defects (Kolterman et al., 1980). However, the postreceptor defect(s) appears to develop only in certain tissues, such as adipocytes, and not other tissues, such as hepatocytes (Kolterman et al., 1980; Ciaraldi et al., 1981). The latter observation may reflect the so-called permissive effect of insulin on the liver; in the absence of other influences, glucose metabolism by the liver is related to the prevailing glucose concentration, provided that a nominal amount of insulin is present (Davidson, 1981). A close correlation has been observed between the extent of hyperinsulinaemia and the reduction in insulin receptor concentration in mildly hyperinsulinaemic obese subjects. This observation, plus *in vitro* evidence reviewed elsewhere (Olefsky, 1981a), suggests that the hyperinsulinaemia may be responsible for the reduced receptor site concentration in mild obesity, due to insulin induced receptor loss. In more severely insulin resistant obese subjects, the postreceptor defects take prominence (Kolterman et al., 1980).

Insulin receptor defects in diabetes mellitus

The term diabetes mellitus is acknowledged as an 'umbrella' term under which shelter a multitude of aetiologically and pathogenically distinct conditions (Cudworth,

1978; Pyke, 1979). The common feature of these conditions is an inappropriately raised plasma glucose concentration. A recently revised classification of diabetes has stressed the heterogeneity of this disorder (National Diabetes Data Group, 1979). Insulin receptor binding studies contribute to this view providing evidence that heterogeneous defects of insulin receptor function occur in diabetes. Not all diabetics exhibit insulin receptor defects, but where such defects exist they appear to be associated with insulin resistance, manifest by the coexistence of hyperglycaemia and hyperinsulinaemia. Thus consideration of such defects should be linked with an examination of postreceptor events and the biological actions of insulin. Available data suggest that most non obese subjects with impaired glucose tolerance (IGT) and all non obese type 2 diabetics with raised basal glucose concentrations exhibit insulin resistance (Ginsberg et al., 1975; Reaven et al., 1976; Olefsky and Reaven, 1977; Kolterman et al., 1981). In general, the severity of insulin resistance in IGT and type 2 diabetic subjects increases with the magnitude of the basal hyperglycaemia, but there is no correlation betweem insulin resistance and the insulin response to a glucose challenge (Olefsky and Reaven, 1977; Kolterman et al., 1981).

Obesity is common among sugjects with IGT and less severe forms of type 2 diabetes, and obesity aggravates the insulin resistance in these subjects (Bar et al., 1976; Reaven and Olefsky, 1978; Kolterman et al., 1981). Circulating levels of insulin antagonists are not raised sufficiently to account for the degree of insulin resistance in most IGT and type 2 diabetic subjects (Olefsky, 1976a;

Olefsky and Reaven, 1977; Reaven and Olefsky, 1978). Thus, attention has been directed towards possible defects at the level of the membrane receptor and cellular postreceptor pathways to account more fully for the insulin resistance in diabetes.

Insulin receptor binding in IGT

In subjects with IGT the dose response curve for the biological action of insulin has been assessed by the euglycaemic clamp technique. In this situation, the suppressive effect of insulin on hepatic glucose output was found to be impaired at submaximal, but not at maximal insulin concentrations. Thus the curve was displaced to the right in these subjects, but a normal maximum biological effect of insulin was achieved (Kolterman et al., 1981). This indicates that the insulin resistance shown by the subjects with IGT is due to a decrease in insulin sensitivity, and implies a defect located predominantly at the level of the insulin receptor.

In this study, the degree of insulin insensitivity in subjects with IGT correlated with a decrease in the concentration of adipocyte insulin receptors, while the affinity of the receptor was normal. A previous investigation noted a similar decrease in the concentration of monocyte insulin receptors of subjects with IGT (Olefsky and Reaven, 1977). Again there was no change in receptor affinity. Hence, those subjects with IGT and without insulin resistance exhibit normal receptor function, whilst those with insulin resistance show a proportionate decrease in receptor site concentration. This has fostered the hypothesis that insulin resistance (or more precisely insulin insensitivity) in subjects with IGT might be accounted for almost totally by

a reduced concentration of insulin receptor sites (Kolterman et al., 1981; Olefsky, 1981a).

Insulin receptor binding in subjects with type 2 diabetes mellitus

Using the euglycaemic and hyperglycaemic clamp techniques, Kolterman et al. (1981) observed that the dose response curve for the biological action of insulin was displaced to the right in obese and non obese type 2 diabetics. In addition to this reduction in insulin sensitivity, most type 2 patients showed a decrease in the maximum biological action of insulin. This degree of insulin unresponsiveness, implies a postreceptor defect and parallels closely the level of basal hyperglycaemia in the more severely hyperglycaemic patients. In these severely hyperglycaemic type 2 patients the findings suggest a postreceptor defect as the predominant abnormality. A continuum of defects is therefore apparent among type 2 diabetics; 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.

The receptor defect in type 2 diabetics has been identified in several cell types as a decrease in receptor site concentration, while receptor affinity is unchanged (Table 1).

Obese type 2 diabetics: while obesity per se causes a reduced concentration of insulin receptors, and aggravates insulin resistance in subjects with IGT and mild type 2 diabetes, in severely insulin resistant type 2 diabetics an additive effect of obesity is not observed. This suggests that the receptor concentration is already markedly compromised in severe type 2 diabetes, and obesity exerts no further effect (Kolterman et al., 1981). Although a post-

Table 1. Effect of impaired glucose tolerance (IGT) and type 2 diabetes on insulin receptor binding

*= decrease; 0 = unchanged.

Condition	Cell type studied	Total specific binding	Receptor site conc.	Receptor affinity	Authors
Non obese IGT	adipocytes	*	*	0	Kolterman et al. (1981)
"	monocytes	*	*	0	Olefsky and Reaven (1977)
Non obese type 2	adipocytes	*	*	0	Kolterman et al. (1981)
"	erythrocytes	*	*	0	Robinson et al. (1979) De Pirro et al. (1980b)
"	monocytes	*	*	0	Olefsky and Reaven (1974, 1976a, 1976b, 1977) Goldstein et al. (1975) DeFronzo et al. (1979)
Obese type 2	adipocytes	*	*	0	Kolterman et al. (1981)
"	cultured lymphocytes	*	*	0	Helderman and Raskin (1980)
"	monocytes	*	*	0	Bar et al. (1976) Beck-Nielsen et al. (1979b)

receptor defect appears to account for the more severe component of insulin resistance in the type 2 diabetics, the maximum suppressive effect of insulin on hepatic glucose output was not impaired. The postreceptor defect(s) in these patients may therefore reside in insulin target cells other than the hepatocyte.

Insulin receptor binding during treatment of subjects with type 2 diabetes

(i) Diet

In the treatment of obese type 2 diabetics, fasting for 2 to 3 days improves insulin sensitivity and this is accompanied by an increase in monocyte insulin receptor affinity. The receptor concentration is not altered, however, despite a considerable reduction in the circulating insulin concentration (Bar et al., 1976). An increase in monocyte insulin receptor affinity has also been noticed after treatment of obese type 2 diabetics with a calorie restricted diet (1200 kcal) for 10 days (Beck-Nielsen et al., 1979b). Insulin sensitivity was improved and the basal insulin concentration reduced during treatment. In contrast, calorie restriction for a month or longer, while associated with improved insulin sensitivity and lower basal insulin concentrations, was accompanied only by an increase in receptor site concentration (Bar et al., 1976; Beck-Nielsen, 1978). A similar pattern of changes in monocyte and adipocyte receptor status has been noted during short term and long term calorie restriction in obese non-diabetics (Bar et al., 1976; Beck-Nielsen et al., 1976; Kolterman et al., 1979).

(ii) Oral hypoglycaemic agents

It is now evident that the hypoglycaemic effect of chronic treatment with sulphonylureas is achieved at least

in part by extrapancreatic actions. Evidence for this derives from observations that the hypoglycaemic effects of sulphonylureas persist after the insulinotropic effect is lost (Lebovitz and Feinglos, 1978). A plausible explanation for these observations would be that sulphonylureas improve the hypoglycaemic action of endogenous insulin. Consistent with this view, both chlorpropamide and glibenclamide increase the concentration of insulin receptors in monocytes, and this is associated with enhanced insulin sensitivity (Olefsky and Reaven, 1976b; Sorensen et al., 1978; Beck-Nielsen et al., 1979b). Studies in normal rats (Bachmann et al.,1979), normal mice (Feinglos and Lebovitz, 1978) and genetically obese-diabetic (ob/ob) mice (Greenstein, 1979) have shown that sulphonylureas increase the concentration of insulin receptors in both hepatocytes and liver plasma membranes. Addition of the sulphonylurea glyburide to cultured human fibroblasts has shown the effect to be direct and rapid. The number of insulin receptor sites was increased after 6 hours in a dose-dependent manner, and the down-regulatory effect of insulin was prevented (Prince and Olefsky, 1980).

Metformin, a biguanide, produces its hypoglycaemic effect in type 2 diabetics by actions at several extra-pancreatic sites (Hermann, 1979). Recent studies have shown that metformin increases the concentration of erythrocyte insulin receptors in normal subjects (Holle et al., 1981). This study was, however, performed on a small group of volunteers, with a short period of administration of the drug. Thus, the biguanides have not received the same attention as the sulphonylureas and there is room for more thorough investigations. It is tempting to speculate that

an increased concentration of insulin receptors might account for the reduction of insulin requirement in type 1 and type 2 diabetics treated with a combination of insulin plus either a sulphonylurea or metformin (Hermann, 1979; Bachmann et al., 1981; Eaton et al., 1981; Slama et al., 1981). The work presented in this thesis will provide an understanding of the mechanisms by which oral hypoglycaemic agents, in particular metformin, achieve their therapeutic effects.

Insulin receptor binding in type 1 diabetics: effects of treatments

Since type 1 diabetics are necessarily treated with insulin immediately upon diagnosis, few studies have examined insulin receptor status in these patients before treatment. Two studies using monocytes have evaluated a limited number of patients at diagnosis, and both studies indicate a heterogeneity of receptor defects.

Some type 1 patients have a reduced binding capacity, whereas others show an increased binding capacity (Pedersen et al., 1978; Fantus et al., 1981). In poorly controlled insulin-treated type 1 diabetics the concentration and affinity of insulin receptor sites in monocytes and cultured lymphocytes is normal (Pedersen et al., 1978; Helderman and Raskin, 1980), an observation that is consistent with normal insulin sensitivity (Ginsberg, 1977a).

Insulin treatment does not produce consistent changes in receptor activity in type 1 diabetics. Pedersen et al. (1978) noted two distinctly different types of response after 10 days of insulin treatment in newly diagnosed patients. In those patients with an increased receptor binding capacity at diagnosis, insulin reduced binding to

normal. However, in patients with a reduced receptor binding capacity at diagnosis, insulin produced no change in binding. In another study, Fantus et al. (1981) treated newly diagnosed and poorly controlled patients with insulin for periods varying from 5 to 78 days. In general the insulin treatment decreased binding to monocytes, due to a decrease in both the concentration and affinity of receptor sites. This was accompanied by an improved metabolic control.

Two case study reports have appeared concerning type 1 diabetics. Bar et al. (1979) reported a poorly controlled insulin treated diabetic with a reduced monocyte receptor concentration. Eight days after cessation of insulin treatment there was a marked increase in receptor affinity, associated with a small improvement in glycaemic control. Eaton et al. (1981) described two poorly controlled insulin treated diabetics with a reduced concentration of erythrocyte insulin receptor sites. Addition of tolbutamide to the treatment regime and a reduction in the insulin dose increased the receptor concentration and ameliorated the hyperglycaemia. Although the reduced insulin dose might lift the down-regulatory effect of insulin, it is possible that the sulphonylurea directly increased the receptor concentration and prevented down regulation by insulin.

KETOACIDOSIS: In newly diagnosed type 1 patients, no correlation has been found between monocyte insulin receptor binding, plasma insulin concentrations and the concentration of circulating ketoacids. However, in insulin treated patients, a positive correlation was observed between receptor affinity and ketoacid concentration (Pedersen et al., 1978). A decrease in pH over the range observed in ketoacidosis decreases receptor binding affinity (Gavin et al., 1973), but prior exposure to low pH does not appear to

alter receptor binding when the radioreceptor assay is conducted at normal pH (Fantus et al., 1981).

With regard to insulin treatment, it is noteworthy that human biosynthetic insulin shows the same receptor binding characteristics, and has the same potency of biological action as pork insulin. This has so far been demonstrated using rat adipocytes (Olefsky, 1981b;Sonne et al., 1981) and human monocytes (Petersen et al., 1981).

Gestational diabetes

In two studies it has been noted that the insulin receptor site concentration of placental membranes is reduced in gestational diabetics. This has been attributed at least in part to the down regulation of receptors by high circulating insulin concentrations (Harrison et al., 1977; Duran-Garcia et al., 1979).

Contributions made by animal models of diabetes to understanding insulin receptor binding mechanisms

Many spontaneous syndromes of inappropriate hyperglycaemia have been described (Herberg and Coleman, 1977; Bray and York, 1979; Herberg, 1979). Insulin receptor status has been examined in the obese-diabetic (ob/ob) mouse, which exhibits moderate hyperglycaemia, marked hyperinsulinaemia and severe insulin resistance (see page 152). Consistent with obesity and type 2 diabetes in humans, this model shows a reduced concentration of insulin receptors and a normal receptor affinity in a variety of its cell types (Freychet, 1976a). A postreceptor defect in these mice has also been identified (Le Marchand-Brustel et al., 1978). 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 (Baxter and Lazarus, 1975; Kern et al., 1975). However, insulin binding to hepatocytes of Zucker fatty (fa/fa) rats was only increased above that of lean rats in the fasting state (Broer et al., 1977).

In contrast, the Chinese hamster provides an example of insulin deficient diabetes, and displays an increased concentration of liver membrane insulin receptors (Hepp et al., 1975). Insulin deficient diabetes induced by streptozotocin also increases the insulin receptor concentration of hepatocytes, muscle cells and adipocytes in rats and mice (Davidson and Kaplan, 1977; Schoenic et al., 1977; Kasuga et al., 1978; Wieringa and Krans, 1978; Duckworth et al., 1979; Kobayashi and Olefsky, 1979). In these animals receptor affinity is usually normal, although severely hyperglycaemic streptozotocin diabetic rats show an increased affinity of adipocyte and soleus muscle insulin receptors (Kobayashi and Olefsky, 1979; Le Marchand and Freychet, 1979). Insulin binding to liver plasma membranes in streptozotocin diabetic rats have been corrected by insulin treatment (Davidson and Kaplan, 1977), and may therfore support the data of Pedersen et al. (1978) in which type 1 diabetics with an increased binding capacity showed a return to normal binding following insulin therapy.

Possible causes of insulin receptor defects in diabetes

The cause of a reduced concentration of insulin receptor sites in insulin resistant subjects with IGT and type 2 diabetes 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 receptor sites in non-diabetic obese persons. Hyper-

insulinaemia at least partly offsets the receptor loss in non-diabetic obese subjects, although it has been argued that a raised insulin concentration is the cause of the receptor loss (Kolterman et al., 1980). Down regulation is likely to operate in subjects with IGT and type 2 diabetics with raised basal insulin concentrations, however, this mechanism does not explain the receptor loss in patients with normal or reduced insulin concentrations.

A recent report has described the effect of insulin treatment for 1 to 8 weeks in a group of insulin resistant hypoinsulinaemic, non-obese, type 2 diabetics (Ginsberg and Rayfield, 1981). In one third of these patients a normal biological action of insulin was regained after insulin treatment. Gross changes in insulin antagonists were not observed, suggesting that the third who benefited from the insulin treatment might have corrected a previous receptor or postreceptor defect. This raises the possibility that insulin deficiency might adversely affect insulin receptor function in some type 2 diabetics. The study emphasizes the heterogeneity amongst patients classed as type 2 diabetics and leaves open the possibility that greater or more protracted insulin treatment might improve control in a larger proportion of type 2 diabetics.

Heterogeneity of receptor status has been demonstrated in type 1 diabetes (Pedersen et al., 1978). Even though all type 1 diabetics are insulin deficient, only a proportion show increased receptor binding. That insulin deficiency per se might contribute to the increased receptor binding is suggested by the normalisation of receptor binding and the instatement of good control during rigorous insulin therapy, although in type 1 diabetics with a reduced

receptor binding capacity, insulin alone does not consistently correct the receptor defect or achieve good control.

It is evident that untreated diabetic conditions with insulin resistance are generally associated with abnormal insulin receptor status. Whether the receptor fault is the primary defect remains to be established, but it is notable that the correction of abnormal insulin receptor function is commensurate with good metabolic control in both type 1 and type 2 diabetics.

An intriguing observation on the receptor defect in type 2 diabetes has been reported by Helderman and Raskin (1980). They cultured T lymphocytes which do not manifest an insulin receptor *in vivo* but show a receptor *in vitro* after activation by a mitogen or antigen. Thus it appears that insulin receptors of T lymphocytes are not subject to the regulatory influences of the *in vivo* environment Activated T lymphocytes of type 1 diabetics showed a normal concentration and affinity of insulin receptor sites. Activated T lymphocytes of type 2 diabetics, however, displayed a reduced concentration of receptor sites with normal affinity. The authors concluded that T lymphocytes from type 2 diabetics are either genetically programmed to exhibit fewer insulin receptor sites, or the cells from these patients have been conditioned by a higher circulating insulin concentration in their donors, and compensate by down regulation.

A contrary view has been expressed by Howard et al. (1981) who cultured skin fibroblasts from type 2 diabetic Pima Indians. Although insulin receptor status was not measured in this study, it was observed that fibroblasts showed a normal sensitivity to the biological action of

insulin. Hence these workers concluded against inherent differences in cell sensitivity in type 2 diabetics.

To summarise, it is clear from the literature presented so far that both the concentration and affinity of insulin receptor sites are subject to continual regulation by a multitude of acute and chronic influences. Although insulin plays an important role in the regulation of its receptor many other factors play their part. Evaluation of insulin receptor status in diabetics emphasizes the heterogeneity of this disorder, not only between, but also within the main types of diabetes. Discrimination between insulin insensitivity and insulin unresponsiveness has demonstrated a spectrum of defects in subjects with IGT and type 2 diabetics. The former exhibit a reduced concentration of insulin receptor sites which appears to account for the decrease in insulin sensitivity. A reduced concentration of receptor sites is also observed in type 2 diabetics, but as the severity of insulin resistance increases a postreceptor defect becomes evident. Type 1 diabetics display variable receptor abnormalities: insulin receptor binding is increased in some and decreased in others. Whether the insulin receptor defects in diabetes reflect a primary lesion awaits clarification, but it is notable that the correction of abnormal receptor function is commensurate with good metabolic control in both type 1 and type 2 diabetics.

Thus, the insulin receptor is far from being an inert structure, playing as it does a central role in the modulation of insulin action and thus in the regulation of intermediary metabolism. In those situations where a defect of insulin action has been located, such as obesity and

diabetes mellitus, the expected change in receptor activity has been confirmed. The relative importance of the two facets of receptor activity, namely the receptor binding and postreceptor binding events, to the development of insulin resistance remains to be fully established. Only when the primary cause of the insulin resistance seen in obesity and diabetes mellitus is established, can a possible therapeutic programme be attempted. Consequently, this thesis includes a profile of insulin receptor binding and insulin action during the development of the obesity syndrome in the obese, hyperglycaemic (ob/ob) mouse.

In addition, this thesis includes an investigation of the mode of action of hypoglycaemic agents (with special reference to metformin) used in the treatment of type 2 diabetes. Relating any effects of these drugs on insulin receptor activity to their therapeutic actions may then aid the development of further antidiabetic agents and establish the usefulness of such drugs in the treatment of diabetes mellitus.

CHAPTER TWO

MATERIALS AND METHODS

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]aminoethanesulphonic acid) (TES), tricine, streptozotocin, rabbit liver glycogen - Type V, collagenase Type II and Type IV, pyruvate and bacitracin were purchased from Sigma Chemical Co. Ltd., Dorset. Bovine serum albumin Fraction V (Lot numbers 330 and 335, insulin free) was purchased from Miles Laboratories Ltd., Slough. Monocomponent Porcine insulin (Lot number S8391272) was purchased from Novo Industria A/S, Copenhagen, Denmark. Na ^{125}I and U-^{14}C-glucose were purchased from Amersham International, Amersham. Ficol Paque and Sephadex G50 were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden and sodium pentobarbitone (Sagatal) was purchased from May and Baker Ltd., Dagenham. Protosol and Econofluor were purchased from New England Nuclear, Southampton and insulin antiserum (guineapig RD12, Lot number K4454) was obtained from Wellcome Reagents Ltd., Beckenham.

Pure metformin hydrochloride (Batch 304) was supplied by Rona Laboratories Ltd., Hitchin and glibenclamide (Batch U002) was provided by Hoechst Pharmaceuticals, Frankfurt, GDR.

All other reagents were of analytical grade, purchased from British Drug Houses Ltd., Dorset. Distilled water was used throughout.

The Radioiodination of Insulin

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

of hormone must be kept within the physiological range for that hormone; for insulin this is often as low as 10^{-10} M. A suitably high number of counts must also be accumulated for meaningful measurements to be made. In addition, the tracer should retain the full biological activity and receptor binding properties of the native hormone.

Several methods are currently available for the radioiodination of proteins but no single method has been found to be completely satisfactory and reliable (Hunter and Greenwood, 1962; Miyachi et al., 1972; Bolton and Hunter, 1973; Redshaw and Lynch, 1974). However, the most widely used labelling methods are modifications of the Chloramine-T technique developed by Hunter and Greenwood (1962). This technique was originally designed to yield a majority of monoiodinated protein, minimizing damage to the protein at the same time.

Chloramine-T is the sodium salt of the n-monochloro derivative of p-toluene sulphonamide. In aqueous solution it breaks down to form hypochlorous acid, a mild oxidising agent. Na ^{125}I (in NaOH solution) is oxidised by chloramine-T in the presence of insulin and ^{125}Iodine is incorporated into the tyrosine residues of the insulin molecule. Excess chloramine-T is reduced by the addition of sodium metabisulphite and free iodine is reduced to iodide. Phosphate buffer containing a protein, such as albumin, is also included in the reaction volume to act as a carrier for the labelled insulin and the latter is then separated from unreacted iodide by gel filtration on Sephadex G50. The concentrations of chloramine-T are kept low to minimize damage to insulin. The reaction time is also reduced (15-20 seconds) to minimize the exposure of insulin to the

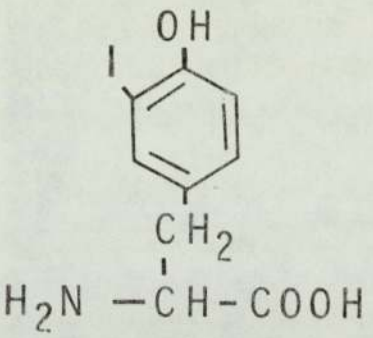
chloramine-T.

The chloramine-T reaction has a pH optimum of pH 7.4 and yields are reduced below pH 6.5 and above pH 8.5 (Freedlender, 1969; Ganguli and Hunter, 1971). However, the Na ^{125}Iodine is obtained from Amersham International suspended in NaOH at pH 8-10 and therefore the pH has to be adjusted to pH 7.4 with phosphate buffer (0.5M) before use.

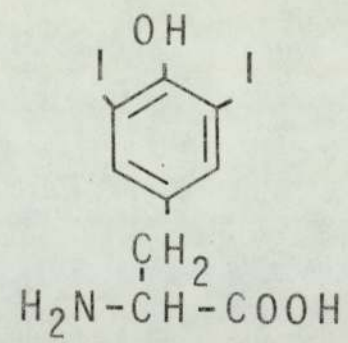
The ^{125}Iodine atoms substitute ortho to the hydroxyl group in the phenolic ring of tyrosine (Figure 8).

Figure 8.

monoiodotyrosine



di-iodotyrosine



It is well known that different tyrosine residues in a protein show different degrees of reactivity in the iodination process, depending on the micro-enviroment of each individual sidechain of the protein (Seon et al., 1970). Also those residues on the surface of the molecule iodise most readily. It has been shown that in the case of insulin one tyrosine residue, A14, is iodinated preferentially (Freedlender and Cathou, 1971). 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 by isolated fat cells (Freychet, 1971a).

Iodination Procedure

The following reactants were added to the vial contain-

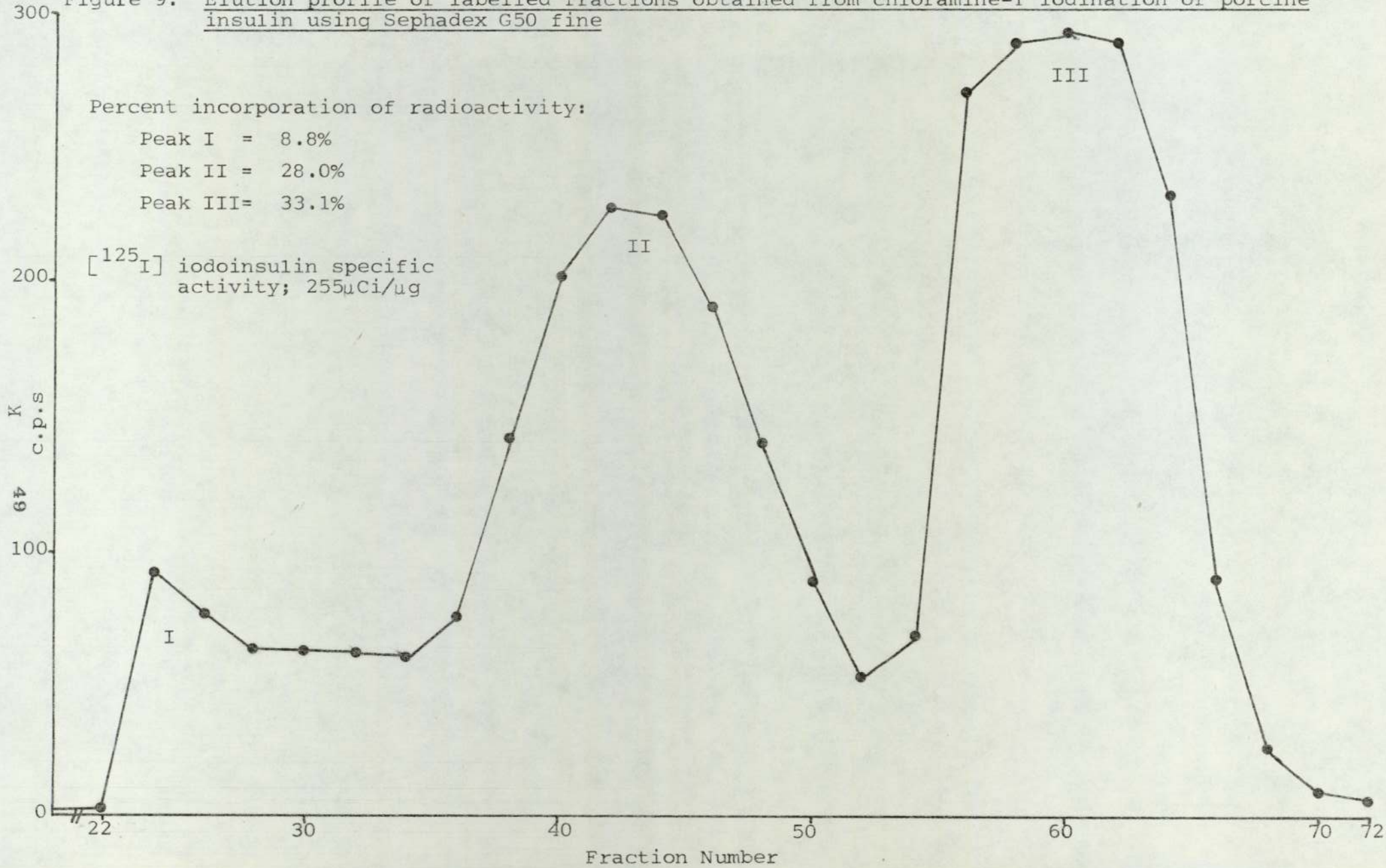
ing Na ^{125}I, in the order shown below, the contents were mixed gently after each addition. Best results were obtained if the radionuclide was no more than three days old.

10µl	Na ^{125}I solution, 1mCi (100mCi/ml)
100µl	0.5M phosphate buffer, pH 7.4
10µl	Porcine monocomponent insulin (0.25mg/ml in 0.01N HCl)
20µl	Chloramine-T (0.25mg/ml in 0.05M phosphate buffer, pH 7.4)
	15-20 seconds
20µl	Sodium metabisulphite (0.5mg/ml in 0.05M phosphate buffer, pH 7.4)
	15 seconds
200µl	Bovine serum albumin (25mg/ml in 0.05M phosphate buffer, pH 7.4)

The labelled insulin was then separated from the unreacted iodide and damaged insulin by gel filtration, using Sephadex G50, fine. The interior of the column (Pharmacia, K15/30) was pretreated with 1ml of 5% bovine serum albumin to reduce adsorption of insulin. The reaction products were eluted with 0.05M phosphate buffer, pH 7.4, containing 0.5% bovine serum albumin and 0.1% sodium azide as a bactericide, at a flow rate of 0.75ml/minute and collected in disposable polystyrene tubes (LP3, Luckhams Ltd.), at one minute intervals. Each tube was then counted for 3 seconds (ICN GAMMASET 500), a typical elution profile is shown in Figure 9.

Three distinct peaks are observed. Peak 1 represents a high molecular weight fraction probably containing aggregates of the insulin molecule (Freychet, 1971a). Peak 2 represents the monoiodinated insulin and Peak 3 contains the unreacted

Figure 9. Elution profile of labelled fractions obtained from chloramine-T iodination of porcine insulin using Sephadex G50 fine



iodide. Figure 10 shows the elution profile of a sample of A14-monoiodoinsulin (a gift from Novo Laboratories) compared with that of a sample taken from peak 2.

The first six tubes from Peak 2 were pooled for use in the radioreceptor assays and were assessed for reaction damage to the insulin molecule as described below.

Assessment of iodination damage to tracer

In preliminary studies the ^{125}I-insulin was assessed for iodination damage to the insulin molecule using trichloroacetic acid precipitability, charcoal adsorption and binding to an excess of guinea pig insulin antiserum,

Trichloroacetic acid (TCA) precipitable radioactivity was determined using a 25µl sample of the pooled (peak 2) ^{125}I-insulin fraction diluted to a final protein concentration of 4.7mg/ml with 0.5% bovine serum albumin (Fraction V). 500µl of insulin solution, plus 500µl of cold 10% TCA, was centrifuged at 1500g for 10 minutes. The supernatant was removed and the pellet washed once with 1ml of 5% TCA, before being counted for radioactivity.

Charcoal adsorption was assessed in the same way, by diluting 25µl of the tracer solution to 400µl with 0.5M phosphate buffer, pH 7.4, and adding 10mg of dextran coated charcoal suspended in cold 0.5M phosphate buffer, pH 7.4

The immunological competence of the tracer was assessed using a 10µl aliquot of the ^{125}I-insulin diluted to 500µl with 0.05M phosphate buffer, pH 7.4. The diluted sample was incubated for 48 hours at 4^{o}C in the presence of an excess of insulin antiserum (1:40,000 GPBL/3). After incubation the bound and free insulin fractions were separated using centrifugation through dextran coated charcoal.

Labelled insulin was assessed as suitable for use in

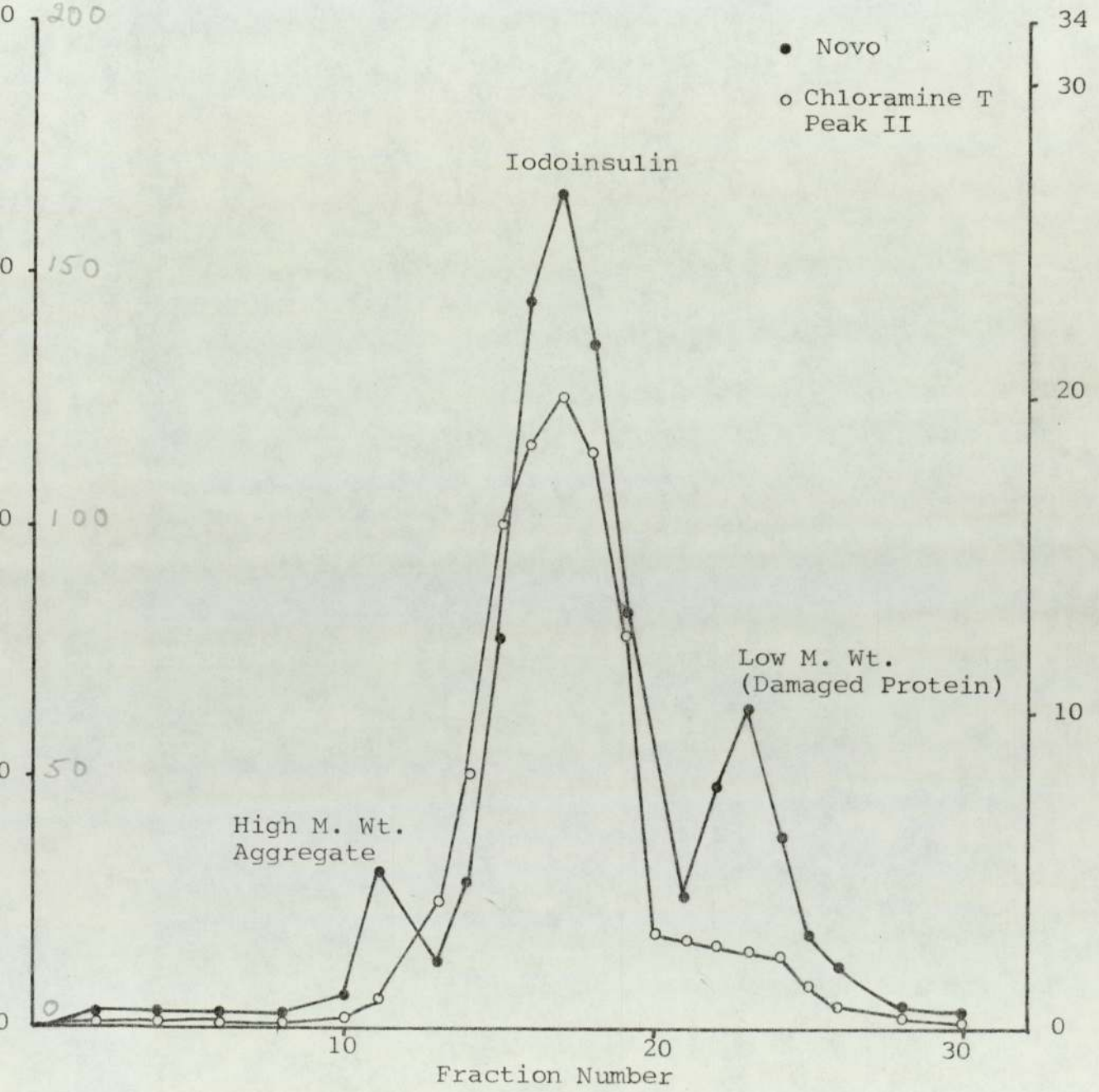


Figure 10. Chloramine-T monoiodinated insulin and iodinated insulin from Novo Laboratories , eluted on Sephadex G50 fine, collected in 1ml fractions and counted in a Gamma Counter.

radioreceptor assays when TCA precipitability was greater than 95%, binding to charcoal was greater than 90% and binding to an excess of insulin antiserum was greater than 85% (Sonksen and Refetoff, 1971).The ^{125}I-insulin obtained in Peak 2, satisfied these criteria and was subsequently used in binding studies. However, routine assessment of ^{125}I-insulin only entailed the TCA precipitation test.

As mentioned earlier (see page 48) a cause of damage to the insulin molecule that can be easily avoided is 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 was of the order of 87%. The reason for this deterioration is not understood, but it may be that irradiation products are higher in the older iodide, causing damage to the tracer produced.

Determination of specific activity

A 10µl aliquot of the reaction mixture was taken prior to separation on the Sephadex column. To this was added 400µl of column eluent and 500µl of cold 10% TCA. The solution was counted and then centrifuged for 10 minutes at 2000g, to precipitate the protein bound radioactivity. The supernatant was then removed and the procedure repeated. The precipitate was then counted.

The counts obtained for the precipitate represent the activity that is incorporated into insulin. The specific activity can then be calculated, using the formula below, since we can determine the amount of radioactivity bound to insulin and we already know the total amount of insulin present in the reaction mixture (2.5µg).

$$\text{Specific activity } (\mu Ci/\mu g) = \frac{\frac{\%TCA \text{ precipitated counts}}{100} \times 1mCi}{2.5\mu g}$$

The specific activity of ^{125}I-insulin produced by this method was usually of the order of 200-250μCi/μg, corresponding to an incorporation level of 0.5-0.6 molecules of iodine per molecule of insulin. These values suggest the production of monoiodinated insulin because only specific activity of over 350μCi/μg indicate di- or triiodination (Yalow, 1976). The presence of only one iodine atom per insulin molecule ensures a minimal effect on insulin's activity (Izzo et al., 1964; Rosa et al., 1966) and helps to prolong shelf life (Schneider et al., 1976; Starr and Rubenstein, 1974). 50μl aliquots of pooled peak 2 fractions were transferred to LP3 tubes and stored at -20°C. Tracer prepared in this way was suitable for use in receptor binding studies for upto two weeks, after this time self-irradiation damage is too high (Berson and Yalow, 1973).

Evaluation of an alternative iodination method

In an attempt to iodinate insulin under milder conditions, in the hope of reducing damage to the insulin molecule further, two iodinations were performed using the recently developed Enzymobead preparation (Bio-Rad Laboratories, Watford). It had been suggested that a possible advantage of this method is that the labelling occurs at the surface of the insulin molecule, further reducing the chance of iodination of the biological site of the insulin molecule, on the β chain. The Enzymobead reagent consisted of lacto-peroxidase and glucose oxidase linked to a solid phase support of hydrophilic spheres. The addition of glucose causes the release of a small amount of hydrogen peroxide

at a steady rate, generated by the glucose oxidase. The lactoperoxidase in turn catalyses the peroxide oxidation of labelled iodide to iodine. The labelled iodine then reacts with insulin to produce radioiodinated insulin (Murphy, 1976).

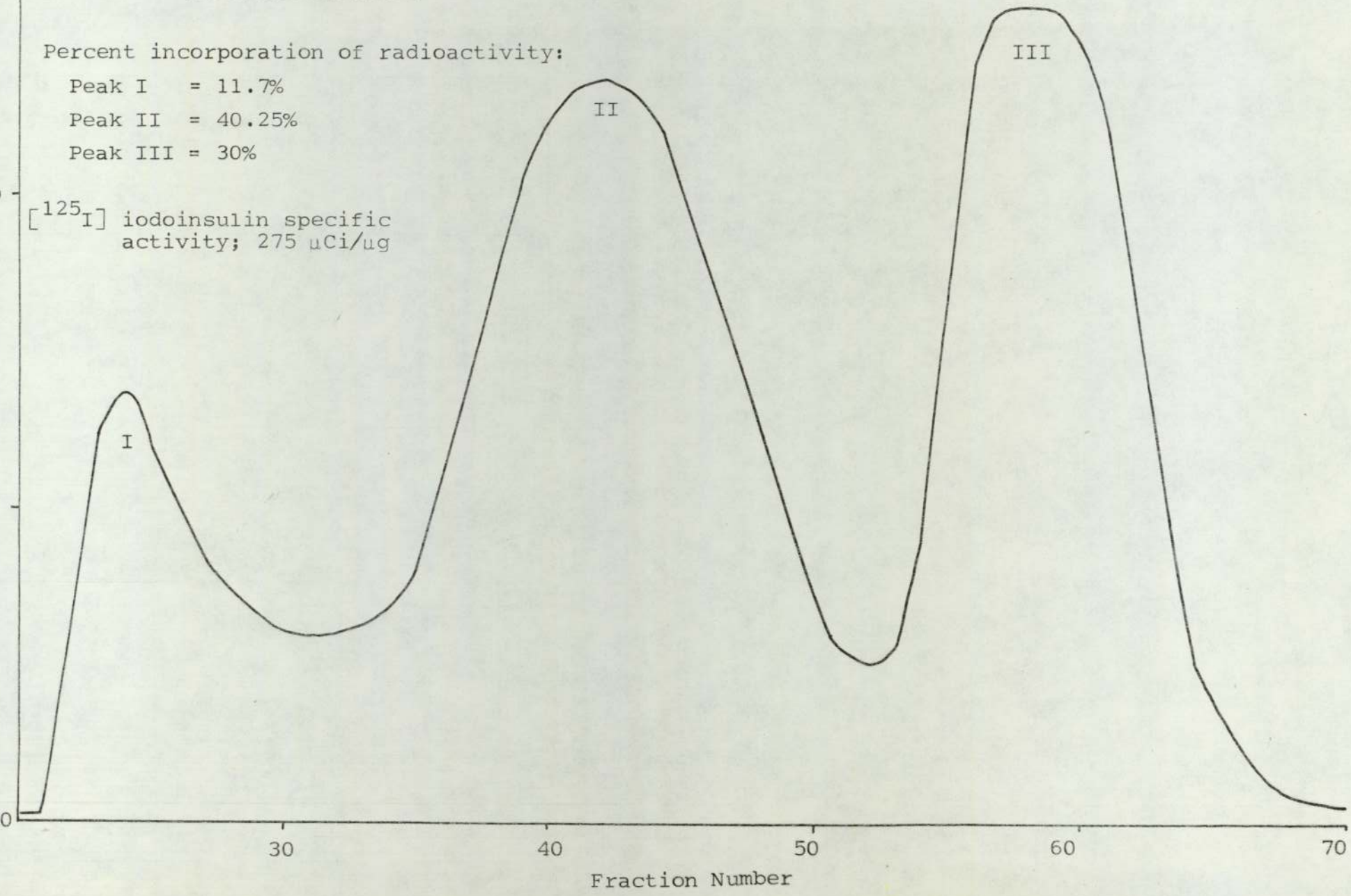
Procedure. The following reagents were added to a 1.5ml plastic microfuge tube:

0.2M phosphate buffer, pH 7.4	50µl
2.5µg Porcine monocomponent insulin in azide free buffer	25µl
Enzymobead reagent	50µl
1.0mCi $Na^{125}I$	10µl
1% β-D-glucose	25µl

The reagents were mixed and the iodination was allowed to proceed for 25 minutes. The reaction was quenched by centrifugation in a Beckman microfuge for two minutes, the supernatant was then applied to a G50 Sephadex column in the usual way.

Results. Figure 11 shows the elution profile of the reaction products of the Enzymobead technique. The profile is comparable with that obtained with the chloramine-T method, except that there is a greater incorporation of iodine into insulin. This is not necessarily desirable as this may lead to a higher degree of iodination ie. di- or triiodination. Also the proportion of damaged insulin was not decreased by this method but in fact was slightly raised ie. 11.7% damage with Enzymobeads and 8.8% damage with the chloramine-T method. Whilst the specific activity gained is satisfactory, this method may be more useful for labelling more labile proteins, such as glucagon. The chloramine-T method was the method of choice for all subsequent iodinations.

Figure 11. Elution profile for labelled porcine insulin produced by Enzymobead iodination using Sephadex G50 fine



Preparation of cells/tissues for receptor binding studies

a) Isolation of mouse hepatocytes

Procedures for the isolation of viable rat hepatocytes are well established and the various techniques used produce a high yield of cells with greater than 90% viability (Berry and Friend, 1969; Seglen, 1973; Juul & Jones, 1982). Howeve the same cannot be said for the mouse hepatocyte and the methods available in the literature for their isolation provide cells with between 70 and 80% viability (Capuzzi et al., 1971; Kahn et al., 1973). In the present study a modification of the method of Kahn et al. was used to prepare hepatocytes.

Procedure. The apparatus used in this procedure is represented diagrammatically in Figure 12. The mouse is anaesthetised by an intramuscular injection of sodium pentobarbitone (Sagatal) and an incision is made in the abdomen to expose the hepatic portal vein. One ligature is placed around the vein above the entry of the mesenteric artery and another is placed distal to the site of cannulation, above the entry of the splenic artery. The latter ligature is secured and a small incision is made above this tie and the PP10 cannula is inserted. The other ligature is then tied to maintain the position of the cannula.

Perfusion then commences with 30ml of HEPES buffer containing heparin and EDTA (Table 2), as soon as the liver blanches the vena cava is cut to allow a flow through the liver. EDTA is included to remove calcium from the liver, digestion of the liver is improved if calcium is removed from the liver prior to perfusion with collagenase-containing buffer (Seglen, 1972). It has been proposed that the dispersion promoting effect is caused by the removal of a

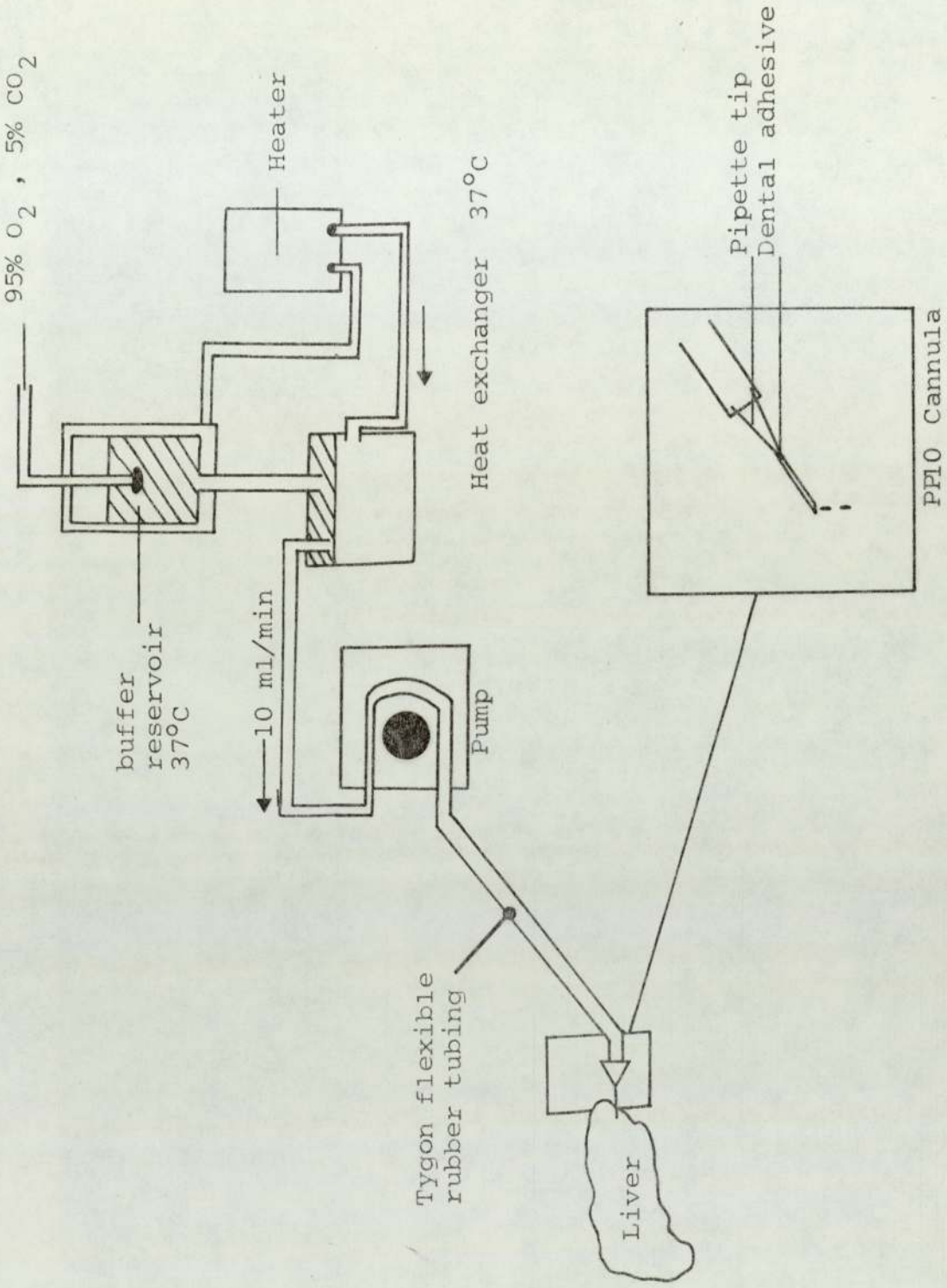


Figure 12. Liver perfusion system for the isolation of mouse hepatocytes.

Table 2. Optimal incubation criteria for insulin receptor binding assays using mouse hepatocytes, human erythrocytes and mouse soleus muscle.

Cell or tissue	Time (Hours)	pH	Buffer	Separation Medium
Mouse Hepatocyte	2	7.8	Buffer 4 + 0.5% BSA + 8 mg/ml bacitracin	Cold Buffer 4
Human Erythrocyte	3	8.0	Buffer G + 1% BSA	Di-n-butyl phthalate
Mouse soleus muscle	4	7.4	Krebs-Ringer Bicarbonate + 0.5% BSA	Cold Saline

calcium dependent hepatic adhesion factor (Modjanova and Malenkov, 1973). The heparin is included to ensure that blood clots do not form and reduce the flow of perfusate in the liver.

The EDTA buffer is followed by 30ml of a calcium free buffer (Table 3), as collagenase is a calcium dependent enzyme it is necessary to remove EDTA before perfusing with collagenase. The flow rate used for all the buffers is 7ml/minute, this was found to be optimal for the production of viable hepatocytes. A fast flow rate is necessary to maintain the buffer temperature at 37°C, to optimise collagenase activity and also to minimise exposure of the hepatocytes to collagenase. If flow rates are too high mechanical disruption of the liver cells is increased, thus reducing viability.

The third perfusate contains calcium and collagenase (0.5mg/ml), and this perfusion continues until the liver has swollen to double its original size. If the liver is prodded gently with forceps it will appear loose and soft when digestion is complete. The collagenase concentration used is only half that suggested by Kahn et al. and it is probably the presence of calcium in the collagenase buffer combined with the increased flow rate (Kahn et al. used 3-4 ml/min) that allows this reduction of collagenase. In addition, expensive hyaluronidase was not included in the digestion buffer, and this had no detrimental effect on cell yield or viability, as previously suggested by Wagle (1974). When digestion is complete the liver is removed gently, rinsed in cold buffer 4 (Table 3) and gently teased with blunt forceps to release the dispersed liver cells. Any connective tissue can be removed at this stage. The

dispersed cells are filtered through a 100μm nylon mesh (Sericol, U.K.), to remove undigested hepatic tissue, and then gassed for 30 seconds (O_2:CO_2, 95%:5%). The digestate is centrifuged at 100g for five minutes at 4°C (MSE Chilspin) to remove non-viable cells and Kupffer cells. The cell pellet is washed once in buffer 4 and then resuspended in incubation buffer (buffer 4 containing 0.5% BSA and 0.08% bacitracin) and filtered through a 60μm nylon mesh.

Aliquots of the cell suspension are taken to assess cell viability and cell concentration, the latter is adjusted to 2×10^6 cells/ml with incubation buffer. Cell viability is routinely assessed using the trypan blue exclusion test. 20μl of the cell suspension is added to 400μl of buffer 4 and 500μl of 0.4% trypan blue, after two minutes the cells are examined and those showing a blue stain throughout the cell are taken as non-viable. Cells with an intact plasma membrane will exclude the dye molecule and are thus taken as viable. The cells produced in the above method routinely exhibited viability of greater than 75%.

b) Preparation of erythrocytes for use in receptor binding assays

Isolated circulating human erythrocytes have been shown to possess specific insulin receptors (Gambhir et al., 1977; Gambhir et al., 1978) and since they are readily available in large quantities they are of great use in clinical trials involving radioreceptor assays. As little as 10-15ml of blood is required to perform the assay, giving it a major advantage over the monocyte radioreceptor assay which often requires 100ml or more (Schwartz et al., 1975).

The erythrocyte does however have certain drawbacks, a major one is that glucose metabolism in the erythrocyte is not dependent on insulin concentration, whereas insulin does regulate glucose metabolism in the monocyte. Despite the fact that neither the monocyte nor the erythrocyte are insulin target tissues, they do bear insulin receptors. Moreover, there is substantial evidence to suggest that the insulin receptor status of these cells may reflect the receptor activity of the major target tissues for insulin ie. fat, muscle and liver (Olefsky, 1976a;Dons et al., 1981a). The erythrocyte was thus chosen as the receptor model in the clinical trial of metformin. The method used for the isolation of the erythrocytes, as well as the radioreceptor assay procedure, were essentially that of Gambhir and colleagues (Gambhir et al., 1978).

Procedure. A 12ml sample of blood was taken from the antecubital vein into a heparinised tube. 6ml of blood was inserted into two plastic tubes and centrifuged (10 minutes at 400xg, 20°C) and the resulting plasma layers removed. Both cell pellets were resuspended in an equal volume of physiological saline (0.9g/100ml) and layered onto 3ml of a Ficol-Hypaque mixture (Ficol-Paque, Pharmacia, U.K.). The contents of each tube was centrifuged for 20 minutes at 400 xg , at room temperature. The erythrocytes were thus separated from the other cells on a density basis. The saline, mononuclear cells, Ficol-Paque, granulocyte phases and the upper layer of the erythrocyte phase were aspirated off. Each red cell pellet was resuspended in an equal volume of saline and the above procedure repeated. Each erythrocyte pellet was then resuspended with two volumes of buffer G (Table 4) to equilibrate the cells and the contents

Table 3. Buffers used for the hepatocyte isolation are given below, all quantities are in grams per litre unless otherwise stated. Buffer 4 is according to Segler (1973). The binding incubation buffer was buffer 4 supplemented with 1% BSA and 0.8% bacitracin. BSA was present to reduce adhesion of insulin to the incubation vessel and thus reduce non specific binding. Bacitracin is present to reduce extracellular insulin degradation. Several organic buffers (HEPES, TES, TRICINE) are included in buffer 4 to maintain pH throughout the binding assay.

	Buffer 1	Buffer 2	Buffer 3	Buffer 4
NaCl	8.30	8.30	8.30	4.00
KCl	0.50	0.50	0.50	0.40
$CaCl_2.2H_2O$	-	-	1.70	0.18
$MgCl_2.6H_2O$	-	-	-	0.13
KH_2PO_4	-	-	-	0.15
Na_2SO_4	-	-	-	0.10
HEPES	2.40	2.40	2.40	7.20
TES	-	-	-	6.90
TRICINE	-	-	-	6.50
1M NaOH (ml/l)	7.50	7.50	8.00	50
Heparin (ml/l) (200 U/ml)	1.00	-	-	-
EGTA	0.49	-	-	-
Collagenase	-	-	0.50	-
pH	7.4	7.4	7.5	7.8

Buffer G	
HEPES	11.91
Tris	6.05
$MgCl_2.6H_2O$	1.78
EDTA	0.58
D-glucose	1.80
NaCl	2.92
KCl	0.37
BSA	10.00
Bacitracin	8.00
$CaCl_2$	1.47
pH	8.0

Table 4. Buffer G is used in the isolation of erythrocytes and in the erythrocyte insulin binding assay (Gambhir et al., 1978). Gambhir and co-workers recommend 0.1% BSA in the buffer G, this has been increased to 1% to minimise tracer adhesion to incubation tubes, cell debris etc. This did in fact reduce non specific binding from above 20% with buffer G supplemented with 0.1% BSA, to approximately 15% with buffer G and 1% BSA. Bacitracin is again present to reduce any extracellular insulin degradation, though this is not a major problem with the erythrocytes. All quantities are in grams per litre.

centrifuged for ten minutes at 400g, at 20°C. The supernatant was removed and the sedimented cells of the two tubes combined and resuspended in buffer G to a total volume of 11ml. The erythrocyte cell count was estimated using a Coulter Counter (Coulter Electronics, Model ZBI) and was adjusted to 4.4×10^9 cells/ml.

As mentioned in the introduction, insulin receptor concentration varies with the age of the erythrocyte (Dons et al.,1981b) and this should be taken into account when designing a clinical trial. For this reason the volunteers in the trial acted as their own controls (see page 126) and so an estimation of reticulocyte population and red cell age were not necessary.

Cells were more than 95% viable as judged by the trypan blue exclusion test.

c) Isolation of the mouse soleus muscle

The soleus muscle is a slow twitch red skeletal muscle, in which it is possible to measure both insulin binding and insulin dependent postreceptor effects (Le Marchand and Freychet, 1978; Le Marchand-Brustel et al., 1978). The procedure adopted for the isolation of mouse soleus muscle is that described by Maizels et al. (1969).

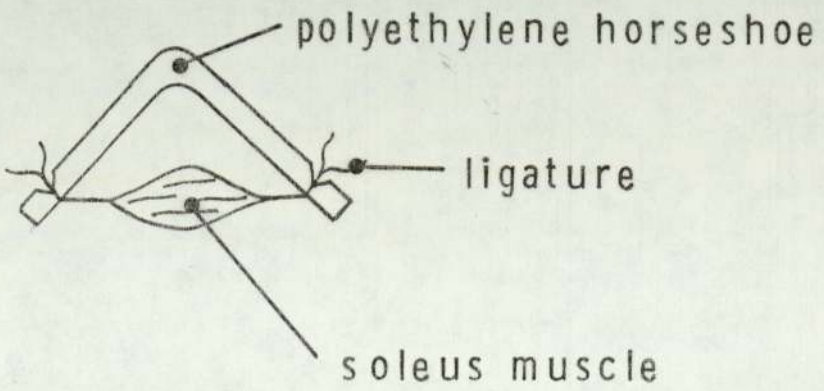
Procedure. Fed mice were killed by cervical dislocation and the soleus muscles from the right and left hind limbs removed as follows. The hind limb was excised from the body using scissors and the overlying skin removed by making a cut around the ankle. The limb was anchored to a dissecting tray with pins inserted into the paw and upper portion of the limb, so as to expose the achilles tendon and the latter was then severed distal to the suture. The gastrocnemius and soleus muscles were gently teased away from the under-

lying tissue. It was important not to damage the muscle in any way since trauma has been shown to hinder normal muscle metabolism (Chaudry and Gould, 1969) and invalidate any studies of the biological effects of insulin.

A second suture was then placed around the proximal tendon of the soleus muscle and the tendon was cut proximal to the suture. The soleus muscle was then peeled away from the gastrocnemius by blunt dissection and the gastrocnemius muscle was cut away from the achilles tendon to free the soleus muscle completely.

Removing the soleus muscle in this way involves severing of the tendons at each end of the muscle, leaving the muscle intact. The muscles were weighed, muscle weights varied with the age of the animal and were in the range 7-15mg, and their tendons secured across the open ends of a polyethylene horseshoe (Figure 13) with 4/0 non capillary braided suture (Wright Dental Supplies Ltd., Birmingham), to maintain the muscle under slight tension during incubation and thus mimic the normal physiological posture of the muscle. Isolated muscles were rinsed in saline (0.9g/100ml) and then used in either insulin receptor binding or postreceptor studies.

Figure 13

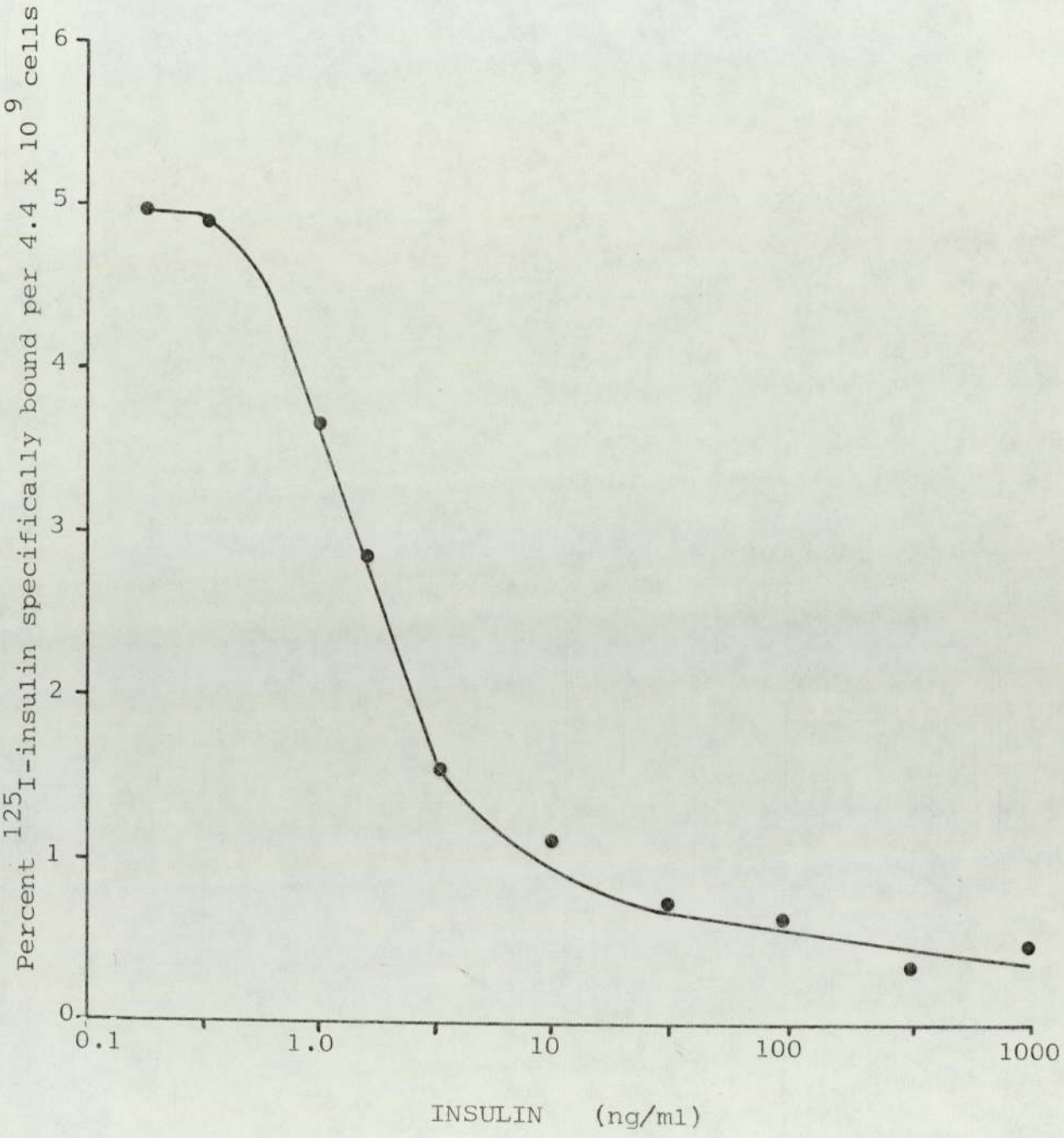


The radioreceptor assay for insulin

As described in the introduction, the insulin receptor is a glycoprotein with an apparent molecular weight of 350,000 possibly consisting of 4 subunits (2α, 2β) and is located in the plasma membrane of target cells. More recent reports have suggested that the receptor is a protein kinase, where activity may be regulated by phosphorylation-dephosphorylation reactions (Kasuga et al., 1982). Receptors have been identified in a variety of cells and tissues including adipocytes, hepatocytes, placenta, fibroblasts and circulating blood cells. Although the role of the insulin receptor in certain of these cells, notably the erythrocyte and placenta, is unclear they have been used widely in receptor studies and appear to reflect receptor status in the major target tissues.

The principles behind the techniques used to quantitate the binding of insulin to its receptor on the plasma membrane of target tissues are very similar to those of radioimmunoassay. A receptor bearing cell or tissue preparation is incubated with a fixed amount of radioactively 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 14). Non specific binding of the labelled insulin, to the sides of the incubation vessel, cell debris etc., must be taken into account and subtracted from values obtained for total binding to provide the level of specific binding to the insulin receptor. Therefore in each assay one incubation vial will contain cells, tracer and a supraphysiological

Figure 14. Inhibition curve for ^{125}I-insulin binding to a normal human erythrocyte preparation.



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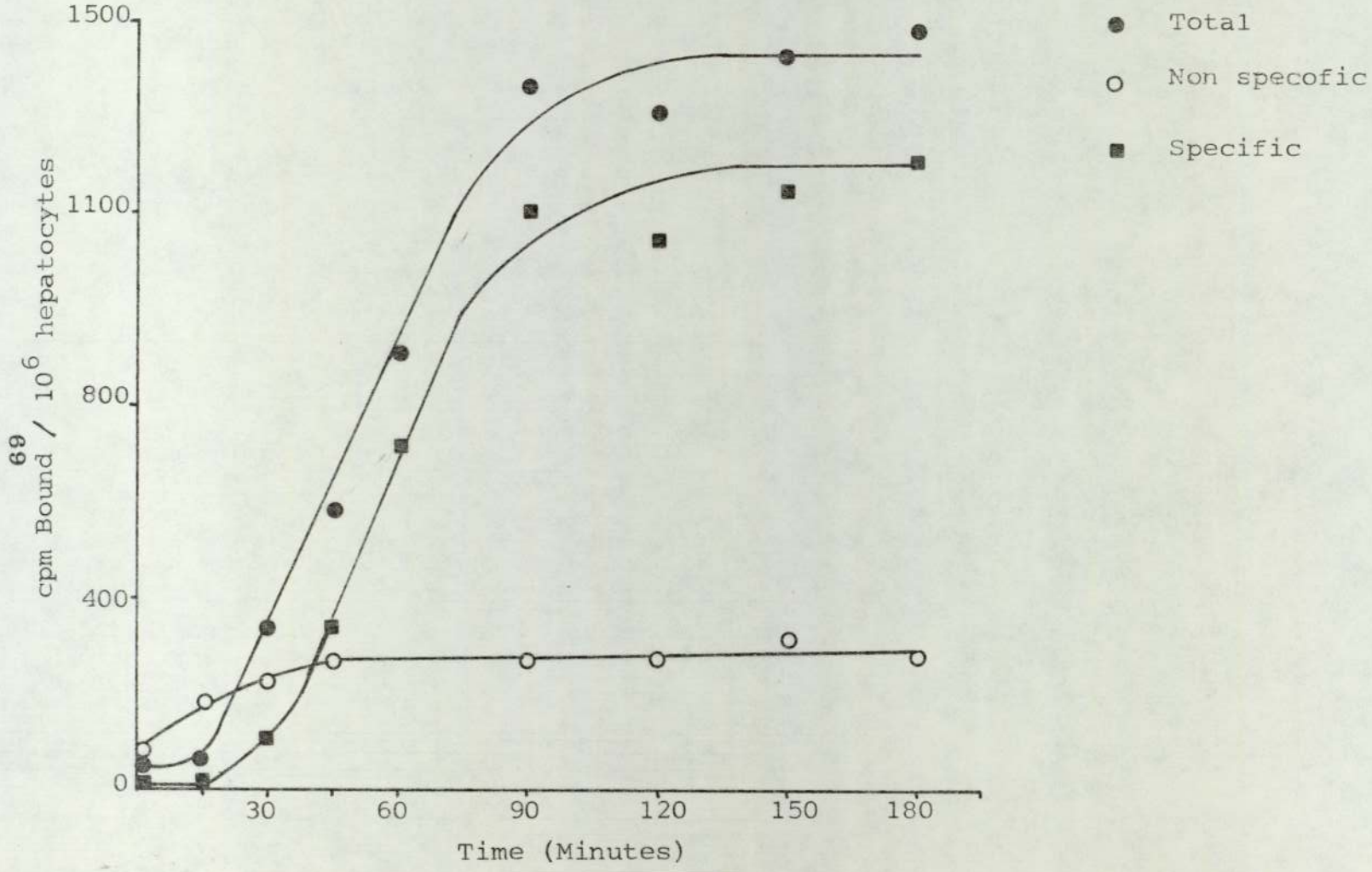
concentration of unlabelled insulin and the activity in this tube represents non specific or non physiological binding (Figure 15). This precaution cannot however take into account any effects of contaminating cells eg. Kupffer cells in the hepatocyte preparation, on insulin binding kinetics. It is therefore important to use a cell preparation that is as homogeneous as possible.

The incubation is allowed to reach a steady state (Figure 15), when the rate of association of insulin with its receptor is approximately equal to the rate of dissociation. At steady state the bound labelled insulin is separated from unbound (free) labelled insulin and counted on a gamma counter (GAMMA SET 500, ICN). The bound and free fractions are usually separated by centrifuging the cell suspension through ice cold buffer or inert oil, depending on the cell type used.

The time taken to reach steady state binding varies greatly between cell types and although raising the incubation temperature will shorten this time, the level of binding will be reduced (Figure 16). The reason for the inverse correlation between insulin binding and temperature is not known for certain, but is thought to be caused by increased dissociation of insulin from the receptor at higher temperatures (De Meyts et al., 1976). The optimal binding of insulin to its receptor also exhibits a sharp pH optimum (Figure 17) of between 7.8 and 8.0 for most cells and tissues investigated. Other factors affecting the binding of insulin to its receptor, as well as information on the binding characteristics of insulin receptors from a variety of tissues and species, are detailed in a review by Ginsberg (1977b).

Figure 15. The binding of [^{125}I] monoiodoinsulin to mouse hepatocytes at 20°C



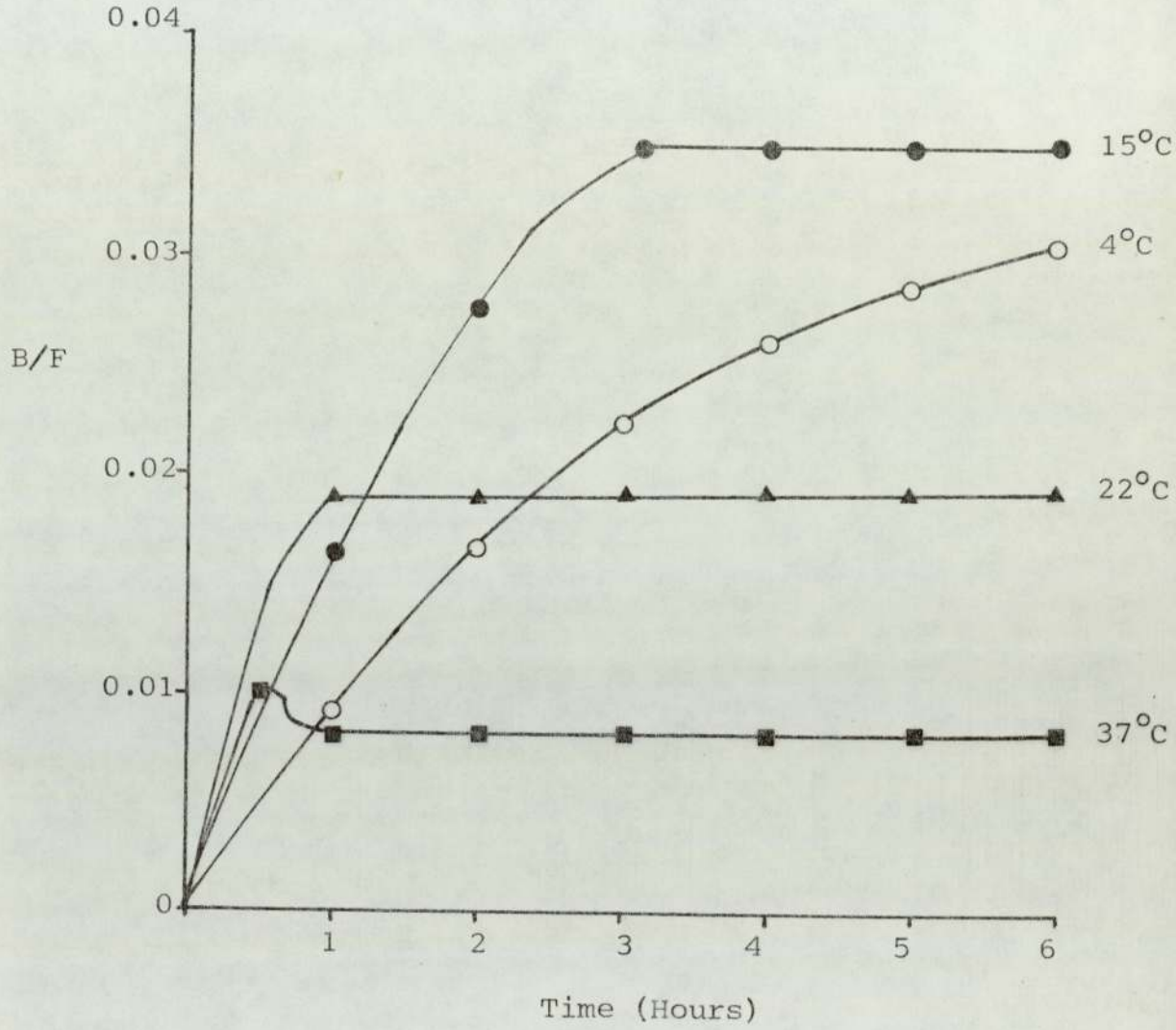


Figure 16. Effect of temperature on binding of insulin to avian erythrocyte membranes. (Ginsberg, 1977b).

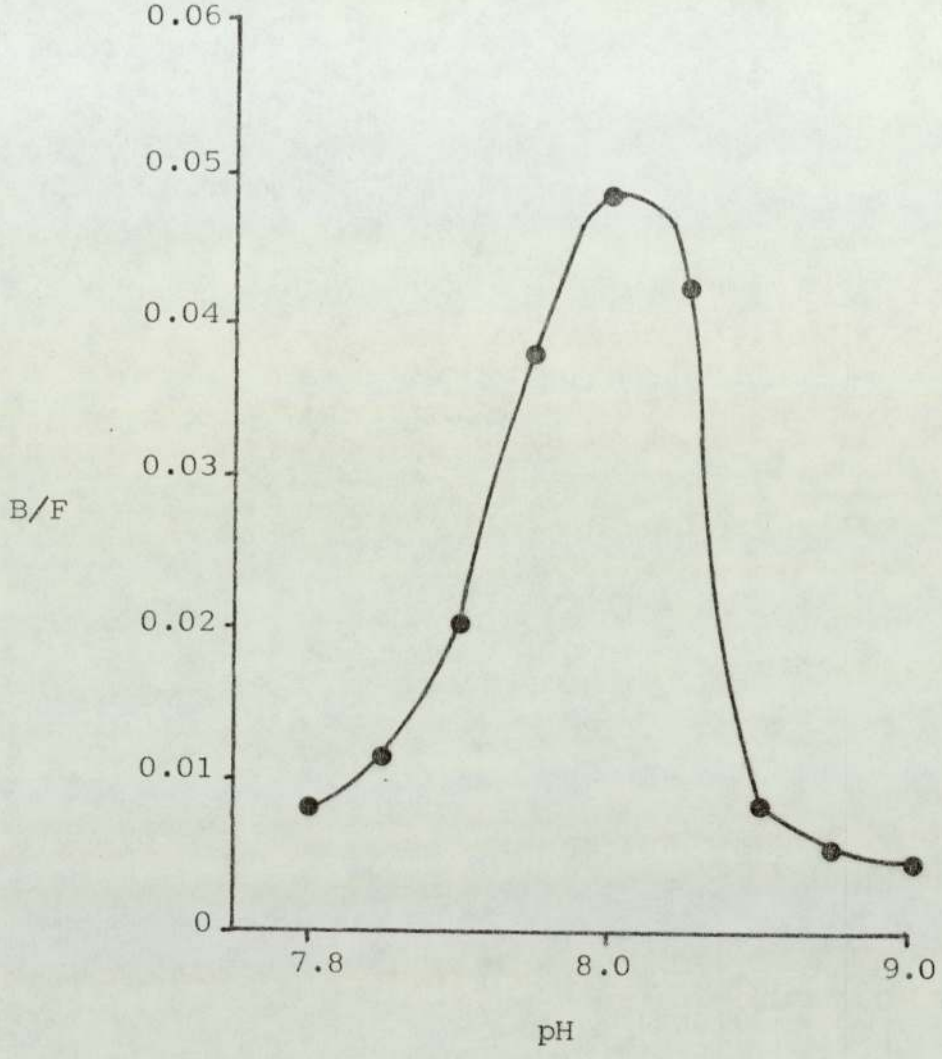


Figure 17. Effect of pH on insulin binding to avian erythrocytes, incubated for 3 hours at 15°C. (Ginsberg, 1977b).

To obtain a reproducible radioreceptor assay the physical criteria of incubation must not only be constant between assays, but should preferably be optimal for the cell or tissue used. The optimum binding criteria were determined for each of the three preparations used in the present work (Table 2).

Fed mice were used throughout the animal studies in an attempt to observe insulin receptor status under normal physiological conditions. However, this does in itself present problems because insulin levels in mice allowed food ad libitum will show greater variability than those in fasted mice. As the level of circulating insulin affects receptor activity (Soman and De Fronzo, 1980; Insel et al., 1980), any variation in plasma insulin concentration will be reflected in an alteration in the level of insulin binding. In order to minimise this variability the receptor binding assays were carried out at the same time each day (10.00h) and circulating plasma insulin and glucose levels were also monitored.

Experimental details of the binding assays used for hepatocytes, erythrocytes and isolated soleus muscle preparations are provided below. This is followed by a description of the methods used for the computation and analysis of insulin receptor binding data.

The isolated mouse hepatocyte insulin binding assay

The method used is a modification of the technique originally described by Kahn and colleagues (1973). Mouse hepatocytes were isolated as previously described (see page 56) and were suspended in incubation medium, consisting of buffer 4 (Table 3) supplemented with 1% BSA (to reduce non specific binding) and 0.08% bacitracin. 400µl aliquots

of the cell suspension were dispensed into 1.5ml microfuge tubes (PPR15, Beckman Ltd., High Wycombe) containing 50µl of ^{125}I-labelled insulin (2.5×10^4 cpm or 0.25nM per tube) and 50µl of a range of unlabelled insulin standards. Seventeen different insulin concentrations were used from 0 to 10^5 ng/ml with a concentration of 10^5 ng/ml representing non specific binding (NS). Each incubation was performed in triplicate. The microfuge tubes were sealed, inverted to mix the contents and placed in a water bath at 20°C for two hours. The tubes were gently shaken every twenty minutes to ensure thorough mixing of the cells and insulin. At the end of the incubation period the tubes were transferred to an ice bath and 0.5ml of ice cold buffer 4 containing 1% BSA was added to each cell suspension. Each tube was then spun for one minute at 9,000g in a Beckman microfuge (Type B, Beckman Ltd., High Wycombe). The cell pellet was washed once with 1ml of ice cold buffer 4 and the tip of the tube was excised with a heated scalpel and counted on a gamma counter. The counts present in the non specific tube were subtracted from all tubes to give the number of counts specifically bound.

The hepatocyte contains a large amount of insulin degrading activity eg. proteases (Poole et al., 1982) which is released into the incubation medium by non-viable cells. This problem does not arise to the same extent with rat hepatocyte preparations, as it is possible to achieve high levels of cell viability (Juul and Jones, 1982) and thus reduce insulin degradation to a minimum. In contrast, the mouse hepatocyte preparations used in this work may contain upto 25% non-viable cells and for this reason bacitracin is included in the medium. In the absence of this inhibitor

analysis of binding data would be difficult as the concentration of insulin in the medium would be reduced. Bacitracin is an inhibitor of glutathione insulin transdehydrogenase (Roth, 1981). Degradation of insulin was not completely arrested [there is evidence to suggest that two other degradation pathways exist (Poole et al., 1982)] but was greatly reduced, to only 2-3% over a two hour period.

Erythrocyte insulin binding assay

Erythrocytes were prepared as previously described (see page 60) and a 400µl aliquot of the cell suspension was added to LP3 tubes containing 50µl of ^{125}I-labelled insulin (10,000cpm or 0.1nM per tube) and 50µl of unlabelled insulin. Twelve different insulin concentrations were used ranging from 0 to 10^5 ng/ml, again 10^5 ng/ml represented the non specific binding. Incubations were carried out in duplicate in order to keep the volume of blood required to a minimum (10-12ml). In addition it was found that the erythrocyte receptor assay was less sensitive to increasing insulin concentrations and that the amount of labelled insulin maximally bound (ie. in the absence of unlabelled insulin) per cell was much lower than for hepatocytes. Moreover, non specific binding was found to be high (15-20%) in the erythrocyte compared to under 5% in the hepatocyte. All these factors led to the reduction in the number of insulin concentrations used in the assay; a range of concentrations is necessary for accurate binding data but in the case of the erythrocyte assay the range used appears to be optimal and further concentrations, particularly above 100 ng/ml, offer no further advantage. (The complications associated with the analysis of erythrocyte insulin receptor binding data is discussed later - see page 86).

In the erythrocyte assay the incubation period was limited to 3 hours at 15°C. The temperature for incubation is lower than usual to raise the level of binding of insulin to the erythrocyte, as stated in Chapter 2 (see page 68) the level of insulin binding is inversely related to the incubation temperature. Tubes were shaken gently every 30 minutes during the incubation. At the end of the incubation period all tubes were placed on ice and a 100µl aliquot was transferred from each tube to a 400µl plastic microfuge tube (ETH26, Beckman Ltd., High Wycombe) containing 100µl di-n-butyl phthalate and 100µl of buffer G supplemented with 1% BSA. The Beckman tube was capped and centrifuged at 9000g for 2.5 minutes in a Beckman microfuge. The cell pellet is retained in the bottom of the tube and the unbound tracer remains in the aqueous phase, the two being separated by the di-n-butyl phthalate layer. The tip of the tube was excised with a hot scalpel blade and counted for five minutes in a gamma counter and adjusted to take account of background activity.

The soleus muscle insulin binding assay

The soleus muscle was the choice of skeletal muscle because it is thin and so reduces problems of diffusion of tracer into the muscle. The insulin binding assay used for the soleus muscle is essentially that described by Le Marchand and colleagues (1978). Mouse soleus muscles were isolated as previously described (see page 64) and after weighing placed in plastic vials, containing 2ml of Krebs-Ringer bicarbonate buffer supplemented with 2% BSA and 2mM pyruvate, for a fifteen minute preincubation period. At the end of the preincubation period, each muscle was transferred to an incubation vial containing 3ml of Krebs-Ringer

bicarbonate buffer supplemented with 2% BSA, 2mM pyruvate and ^{125}I-labelled insulin (200-250 μCi/μg; 1.0 ng/ml) in the absence or presence of unlabelled insulin. Vials containing no unlabelled insulin represented total specific binding and this was used to calculate the specific binding fraction. Non specific binding was again estimated using an unphysiological concentration of insulin, in this case 8μM, from each mouse one muscle was used to measure total binding and the other non specific binding. The latter was subtracted from the former to provide values for specific binding.

Vials were gassed with O_2:CO_2 (95:5) for five minutes at the start of the incubation period and at 30 minute intervals thereafter. The vials were incubated for a total of four hours at 20°C (Figure 18), after which time the muscles were washed five times in 3ml of ice cold saline (each wash step consisted of a five minute incubation in the ice cold saline). After the final wash step the muscles were hydrolyzed in 0.3ml of 1.0M NaOH and a 100μl aliquot was removed for the estimation of protein by the method of Lowry et al. (1951) (see Appendix).

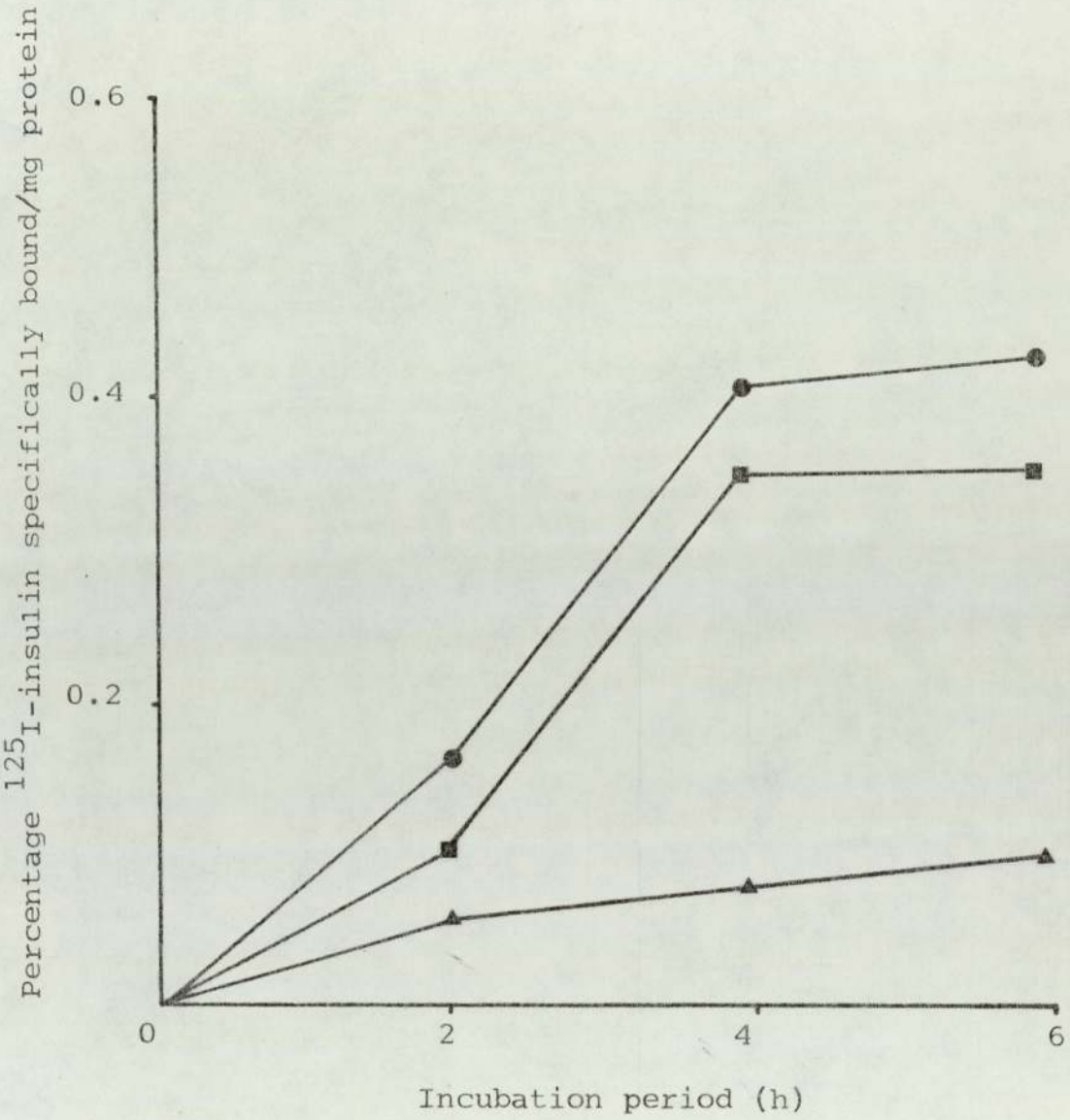
The muscle hydrolysates were then counted and the results were expressed as percent ^{125}I-insulin specifically bound per mg muscle protein.

Analysis of insulin binding data

The methods used to interpret insulin binding data in this thesis, and most commonly in the literature, apply to data obtained at steady state binding. Insulin receptor interactions are generally depicted as simple, reversible, bimolecular equilibriums. The binding of insulin to its receptor does not fit all constraints of this model, but

Figure 18. Time course of binding of ^{125}I-insulin to mouse soleus muscle. Soleus muscles were incubated for upto 6 hours at 20°C, with maximal binding being reached by 4 hours.

● = Total binding ■ = Specific binding ▲= non-specific binding



the following set of equations derived from the law of mass action provide the most adequate interpretation of the insulin binding reaction.

The law of mass action states that, at equilibrium:-

$$[H] + [R] \underset{k_d}{\overset{k_a}{\rightleftharpoons}} [HR]$$

Where:- $[H]$ = free insulin concentration

$[R]$ = concentration of unoccupied receptors

$[HR]$ = concentration of insulin bound to receptor sites

k_a = association rate constant for insulin binding

k_d = dissociation rate constant for insulin from its receptor

Expressions representing the affinity of the receptor for the insulin molecule are given by:-

$$K_a = \frac{k_a}{k_d} = \frac{[HR]}{[H][R]} \quad , \text{ where } K_d = \frac{1}{K_a}$$

The total concentration of insulin receptor sites (R_o) and the total concentration of insulin (H_o) in the system can be represented by the expressions:-

$$R_o = [HR] + [R] , \quad H_o = [HR] + [H]$$

The fraction of total receptors occupied by insulin molecules is termed the fractional occupancy ($\bar{Y}$) and as this is a ratio of the two terms $[HR]$ and (R_o) its value ranges from 0 to 1.0:-

$$\bar{Y} = \frac{[HR]}{R_o}$$

The fraction of unoccupied receptors can therefore be written as:-

$$\frac{[R]}{R_o} \quad \text{or } 1 - \bar{Y}$$

Hence $[HR] = \bar{Y}R_o$ and $[R] = (1-\bar{Y})R_o$

The term $\bar{Y}$ is used to assess receptor occupancy at varying insulin concentrations and forms part of the average affinity profile, which is an analysis designed for systems exhibiting negative cooperativity (De Meyts and Roth, 1975) (see page 17).

The mass action equation for the affinity constant now becomes:-

$$K_a = k_a [H] (1-\bar{Y}) R_o$$

and the equation for the dissociation constant is:-

$$K_d = k_d.[\bar{Y}].R_o$$

However, at equilibrium the law of mass action states that $K_a = K_d$, therefore:-

$$k_a[H] (1-\bar{Y}) R_o = k_d.\bar{Y}.R_o$$

$\bar{Y}$ and R_o are common factors on both sides of the equation, and dividing throughout by $\bar{Y}$ and R_o gives:-

$$\bar{Y} = \frac{k_a[H]}{k_d + k_a.[H]}$$

Divide top and bottom by k_a, remembering that $k_d / k_a = K_d$, then:-

$$\bar{Y} = \frac{[H]}{K_d + [H]} \qquad (1)$$

Divide by K_d to give:-

$$\bar{Y} = \frac{K_a[H]}{1 + K_a[H]} \qquad (2)$$

Equations (1) and (2) are the binding equivalents of the classical Michaelis-Menten equation for enzyme kinetics. A plot of fractional occupancy as a function of the total hormone concentration will give a saturation plot or binding

isotherm. In practice the concentration of bound hormone [HR], rather than occupancy, is plotted against total hormone concentration. At the horizontal asymptote, [HR] = R_o and the concentration of unlabelled insulin required to reduce the level of bound tracer to half its value represents the affinity constant for the dissociation, K_d (ie. [HR] = $R_o/2$, [H] = K_d).

When the binding of ligands to macromolecules does obey the law of mass action, the analysis of equilibrium binding data is straightforward. The most popular method of graphic analysis is that described by Scatchard (1949).

Scatchard analysis:-

If $$\bar{Y} = \frac{[HR]}{R_o} = \frac{[H]}{K_d \quad [H]}$$

Then $$[HR]\,(K_d + [H]) = [H].R_o$$

Which can be developed to:-

$$[HR].K_d = -[HR][H] + [H].R_o$$

Divide by [H] and K_d to give:-

$$\frac{[HR]}{[H]} = \frac{1}{-K_d}.[HR] + \frac{R_o}{K_d} \qquad (3)$$

Equation (3) forms the basis of the Scatchard analysis. Since the equation of a straight line is $y = mx + c$, a plot of [HR] / [H] , (bound/free) versus [HR], (bound) should give a straight line. The slope of this line is $1/-K_d$, $(-K_a)$, the ordinate intercept is R_o/K_d and the abcissa intercept gives receptor concentration, R_o . If the value of R_o is in moles, the number of receptor sites per cell can be expressed as:-

$$\text{Number of sites per cell} = \frac{R_o \times (6.022 \times 10^{23})}{\text{number of cells}} \; *$$

Where * = Avagadros number

However, the Scatchard plot is only linear in a small number of cases, eg. the binding of growth hormone to its receptors on cultured lymphocytes, and the binding of insulin to its receptors yields a curvilinear Scatchard plot with upward concavity (Figure 19). Traditionally this has been interpreted as an indication of the presence of two or more distinct receptor populations, with different but fixed affinities (Kahn, 1975). The intercepts of the two slopes of the Scatchard plot on the x-axis gives receptor number and the slope of those lines represents the affinity of the receptors. For the insulin receptor two distinct receptor populations have been identified, a high affinity, low capacity (R_1) class and a low affinity, high capacity (R_2) class of receptors.

The curvilinearity of the Scatchard plot can also be taken to represent a single, homogeneous group of insulin receptors with varying affinity (De Meyts et al., 1973). 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 conformation 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 enviromental factors such as temperature, pH and ionic milieu (De Meyts and Roth, 1975).

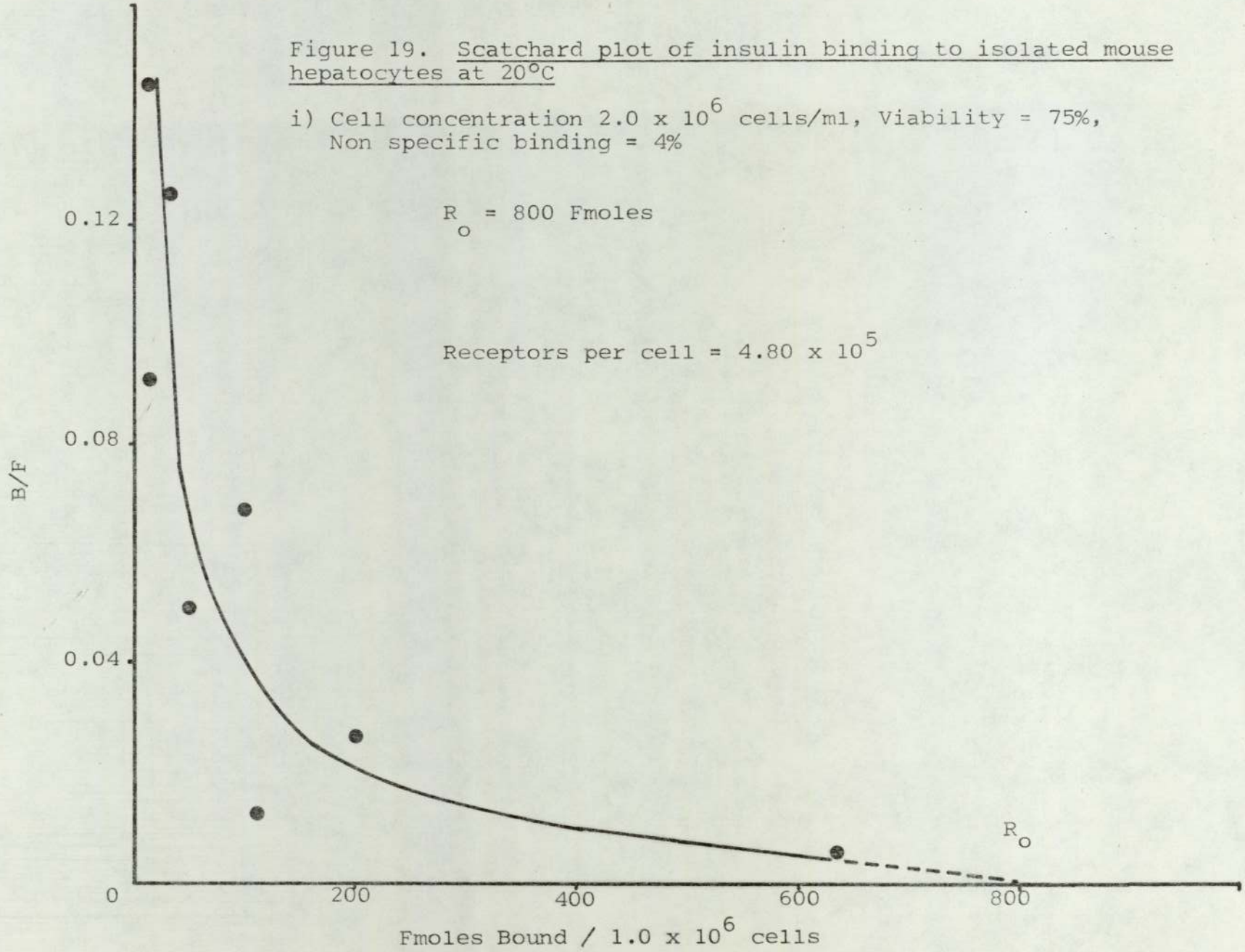
As the Scatchard plot cannot determine the contribution made by the site-site interactions to multiple ligand binding at equilibrium, alternative methods are required. A method of analysis that could indicate the presence of cooperativity is the Hill plot (Hill, 1910). Hill developed this binding parameter to interpret the interaction between

Figure 19. Scatchard plot of insulin binding to isolated mouse hepatocytes at 20°C

i) Cell concentration 2.0 x 10^6 cells/ml, Viability = 75%, Non specific binding = 4%

R_o = 800 Fmoles

Receptors per cell = 4.80 x 10^5



oxygen and haemoglobin.

The Hill equation is given by:-

$$Y = \frac{100.k_x^n}{1 + k_x^n}$$

When applied to the hormone-receptor system the equivalent expression is:-

$$\bar{Y} = \frac{K_a [H]^n}{1 + K_a [H]^n}$$

When n = 1 the above expression reduces to the Michaelis-Menten equation (2), given previously (page 79) and so describes a non cooperative system. To obtain the Hill plot, the equation is made logarithmic and the plot of $\log \bar{Y} / 1-\bar{Y}$ against $\log [H]$ gives a straight line of slope n. If $n>1$, cooperative interactions are positive and if $n<1$ they are negative. The value for the binding data represented as a Scatchard plot in Figure 19 is n = 0.75. Thus cooperativity between mouse hepatocyte insulin receptors is negative, explaining the fall in receptor affinity as the proportion of occupied receptors increases.

The usefulness of the Hill plot is limited and its interpretation beyond that stated previously is questionable (Saroff & Minton, 1972). A new parameter developed by De Meyts and Roth (1975) describes the site-site interactions of the insulin receptor, abandoning the 'affinity constants' of traditional analysis and using instead the average affinity of the receptor sites, $\bar{K}$. If $\bar{K}$, calculated as $(B/F)/R_o - B$ is plotted as a function of fractional occupancy (B/R_o), an 'average affinity profile' is obtained for the binding of insulin to its receptor at varying insulin concentrations (Figure 5, page 17).

This plot reveals that at low insulin concentrations (or occupancy) the receptors are in a limiting high affinity state ($\overline{K}_e$) known as the 'empty site' conformation. As the fraction of receptors occupied increases above a certain threshold, $\overline{K}$ begins to fall off due to increasing negative site-site interactions. A limiting low affinity state ($\overline{K}_f$) of the receptors is eventually reached, known as the 'filled site' conformation. Recently it has been shown that there may be a third conformation of very low affinity and high capacity, known as '$\overline{K}_{super}$' (Gu and De Meyts, 1982). The physiological role of these states in insulin action has not yet been identified, as the $\overline{K}_f$ and $\overline{K}_{super}$ states occur at normally supraphysiological insulin concentrations. Further work is obviously needed to establish a case for negative cooperativity and a function of the various proposed affinity states of the insulin receptor.

It is not necessary to construct an average affinity profile in order to obtain values for $\overline{K}_e$ and $\overline{K}_f$. As shown in Figure 20 they can be determined in the present work.

As the argument for and against negative cooperativity is still not settled, in the present study binding data were analysed assuming both multiple classes of receptors and a single class exhibiting negative cooperativity. Therefore, binding data are represented in terms of high affinity receptor number, low affinity receptor number; total receptor number; $\overline{K}_e$, $\overline{K}_f$ and the 50% inhibition concentration termed the apparent affinity constant, K. The latter is a valid estimate of receptor affinity in comparative studies (Olefsky and Reaven, 1974).
However, in muscle binding studies a full Scatchard plot was not practical since the whole tissue is used. Each

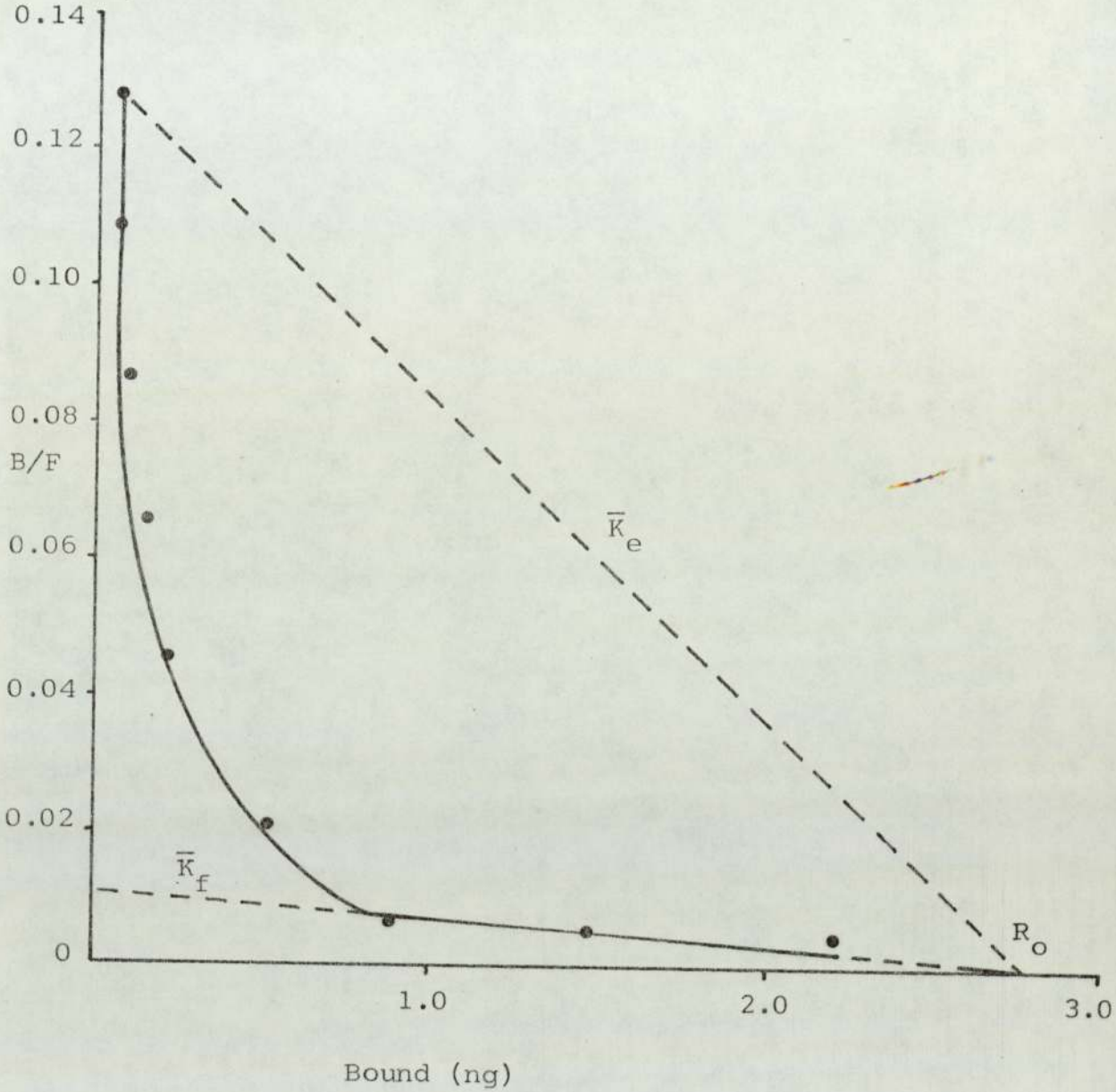


Figure 20. Scatchard plot of ^{125}I-insulin binding to human erythrocytes at 15°C. $\overline{K}_e$ and $\overline{K}_f$ are estimated from the slopes of the lines (-------) indicated on the diagram.

mouse can only provide one value for specific binding (one leg provides a value for total binding and the other non specific binding) and so to produce one Scatchard plot and one value for receptor concentration would require at least ten mice, it is thus expensive as far as animals and time required are concerned.

Early binding studies were performed using a small range of insulin concentrations to show that binding was specific and was inhibited by unlabelled insulin. Several insulin concentrations were also used in a study with streptozotocin treated mice (see page 131), but the aging experiments (see page 163) were performed in the absence or presence of a single concentration of insulin (ie. 8μM, for non specific binding). In this case the level of binding was expressed using the specific cell binding fraction (amount of ^{125}I-insulin specifically bound per mg protein).

In the erythrocyte assay a full Scatchard plot could be produced, but the assay was less sensitive to increasing insulin concentrations (above 100 ng/ml) than the hepatocyte. This led to only two or three points on the horizontal section of the Scatchard curve, making extrapolation to the abcissa more difficult and less reliable than for the hepatocyte. For this reason the plot was linearised by plotting log B/F versus B , which gave the best approximation to a straight line, assessed by linear regression using the least squares method (Figure 21 , also page 203). The doubtful nature of the $\overline{K}_e$ and $\overline{K}_f$ values, and the fact that both affinity states are derived using R_o suggested that the 50% inhibition value, K, should be given for the erythrocyte binding assay.

The Scatchard plots for the hepatocyte assay were

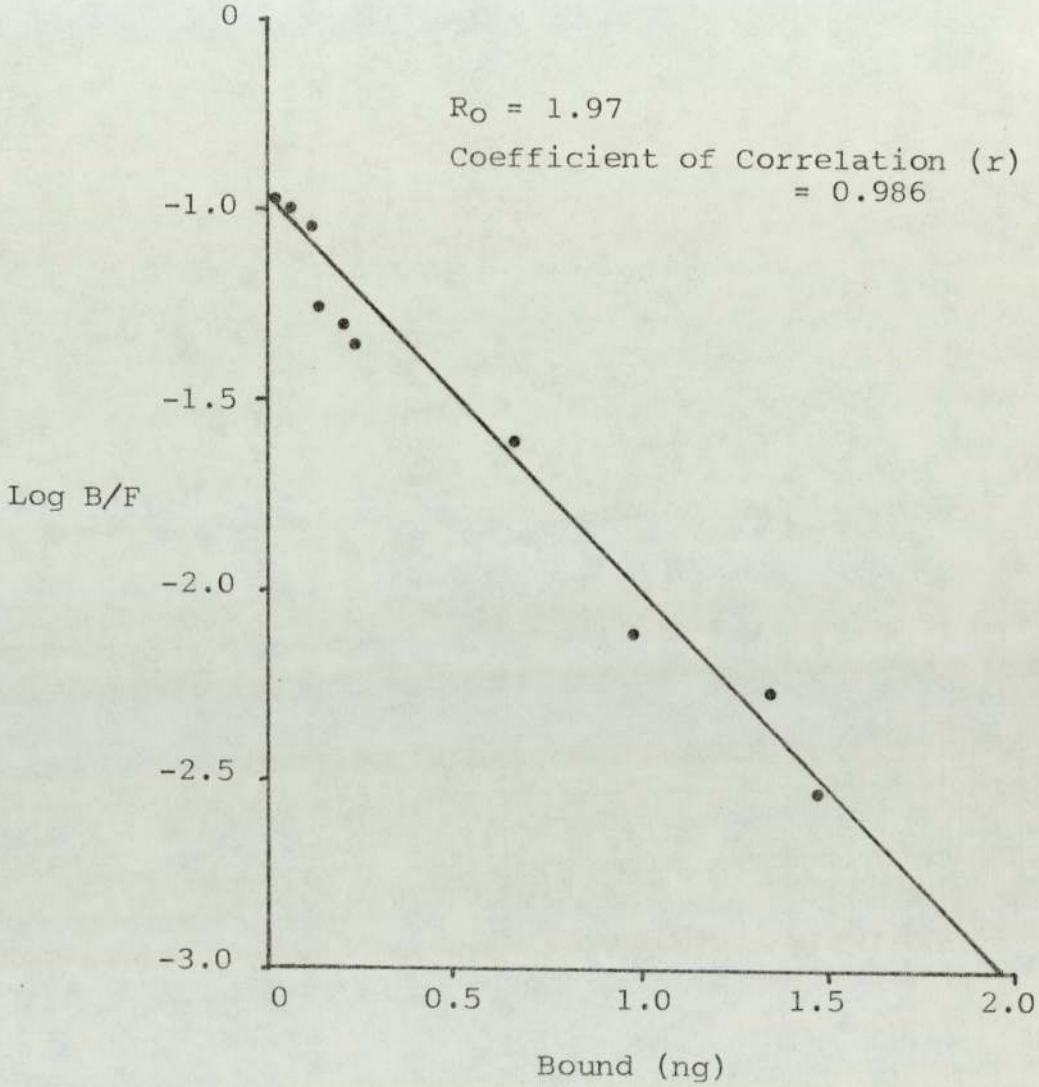


Figure 21. ^{125}I-insulin binding to human erythrocytes at 15°C, plotted as the log of Bound/Free (B/F) versus Bound.

routinely produced using a computer programme and graph plotter, the printout included values for receptor number and the various affinity constants already mentioned, as well as the actual Scatchard graph. The computer programme is fully described in the Appendix and sample printouts are also included (see page 203).

The effect of insulin on the rate of glycogen synthesis in isolated soleus muscle

Skeletal muscle is a major site of insulin action, affecting the rate of glucose uptake and metabolism. Several established techniques were available to monitor the biological effect of insulin, including insulin's effect on glucose uptake, glycogen synthesis and glycolysis. Although the uptake of 2-deoxyglucose provides an estimate of glucose uptake (Kipnis and Cori, 1960) it is desirable to measure this parameter more directly, by measuring the incorporation of ^{14}C-glucose into glycogen (Cuendet et al., 1976). Moreover, Grundleger et al. (1980) have already considered the effect of aging on insulin stimulated muscle glucose uptake and to avoid mere duplication of their work this study monitored insulin effect on the rate of ^{14}C-glycogen formation.

The procedure adopted was based on that originally described by Chaudry and Gould (1969) with some modifications by Cuendet et al. (1976). Initial studies were undertaken to establish the time and insulin dependency of the rate of ^{14}C-glycogen synthesis in isolated mouse soleus muscle preparation.

Time course for the effect of insulin on the rate of ^{14}C-glycogen synthesis

Fed mice were killed by cervical dislocation and soleus

muscles isolated from the right and left hind limbs according to the method of Maizels et al. (1969). The muscles were weighed, attached to polythene holders and individually preincubated in flat bottomed plastic vials (PT2453, Luckham, Sussex), as previously described (see page 64). The vials contained 2ml of Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 2% bovine serum albumin V (KRB-BSA buffer) and unlabelled glucose (5mM). The buffer had been gassed with O_2:CO_2 (95:5) and maintained at 37°C.

After a 15 minute preincubation period, designed to remove endogenous insulin, each muscle was transferred to an incubation vial containing 3ml of KRB-BSA buffer supplemented with glucose (5mM), 1.5μCi U-^{14}C-glucose plus or minus insulin (5nM). In a series of separate experiments muscles were incubated for 30, 60, 90, 120 and 150 minutes. The medium was gently gassed continuously with O_2:CO_2 (95:5) for the first two minutes, after which the vials were sealed with polyethylene stoppers and incubated in a shaking waterbath (90 cycles/minute) at 37°C. At the end of the incubation the muscles were hydrolysed in 0.5ml of 1M sodium hydroxide and glycogen content was determined by the method of Lo et al. (1970). A 50ml sample of the muscle hydrolysate was frozen and stored for protein estimation (Lowry et al., (1951). The rate of glycogen synthesis was expressed as nmoles of glycogen/hour/mg protein, the glycogen produced in the absence of exogenous insulin was taken as the basal rate of glycogen synthesis and was subtracted from that obtained in the presence of added insulin to give the value for the rate of insulin stimulated ^{14}C-glycogen synthesis.

Figure 22 shows the time course of ^{14}C-glycogen synthesis in the mouse soleus muscle preparation and shows that equilibrium is reached by 120 minutes. The plot for glycogen synthesis in the presence of insulin is parallel to that produced under basal conditions where conversion of ^{14}C-glucose into glycogen is caused by endogenous insulin.

Dose dependency of insulin stimulated ^{14}C-glycogen synthesis

Fed mice were killed by cervical dislocation and the soleus muscles isolated, weighed and preincubated as described for the time dependency study (above). The incubation vials contained 3ml of KRB-BSA buffer supplemented with glucose (5mM), 1.5µCi of U-^{14}C-glucose and insulin at concentrations of 0, 0.6, 3.0, 6.0, 30 and 60nM. The vials were gassed with O_2:CO_2 (95:5) for two minutes at the start of the incubation and then sealed with polyethylene stoppers and incubated for 2 hours at 37°C in a shaking waterbath (90 cycles/minute). At the end of the incubation the muscles were hydrolysed in 0.5ml of 1M sodium hydroxide, a 50µl sample of the hydrolysate was removed, stored and frozen for protein estimation (Lowry et al., 1951). The remaining 450µl was used to determine muscle glycogen.

Insulin stimulated ^{14}C-glycogen synthesis (nmole/hour/mg protein) was plotted against insulin concentration (nM) to give the dose response curve seen in Figure 23. Insulin concentrations of 6nM and above produced a maximal rate of glycogen sythesis.

The results obtained for these studies indicate that the isolated mouse soleus muscle is a viable preparation for the demonstration of insulin's postreceptor activity.

Figure 22. ^{14}C-glycogen production by mouse soleus muscles incubated for increasing duration in the absence (o) and presence (●) of insulin (0.3 µg/ml). Values are expressed as nmoles ^{14}C-glycogen produced per minute per mg protein. Values are mean ± SEM,n=6

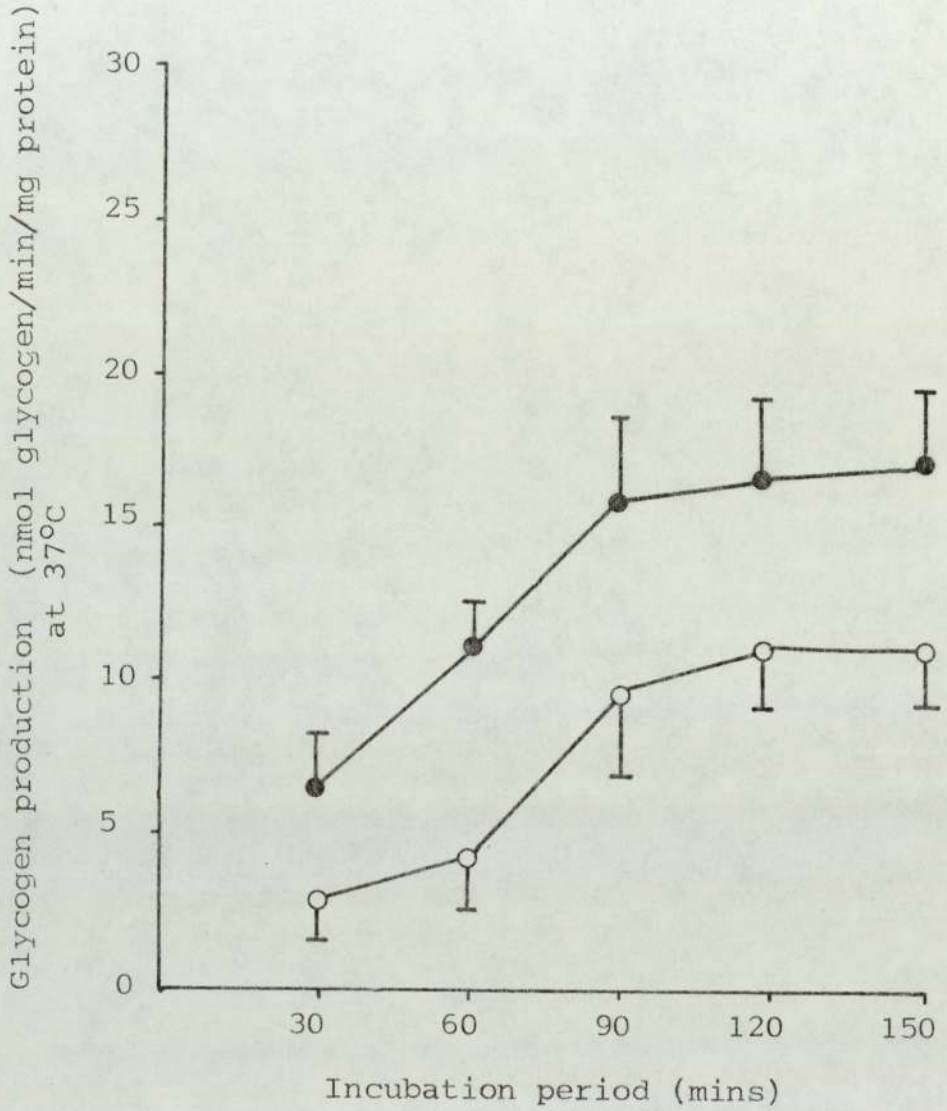
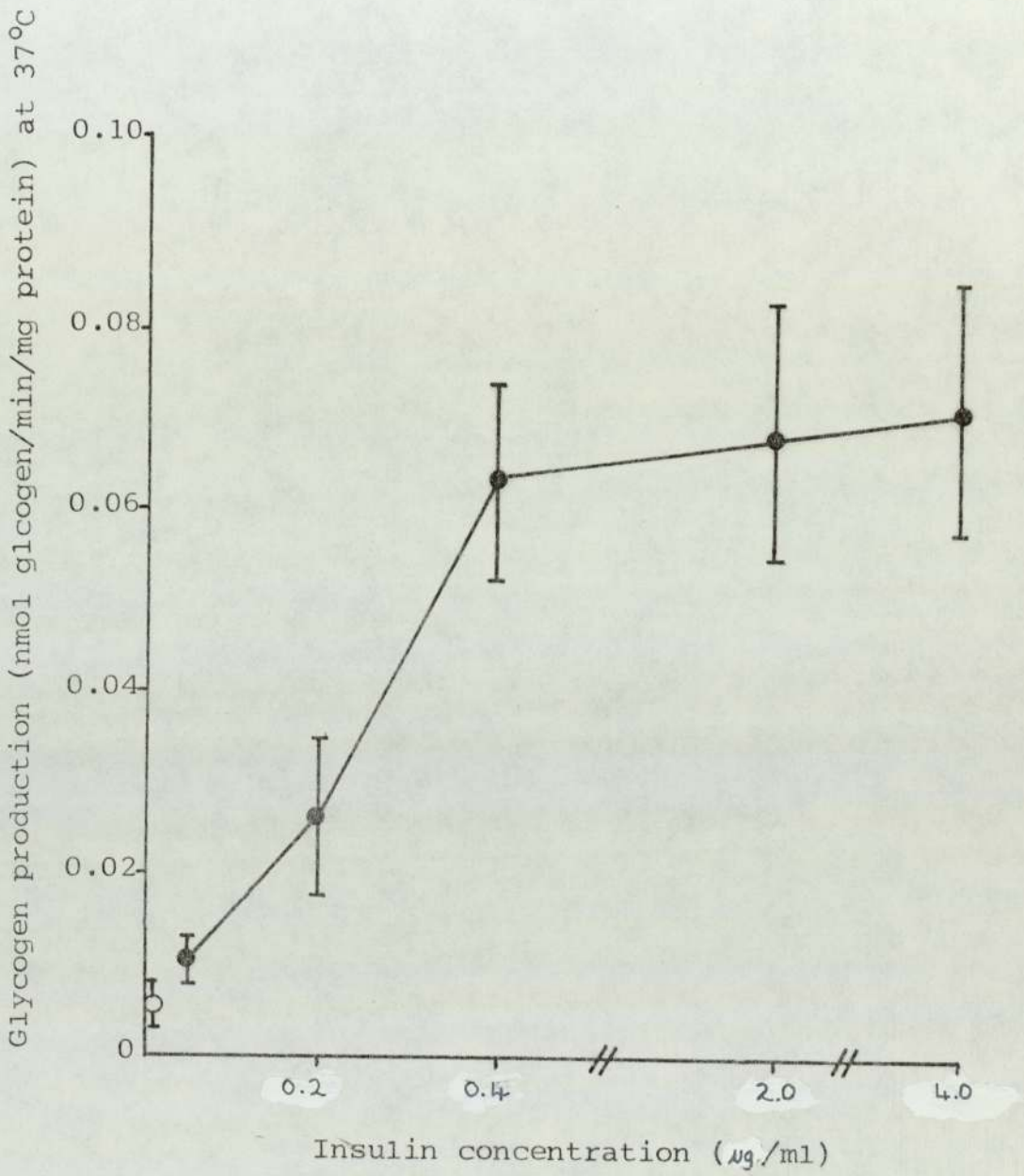


Figure 23. Glycogen production by mouse soleus muscles incubated in the absence (O) and presence (●) of increasing insulin concentrations (0.04, 0.2, 0.4, 2.0 and 4.0 µg./ml). Values are expressed as nmoles ^{14}C-glycogen produced per minute per mg protein. Values are mean ± SEM, n = 6.



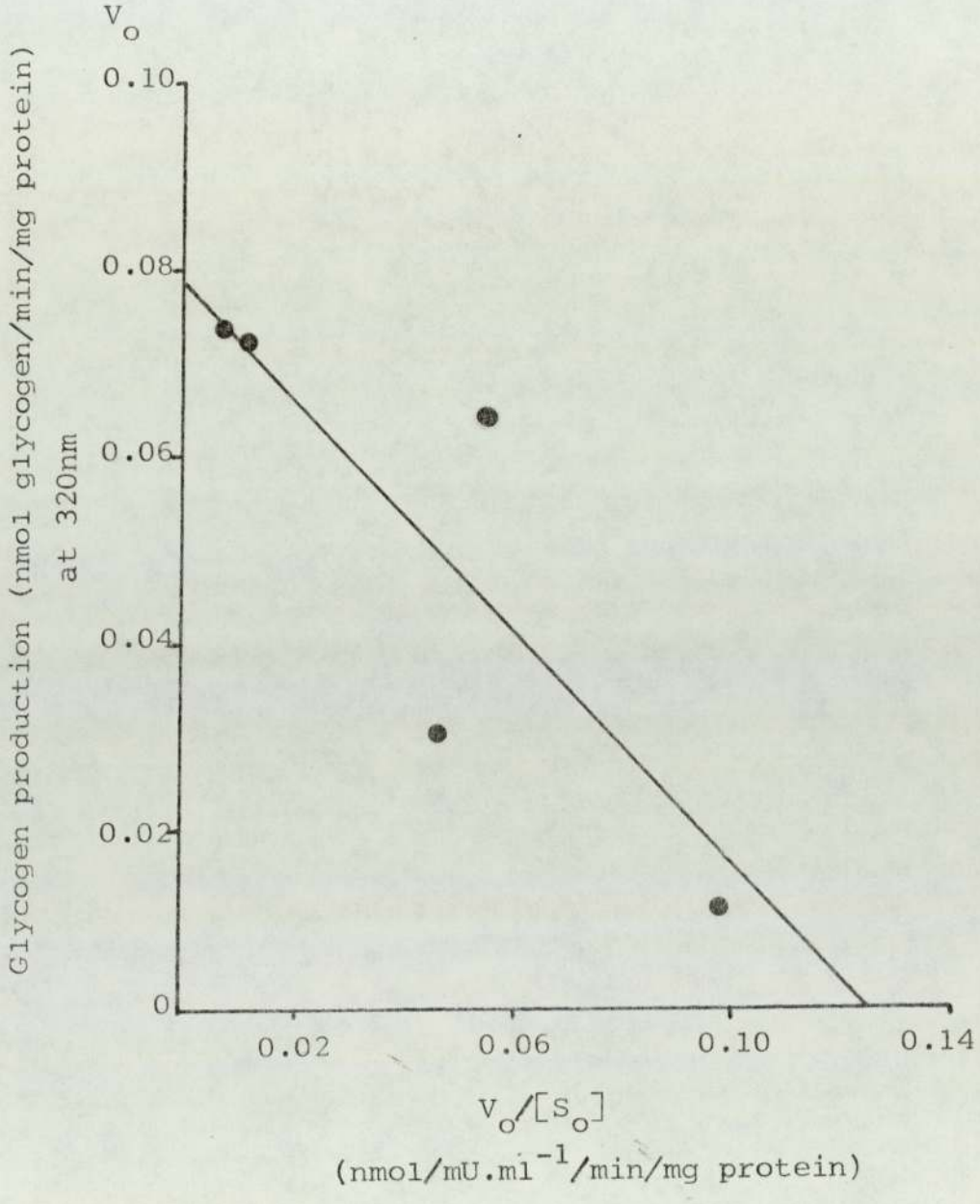
The production of glycogen during the incubation period indicates that glucose uptake is occurring and that glucose is being metabolised to glycogen by the soleus muscle. The stimulatory effect of insulin on the conversion of glucose to glycogen by soleus muscle was confirmed by the observed increase in glycogenesis in the presence of insulin. ^{14}C-glycogen production increased with increasing insulin concentrations and was maximal at insulin concentrations of 6nM and above. An Eadie-Hofstee plot (Figure 24) indicated that an insulin concentration of 3.75nM produced a half maximal increase in the rate of glycogen sythesis. An insulin concentration of 6nM was used in subsequent ^{14}C-glycogen studies, since it has been suggested that a maximal insulin concentration is required to determine insulin responsiveness (see page 29) and hence detect alterations in insulin action at the postreceptor rather than the receptor level (Olefsky, 1981a).

These preliminary observations confirm that the measurement of the rate of ^{14}C-glycogen production in the isolated soleus muscle is both time and dose dependent. In subsequent studies two hour incubations were used in the presence of 6nM insulin.

Statistics

Unless otherwise states Students' t-test was used for statistical comparison and differences between groups were taken to be significant if $p<0.05$.

Figure 24. Eadie-Hofstee plot of V_o versus $V_o/[S_o]$ for glycogen production, where V_o is the rate of glycogen production (nmol/min/mg protein) and $V_o/[S_o]$ is the product of initial reaction rate and the reciprocal of the insulin concentration used (nmol/mU.ml^{-1}/min/mg protein). The intercept on the V_o axis represents V_{max} and the gradient of the line is $-1/K_m$. (Straight line drawn using regression analysis).



CHAPTER THREE

ORAL HYPOGLYCAEMIC AGENTS AND INSULIN RECEPTOR BINDING

Oral hypoglycaemic agents and insulin receptor binding

Orally active hypoglycaemic agents, that are used clinically, can be divided into two main groups, the sulphonylureas and the biguanides. As their mode of action is quite different they will be discussed separately. The general structure of the two groups plus the formulae for glibenc lamide, chlorpropamide and metformin are given in Figure 25.

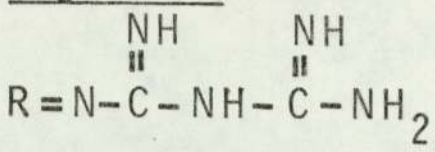
Sulphonylureas

Early work on the hypoglycaemic effect of sulphonylureas was carried out by Loubatieres (1946) and the synthesis of carbutamide in 1955 initiated the clinical trials and the rise in the use of these drugs in the treatment of diabetes mellitus.

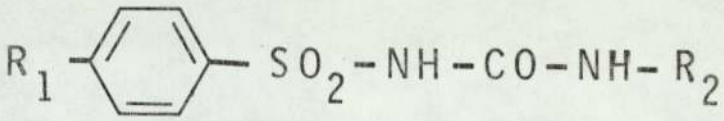
Sulphonylureas have been extensively used in the treatment of NID, type 2 diabetes since the early 1950's. Although the incidence of side effects is low with these drugs, a report by the University group diabetes Program (1970) suggested that tolbutamide may have specific cardiovascular toxicity. However, information regarding the cardiovascular effects of sulphonylureas is still inconclusive, and this question has been adequately reviewed by Levey (1977).

A recent review on the therapeutic usefulness of sulphonylureas (Lebovitz and Feinglos, 1978) has suggested that their application as an antidiabetic drug should be limited to those patients who are forty years or older, of normal weight or obese, with recently diagnosed type 2 diabetes. Furthermore the presence of modest insulin secretion and markedly impaired insulin action would be a good indication for sulphonylurea therapy.

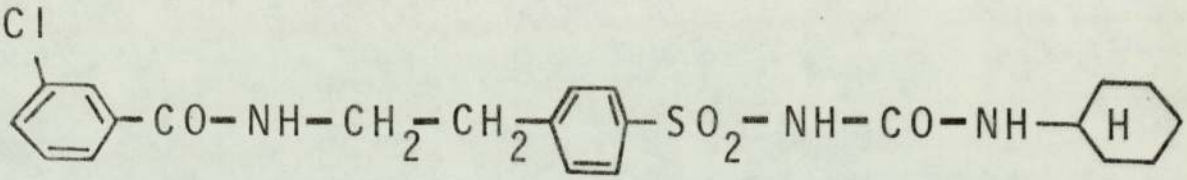
Biguanide



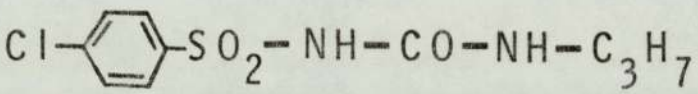
Sulphonylurea



Glibenclamide



Chlorpropamide



Metformin

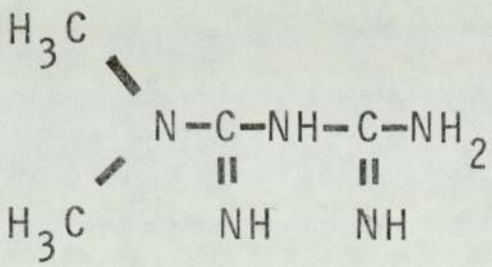


Figure 25. The general structural formulae for sulphonylureas and biguanides, with the specific formulae of glibenclamide, chlorpropamide and metformin.

Sulphonylureas are thought to achieve their antidiabetic effect in two distinct ways, namely an acute pancreatic effect on the β cell, coupled with a chronic extrapancreatic action. Sulphonylureas have been shown to initially stimulate the first phase of pancreatic insulin release (Hellman and Taljedal, 1975; Yalow et al., 1960; Grodsky et al., 1977) thus contributing to the amelioration of hyperglycaemia in the early stage of therapy. However, as treatment progresses the chronic influence becomes inhibitory in nature upon both pancreatic insulin synthesis and secretion (Dunbar and Foa, 1974; Duckworth et al., 1972b; Barnes et al., 1974). As the hypoglycaemic properties of the sulphonylureas persist into the chronic stage of therapy, it seems likely that potentiation of insulin action takes over as the primary mode of action of these drugs. This potentiation of insulin action could be achieved via an effect on the circulating levels of insulin antagonists or alternatively at the level of the insulin receptor itself. Several studies have failed to confirm any effect of sulphonylureas on glucagon secretion, which is not altered in normal or diabetic subjects receiving sulphonylureas (Marco and Valverde, 1973; Kalk et al., 1975).

There is substantial evidence for an effect of sulphonylureas at the receptor level. Sulphonylureas have been shown to increase insulin binding to target cells in vivo (Olefsky and Reaven, 1976b; Feinglos and Lebovitz, 1978; BeckNielsen et al., 1979b) which is accompanied by an improvement in insulin action (Pagano et al., 1982). The improvement in insulin binding is reported to be associated with an increase in insulin receptors in type 2 diabetics, but with an increase in receptor affinity in type 1 diabetic

subjects (Cordera et al., 1982).

In addition to an increase in insulin receptor binding, it is possible that enhancement of insulin action by sulphonylureas might occur at a postreceptor site of insulin action.

Several workers have concentrated on the effect of sulphonylureas at the tissue level, showing a potentiation of insulin action in liver (Blumenthal, 1977) and skeletal muscle preparations (Feldman and Lebovitz, 1969a). Specific effects on target tissues for insulin have also been demonstrated, including an inhibition of liver (Davidoff, 1977) and adipose tissue (Shepherd and Fau, 1977) triglyceride lipase as well as alterations in glucose fluxes in liver and fat cells (Feldman and Lebovitz, 1969b; Shepherd and Fau, 1977). Whether these effects can be attributed to alterations in insulin receptor status and/or modulation of postreceptor mechanisms, remains to be determined.

Biguanides

The first biologically active biguanides were synthesised over a century ago (Rathke, 1879) and despite being in clinical use for some 25 years (Sterne, 1957), they have not enjoyed the same popularity as the sulphonylureas (Van de Kuy, 1980). Much of this can be attributed to the withdrawal in many countries of the early biguanide, phenformin. This was due to the high level of lactic acidotic incidents occurring in patients treated with this drug (Cohen and Woods, 1976; Luft et al., 1978).

Metformin is the only biguanide in clinical use today and although still controversial in some respects, it is being increasingly regarded as a valuable drug in the treatment of obese maturity-onset diabetes (Hermann, 1979).

Lactic acidosis occurs only very rarely (Berger, 1979) and the Canadian authorities have recently discontinued their metformin monitoring programme, which was instigated in 1977 (Biron, 1980).

Both biguanides and sulphonylureas require the presence of insulin for their therapeutic effect (Shen and Bressler, 1977), but unlike the sulphonylureas, biguanides have only an extrapancreatic effect as their primary mode of action (Hermann, 1979; Sterne et al., 1982). Biguanides do not stimulate insulin secretion directly in the clinical situation and in fact it is a regulation to the contrary that usually occurs, indeed metformin will reduce insulin and glucose in the portal blood during an oral glucose tolerance test (Berger and Kunzli, 1970). In non-diabetic pigs, insulin levels were shown to drop in portal blood when the animals were treated with metformin (Kuhl et al., 1976) and in incubated and perfused islets of Langerhans, glucose induced insulin and pro-insulin biosynthesis, has been shown to be inhibited in the presence of high doses of metformin (Schatz et al., 1972a). Data indicating a stimulatory effect with phenformin and metformin have only been obtained with high doses using the perfused pancreas either in isolation or in situ (Loubatieres et al., 1971; Schatz et al., 1972a), and hence appears to be a pharmacological artefact (Sterne and Junien, 1980).

Metformin will reduce previously raised plasma insulin levels in obese diabetics (Vermeulen and Rattiers,1972; Vague, 1976) and obese hyperinsulinaemic non diabetics (Schatz et al., 1972b; Vague, 1976). The effect of metformin on insulin levels is not immediate (Fajans et al., 1960; Madison and Ungar, 1960) and so the overall clinical effect

of metformin is an enhancement of insulin activity producing a fall in blood glucose and an economy of insulin.

The exact mechanism by which biguanides potentiate the action of insulin is not yet certain, but it is known that their primary effect is exerted at the level of the cell membrane (Schafer, 1980). Alterations in metabolic responses occurring secondary to the activity at the cell membrane level are many and varied, including increased muscular glucose uptake (Frayn and Adnitt, 1972; Frayn et al., 1973; Kemmer et al., 1977), decreased intestinal glucose absorption (Lorch, 1971), decreased gluconeogenesis (Meyer et al., 1967, Nattrass et al., 1977), decreased free fatty acid oxidation (Muntoni, 1974) and lipid biosynthesis (Sirtori, 1977; Marquie, 1978). As muscle is a major insulin target tissue and a major site of glucose utilization the effect on muscle glucose uptake and oxidation is thought to be the most important metabolic consequence of metformin therapy (Hermann, 1980).

The action of biguanides at the cell membrane might involve insulin receptors, producing either an alteration in insulin receptor status (number and/or affinity) or alternatively an enhancement of one or more postreceptor mechanisms. Preliminary observations by Holle et al. (1980) with metformin have shown an increase in erythrocyte insulin receptor number both in normal subjects and cultured erythrocytes. The same effect has been demonstrated for phenformin (Cohen et al., 1980). There is no evidence in the literature for any action of biguanides on postreceptor mechanisms.

In 1980, Hermann suggested that there is clearly a need for further investigations into the action of biguanides on

insulin receptors in various cells, together with studies of the metabolic consequences of any altered membrane function.

Aims

On this basis animal studies were initiated to investigate the effects of the biguanide, metformin, on hepatocyte insulin receptor status in both lean homozygous (+/+) and obese (ob/ob) mice of the Aston strain. The latter was used as an animal model for obesity and type 2 diabetes mellitus, for which the biguanides are in the main prescribed. The sulphonylurea, glibenclamide, was used in the studies with lean mice, not only to act as a comparison with the biguanide but also to enable confirmation of the results reported in the literature using other cell models eg. monocytes.

1) The effect of chronic oral hypoglycaemic therapy on insulin receptor status in lean (+/+) mice of the Aston obese mouse colony

Animals. Adult male (10 weeks) lean homozygous (+/+) mice were used in the present study and housed in an air-conditioned room at 22°C ± 2°C, with a regulated lighting schedule of 12hrs dark and 12hrs light. Mice received a standard pellet diet (Mouse breeding diet, Heygate and Sons Ltd., Northampton) and were allowed free access to water.

Experimental protocol.

Two groups of six 10 week old mice were given either metformin (60 mg/kg/day) or glibenclamide (0.4 mg/kg/day) over a subsequent 50 week period. This extended period was used to investigate both the chronic effects of these drugs, and whether any alterations in receptor status occur after extended therapy in aging mice. Both drugs were administered

in the drinking water, and control mice received water alone. The drug doses used were based upon the maximum recommended clinical dose on a weight for weight basis. Fluid intake was monitored, using metabolic cages, and the concentration of the drug was adjusted accordingly, as described by Billingham et al. (1981).

Insulin binding studies

Blood samples (150µl) were obtained from the cut tail tip of fed mice at 10.00hrs on the day of the insulin binding assay. Plasma was separated and assayed for glucose by the glucose oxidase method (Stevens, 1971) using an automatic analyser. Plasma insulin levels were measured using a modification of the double antibody radioimmunoassay technique of Hales and Randle (1963).

Mice were anaesthetized with sodium pentobarbitone (50 mg/kg) by intramuscular injection, and hepatocytes were isolated by a modification of the method of Kahn et al. (1973) as described on page 56. The cell concentration was adjusted to 2.0×10^6 cells/ml and cells were only used if viability was assessed to be >70%. Isolated hepatocytes were then transferred to 1.5ml microfuge tubes (PPR15, Beckman Riic, High Wycombe) and the insulin receptor binding assay was performed as previously described (see page 72). Non specific binding was found to be 4.4 ± 0.5 % (mean ± SEM) of the total binding to hepatocytes. Binding data were analysed using a computer assisted Scatchard plot (see Appendix for programme and sample calculation) (Scatchard, 1949) and presented as high affinity, low affinity and total number of insulin receptors per cell as well as $\overline{K}_e$, $\overline{K}_f$ and the 50% inhibition value, K.

Results

Although experiments were initiated with six mice in each group only four survived, losses were mainly due to fighting.

Table 5 records the body weights, plasma glucose and insulin concentrations and hepatocyte insulin binding data for normal mice treated with metformin (60 mg/kg/day) or glibencalamide (0.4 mg/kg/day) over 50 weeks. Glibenclamide did not significantly alter either body weight or plasma insulin concentrations. Similarly metformin had no significant effect on body weight or plasma insulin concentrations. Both drugs significantly increased plasma glucose concentration. These observations are broadly in agreement with the findings of Billingham et al. (1981). Scatchard analysis of binding data (Figure 26) revealed that hepatocyte receptor number was significantly increased by both drug treatments, by almost two fold with metformin. This increase was due almost entirely to an increase in the number of low affinity receptors. There was no significant change in the number of high affinity receptors. Receptor affinity was unaltered when analysed by the 50% inhibition method (page 84) but 'average affinity' analysis revealed a fall in $\overline{K}_e$ with metformin treatment.

Discussion

Consistent with previous reports using other receptor models (Beck-Nielsen et al., 1979b; Prince and Olefsky, 1980) this study confirms that glibenclamide therapy produces an increase in cellular insulin binding, mediated via an increase in the number of low affinity hepatocyte insulin receptors. In addition, metformin also increased hepatocyte insulin receptor number by raising the concentration of low

Table 5. Chronic effect of metformin (60 mg/kg/day for 50 weeks) and glibenclamide (0.4 mg/kg/day for 50 weeks) on hepatocyte insulin receptor status in normal mice from the Aston obese colony. Values are mean ± SEM of 4 mice. *P<0.05, **P<0.02, ***P<0.01 compared with control group.

	Control	Metformin	Glibenclamide
Body weight (g)	46.0 ± 0.5	49.2 ± 2.3	45.6 ± 2.4
Plasma glucose (nmol/l)	7.4 ± 0.3	10.0 ± 0.4***	10.9 ± 0.7***
Plasma insulin (ng/ml)	2.94 ± 0.59	3.34 ± 0.61	3.30 ± 0.39
Total receptor number (per cell x 10^5)	2.88 ± 0.30	5.29 ± 0.41**	4.39 ± 0.50*
High affinity receptor number (per cell x 10^5)	1.25 ± 0.19	1.19 ± 0.06	1.25 ± 0.17
Low affinity receptor number (per cell x 10^5)	1.61 ± 0.40	4.00 ± 0.41**	2.97 ± 0.54
Affinity constant K_e (nM^{-1})	0.144±0.036	0.072±0.007*	0.120±0.030
Affinity constant K_f (nM^{-1})	0.028±0.010	0.018±0.001	0.030±0.010
Apparent affinity constant (nM)	1.65 ± 0.26	2.13 ± 0.27	1.68 ± 0.03

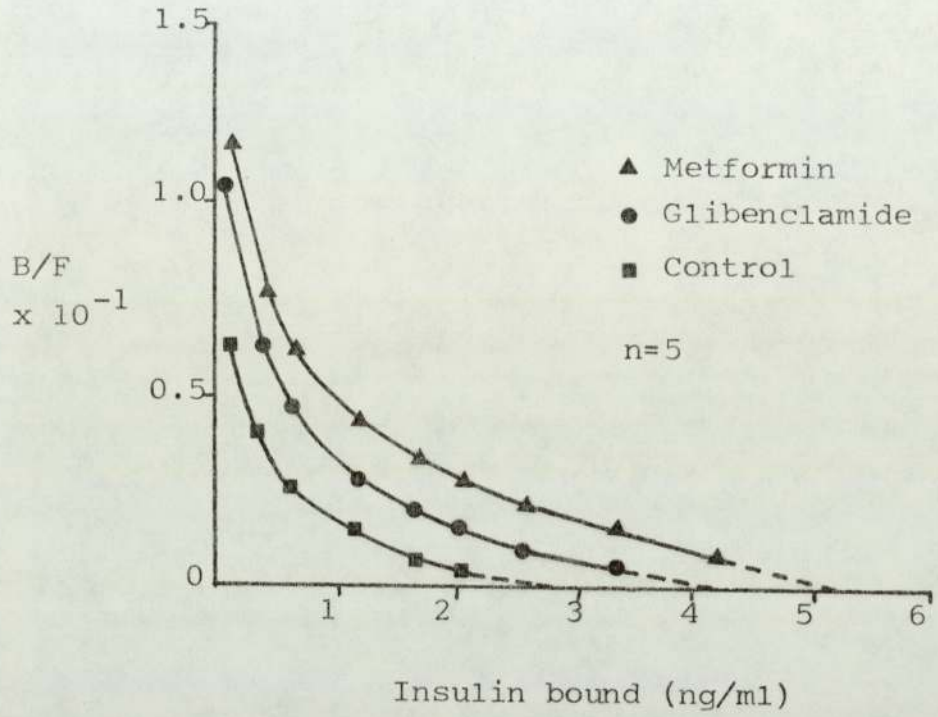


Figure 26. ^{125}I-insulin binding to isolated hepatocytes at 22°C, pH 7.8. Control mice (■) received drinking water only and test mice received metformin (▲) or glibenclamide (●) for 50 weeks.

affinity receptors. The effect of metformin on insulin receptor number was more pronounced (31% greater) than that of glibenclamide. Prince and Olefsky (1980) noted that the increase in receptor number with glibenclamide was slight and Vigneri et al. (1982) observed that metformin increased insulin binding to a greater extent than sulphonylureas. (In fact no significant effect was seen with the sulphonylureas). Biguanides therefore appear to have a greater capacity than the sulphonylureas to modulate the cellular binding of insulin.

It is interesting to note that the *in vivo* work of Holle et al. (1981) describing the effect of metformin on erythrocyte insulin receptors has also demonstrated an increase in insulin receptor number as reflected by an increase in the number of low affinity receptors. In contrast, the work of Vigneri and coworkers (1982) using an *in vitro* cell culture system demonstrated increased receptor affinity without an effect on receptor number.

Conflicting *in vivo* and *in vitro* studies have also appeared in the literature concerning the possible mode of action of sulphonylureas. Whilst Prince and Olefsky (1980) have reported that glibenclamide produced a small increase in insulin binding to cultured fibroblasts, Vigneri et al. (1982) could not reproduce this effect under similar conditions. Maloff and Lockwood (1981) similarly reported no effect for the sulphonylurea, tolazamide, on insulin binding to isolated adipocytes. These workers did, however, show an improved insulin action under these conditions. However, the majority of work in man and animals has demonstrated increased insulin binding with sulphonylureas (Olefsky and Reaven, 1976b; Feinglos and Lebovitz, 1978;

Beck-Nielsen et al., 1979b).

It appears therefore that metformin exerts a direct effect by increasing cellular insulin binding *in vivo* either by increasing insulin receptor affinity or number. This observation could account for some of the hypoglycaemic effects of metformin *in vivo*. The conflicting data for the sulphonylureas is difficult to understand. Finally, despite a normal reduction in receptor numbers with age in the mice (see aging section page 167) isolated hepatocytes were able to respond to the two drugs, indicating that the ability to enhance insulin binding in the hepatocyte is not reduced with age or chronic oral hypoglycaemic therapy. Also a metabolic derivative of sulphonylureas maybe the active potentiator of insulin binding to target cells, thus explaining the lack of any effect in *in vitro* studies. Vigneri has suggested that the chronic administration of sulphonylureas leads to reduced plasma insulin levels and that this may lead to an overall increase in insulin receptor binding. The present study cannot confirm this view since no significant changes in plasma insulin levels were observed. The subsequent reduction in insulin levels seen in the clinical situation with chronic sulphonylurea therapy (Duckworth et al., 1972b)might in part explain the enhancement of insulin binding *in vivo*; in terms of reducing the 'down regulatory' influence of normal circulating concentrations of insulin.

2) *The time dependency of metformins' effect on hepatocyte insulin receptor status in lean (+/+) mice*

The present study was initiated to establish the time course of the effect induced by metformin. Holle et al. (1981) have shown that the increase in erythrocyte receptor

number brought about by metformin occurs within 24 hours *in vitro* and this has been confirmed by Vigneri et al. (1982). However, in their *in vivo* studies Holle and coworkers only examined erythrocyte receptor status after 4 days of treatment. In the present study receptor binding has been investigated after 24, 48, 72 and 96 hours of metformin therapy and in addition, 24 and 48 hours after metformin had been discontinued.

Animals. As for study 1.

Experimental protocol

These studies were carried out with groups of 4, 20 week old lean (+/+) mice. Test mice received metformin (60 mg/kg/24hrs) for 24, 48, 72 and 96 hours followed by examination of receptor status. In addition the drug was withdrawn after 96 hours of therapy and receptor status examined after 24 and 48 hours. The drug was administered in the drinking water as previously described, control mice, and mice taken off metformin, subsequently received drinking water only.

Insulin binding studies

Blood samples (100µl) were obtained from the cut tail tip of fed mice at 10.00h on the day of the insulin binding study. Plasma was separated and assayed for metformin using a GLC technique (Lennard et al., 1978), to ensure that the circulating level of the drug was within the range known to be clinically effective (Catellier et al., 1977; Mehnert, 1969).

Mice were anaesthetized with sodium pentobarbitone (50 mg/kg) by intramuscular injection and insulin binding to isolated hepatocytes was assessed as previously described (page 72). It was decided not to include metformin in the

assay incubation medium since the actual circulating levels of metformin were not then known. In addition it was felt that any effect on receptors developed over 24 hours would not be lost over the 2 hour incubation period. In retrospect, recent reports of conflicting *in vivo* and *in vitro* effects of both biguanides and sulphonylureas on insulin binding (Vigneri et al., 1982) suggest that this was a wise decision.

Binding data were analysed using a computer assisted Scatchard plot and the results have been presented as high affinity, low affinity and total receptor number per cell together with values for $\overline{K}_e$ and $\overline{K}_f$.

Results

Data describing the acute time dependent effect of metformin on hepatocyte insulin receptor status in normal mice have been summarised in Table 6. Normal mice treated with metformin (60 mg/kg/24h) showed a significant increase in the number of low affinity hepatocyte insulin receptors after 24 hours. There was also a significant effect at 72 and 96 hours. There was no significant alteration in the number of high affinity receptors or the affinity constants $\overline{K}_e$ and $\overline{K}_f$.

Metformin levels were highest after 24 hours and remained steady at a slightly lower level upto 96 hours. The high value after 24 hours could probably have been caused by an increased fluid intake at the start of the study. Also, the metformin levels given were from single determinations only, due to the low availability of plasma from the mice. Metformin has a short half life of approximately 3 hours in the circulation (Beckman, 1969) and so was undetectable in the plasma 24 and 48 hours after withdrawal.

Table 6. Acute time-dependent effect of metformin (60 mg/kg/24h), and the effect of withdrawal of metformin (60 mg/kg/24h for 96h) on hepatocyte insulin receptor status in normal mice. Values are mean ± SEM of 4 mice. * $P<0.05$ compared with control group. † plasma samples were pooled to give a single reading. ND=not detectable.

	Plasma Met-formin µg/ml†	Total receptor number per cell x 10^5	High affinity receptor per cell x 10^5	Low affinity receptor per cell x 10^5	Affinity constant $\bar{K}_e$ nM^{-1}	Affinity constant $\bar{K}_f$ nM^{-1}	Apparent affinity constant nM
Control	ND	3.58 ± 0.23	1.07 ± 0.10	2.50 ± 0.05	0.144 ± 0.003	0.032 ± 0.002	2.06 ± 0.10
Metformin 24h	0.41	4.40 ± 0.38	1.15 ± 0.10	3.24 ± 0.32*	0.144 ± 0.007	0.036 ± 0.007	1.95 ± 0.20
Metformin 48h	0.22	5.34 ± 0.57*	1.54 ± 0.21	3.80 ± 0.71	0.108 ± 0.020	0.036 ± 0.010	1.94 ± 0.13
Metformin 72h	0.23	4.56 ± 0.18*	1.29 ± 0.07	3.26 ± 0.22*	0.144 ± 0.001	0.032 ± 0.003	1.89 ± 0.06
Metformin 96h	0.34	5.97 ± 0.76*	1.39 ± 0.03	4.58 ± 0.73*	0.108 ± 0.021	0.025 ± 0.003	1.83 ± 0.07
Metformin withdrawn 24h	ND	6.00 ± 0.88*	1.51 ± 0.20	4.33 ± 0.95	0.144 ± 0.003	0.025 ± 0.003	2.04 ± 0.11
Metformin withdrawn 48h	ND	4.42 ± 0.42	1.30 ± 0.13	3.11 ± 0.38	0.180 ± 0.025	0.036 ± 0.003	1.84 ± 0.08

When metformin had been withdrawn for 24 hours from mice that had previously been treated for 96 hours, the total hepatocyte insulin receptor number was still elevated compared with controls. But 48 hours after withdrawal hepatocyte insulin receptor numbers were appreciably reduced.

Discussion

The plasma metformin levels achieved were less than expected, ie. less than 1 µg/ml, but were still in the weight for weight clinically effective range (Catellier et al., 1977; Mehnert, 1969). This discrepancy may have been due to an increased renal clearance rate in mice or perhaps a fluid intake that was somewhat lower than estimated in the initial fluid intake monitoring study.

An increase was seen in receptor number and this was maintained for upto 96 hours. This rapid effect is consistent with the observations of Holle et al. (1981) on cultured erythrocytes and Vigneri et al. (1982) on cultured fibroblasts, IM-9 lymphocytes, MCF-7 human breast cancer cells and H35 rat hepatoma cells. The increased insulin binding is mediated via an increase in the number of low affinity receptors in this study, with no significant effect on receptor affinity. A similar observation was made by Holle et al. (1981), but Vigneri and colleagues (1982) were able to show an altered receptor affinity and no change in receptor number.

Exactly how metformin is able to influence either receptor number or affinity is not understood. It is thought that the 'de novo' synthesis of insulin receptors is not involved, firstly because receptor number has been shown to be increased in erythrocytes incubated with metformin (Holle et al., 1981), and anucleate erythrocytes cannot

synthesise protein. Secondly, Vigneri and coworkers (1982) have shown that the enhancement of insulin binding by biguanides, metformin and phenformin is not blocked by cyclohexamide, a classical inhibitor of protein synthesis. However, as the clinical significance of a raised total insulin receptor number is doubtful (Kono and Barham, 1971), it seems likely that metformin elicits more significant alterations at the postreceptor level. The persistence of the elevated insulin receptor number, after the withdrawal of metformin, could be explained by the slow reconciliation of postreceptor mechanisms to control levels leading to a protracted enhancement of insulin binding. The possible existence of post insulin receptor effects for metformin has not yet been proven, and has been made the subject of experimental work in this thesis. It is also equally likely that this effect of metformin is confined to animals and would not be seen in human studies. This thesis therefore includes a clinical trial, investigating the effect of metformin on insulin receptor status.

3) The effect of metformin therapy on hepatocyte insulin receptor status of obese hyperglycaemic (ob/ob) mice

The hypoglycaemic effect of metformin is not seen in either non-diabetic humans (Fajans et al., 1960; Sterne, 1969; Hermann, 1973) or normal mice. The hypoglycaemic effect of metformin seen in diabetics is achieved by increasing the peripheral uptake of glucose and decreasing hepatic gluconeogenesis (Hermann, 1980). Normoglycaemia is thought to be preserved in the nondiabetic by a compensatory increase in gluconeogenesis from lactate (Kreisberg et al., 1970; Hermann. 1979). The ability to maintain glucose levels by gluconeogenesis is not present in the diabetic,

enabling metformin to produce its therapeutic effect. On the basis of metformins' known effects in normal and diabetic animals, subsequent studies presented here involve only obese hyperglycaemic animals and diabetic patients since these two groups will provide the most relevant information on the mode of action of metformin.

Metformin is used mainly in the treatment of obese non-insulin dependent patients, with no indication of impaired renal or hepatic function, who have not achieved good control on dietary restriction alone.

As the obese hyperglycaemic (ob/ob) mouse is a legitimate animal model for obesity and type 2 diabetes (Bray and York, 1979) the present study sets out to investigate the effect of metformin on hepatocyte insulin receptor status of the obese mice. In addition insulin hypoglycaemia tests were performed to assess any improvement, following metformin administration, in the severe insulin resistance seen in the obese mice.

Experimental procedure

Male, 20 week old obese mice, from the Aston colony were used. The Aston colony was derived from C57BL/6J mice of The Jackson Laboratory, Bar Harbor, Maine. These were outcrossed with various strains for high litter size and faster growth rate at The Institute of Animal Genetics, Edinburgh. These mice exhibit hyperglycaemia, hyperinsulinaemia, obesity, hyperglucagonaemia and severe insulin resistance. The syndrome is described more fully elsewhere in this thesis (page 152) and also in reviews in the literature (Bray and York, 1979; Bailey et al., 1982).

Obese animals were housed in an air conditioned room at $22^{\circ}C \pm 2^{\circ}C$ with a 12h dark and 12h light lighting schedule,

and fed a standard pellet diet (Mouse breeding diet, Heygate and Sons Ltd., Northampton) and allowed free access to drinking water. 20 week old test mice received metformin in the drinking water (120 mg/kg/day or 240 mg/kg/day) for 4 weeks and control mice received drinking water only. The doses of metformin used in this study were well above the maximum recommended clinical dose because other workers (Bailey and Flatt, unpublished observations) have shown that metformin does not improve insulin resistance in the obese mouse at a clinical dose (60 mg/kg/day).

Insulin hypoglycaemia test

Insulin hypoglycaemia tests were performed at 10.00h on fed obese mice one week prior to the insulin binding studies. Blood samples (40µl) were obtained from the cut tail tip immediately before, and at 15, 30 and 60 minutes after an intraperitoneal injection of monocomponent porcine insulin (4 mg/kg). Plasma was separated and assayed for glucose (Stevens, 1971).

Insulin binding studies

Blood samples (150µl) were obtained from the cut tail tip of fed mice at 10.00h on the day of the insulin binding studies. Plasma was separated and assayed for glucose (Stevens, 1971) and insulin (Hales and Randle, 1963).

Mice were anaesthetized with sodium pentobarbitone (50 mg/kg) by intraperitoneal injection (muscle wasting in the obese mouse makes intramuscular injections impractical) and ^{125}I-insulin binding to isolated hepatocytes was measured as described in a previous section (page 72). However, since the hepatocytes of adult obese mice are larger than those of their lean littermates (Kahn et al., 1973), cell diameter was measured and insulin receptor

number were expressed in terms of μm^2 of cell surface area. Cell size was determined in two ways: a) a direct measurement of cell diameter using a microscope fitted with a graticule and b) using the cell sizing facility on a coulter counter. In the first method calculation of the cell dimensions assumed each cell to be spherical. These two methods gave values that were not significantly different and since the graticule method was convenient this was adopted for routine use.

Results

Table 7 confirms that 20 week old obese mice have a body weight more than double that of their lean littermates (+/+) and were significantly hyperglycaemic and hyperinsulinaemic.

When the values for receptor number derived from a Scatchard plot (Figure 27) are expressed in terms of 'per cell x 10^5', there is no significant difference between the receptor number on obese and lean mouse hepatocytes (Table 7). However, isolated hepatocytes from obese mice (32 ± 2) were significantly larger than those from lean mice (26 ± 1.8). This confirms data originally reported by Kahn et al. (1973). However, when receptor number was expressed in terms of unit cell surface area the receptor number was significantly decreased by 37% for obese hepatocytes compared to lean (Table 7). The decrease in receptor number was proportionally greater for the high affinity (60%) compared to the low affinity receptors (27%). The reduction in high affinity receptors was partially compemsated by an increase in the value for $\overline{K}_e$, nochange was seen in $\overline{K}_f$ or the apparent affinity constant.

Metformin administration (120 mg/kg/day) did not

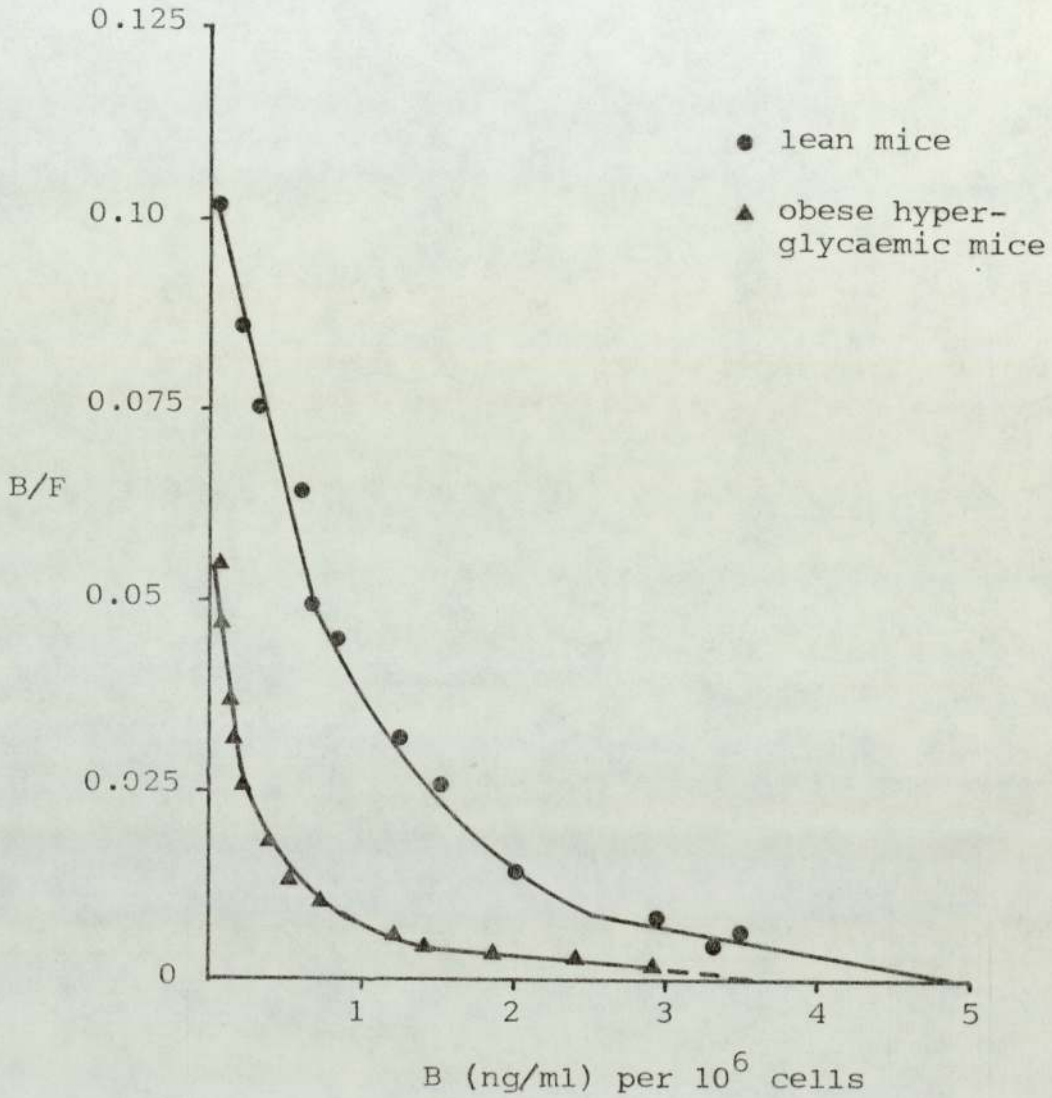


Figure 27. ^{125}I-insulin binding to isolated hepatocytes of lean and obese hyperglycaemic (ob/ob) mice at 22°C, pH 7.8.

significantly alter body weight, plasma glucose, plasma insulin, insulin receptor number, $\overline{K}_f$ or the apparent affinity constant compared to obese control mice. However, there was a slight reduction in the value for $\overline{K}_e$ (Table 7). Metformin at the higher dose (240 mg/kg/day) produced an increase in total receptor number (85%) that was accounted for by increases in both high (63%) and low (86%) affinity receptors. However, the value for $\overline{K}_e$ was again reduced, despite no alteration in the value for $\overline{K}_f$ or the apparent affinity constant. Although the reduction in $\overline{K}_e$ would affect the degree to which binding was increased, it did not obliterate the effect of the increased receptor number (Table 7). Neither dose of metformin significantly affected the hypoglycaemia induced by a single dose of insulin (Table 8), but the higher dose did reduce plasma insulin levels (39%) whilst maintaining the plasma glucose concentration (Table 7).

Although the obese hyperglycaemic (ob/ob) mouse is generally accepted as a model for maturity onset diabetes, the results of this study enforce the need for caution in the extrapolation of animal data for the human situation. Metformin is undoubtedly a valuable therapeutic agent in the treatment of obese type 2 diabetic patients, producing hypoglycaemia and improved insulin sensitivity. Information describing the effect of metformin on insulin receptor status in type 2 diabetics is not available in the literature, although metformin has been shown to improve insulin binding, by increasing the number of low affinity receptors, in normal subjects (Holle et al., 1981). In the present study the apparent lack of effect of a clinically related dose of metformin on insulin receptor status and action in obese

Table 7. Chronic effect of metformin (120 and 240 mg/kg/day for 4 weeks) on hepatocyte insulin receptor status in obese hyperglycaemic (ob/ob) mice. Values represent mean ± SEM of 4 mice. * P<0.05, **P<0.01 compared with obese control group.

	Body weight g	Plasma glucose nmol/l	Plasma insulin ng/ml	Total receptor number per cell x 10^5	Total receptor concentration per μm^2 cell surface area
Lean (+/+) control	40.5 ± 1.1**	7.3 ± 0.4*	2.1 ± 0.3**	3.58 ± 0.23	168 ± 9**
Obese (ob/ob) control	102.7 ± 4.5	11.5 ± 0.3	34.4 ± 1.8	3.44 ± 0.30	106 ± 12
Obese (ob/ob) Metformin 120 mg/kg/day	96.0 ± 5.8	12.5 ± 0.8	36.6 ± 2.5	4.42 ± 0.26	137 ± 6
Obese (ob/ob) Metformin 240 mg/kg/day	100.8 ± 4.9	10.1 ± 0.6	21.1 ± 1.6**	6.31 ± 0.47**	196 ± 12**

Table 7. (continued)

High affinity receptor concentration per μm^2 cell surface area	Low affinity receptor concentration per μm^2 cell surface area	Affinity constant $\bar{K}_e$ nM^{-1}	Affinity constant $\bar{K}_f$ nM^{-1}	Apparent affinity constant nM
48 ± 5**	120 ± 2**	0.144 ± 0.003**	0.032 ± 0.002	2.06 ± 0.10
19 ± 3	87 ± 6	0.210 ± 0.007	0.025 ± 0.007	1.79 ± 0.10
25 ± 3	103 ± 12	0.110 ± 0.003**	0.020 ± 0.001	1.85 ± 0.15
31 ± 4*	162 ± 9**	0.072 ± 0.003**	0.021 ± 0.003	1.83 ± 0.08

Table 8. Chronic effect of metformin (120 and 240 mg/kg/day for 4 weeks) on the hypoglycaemic response to insulin (100 U/kg , IP) in obese hyperglycaemic (ob/ob) mice. Values are mean ± SEM of 4 mice. % represents the percentage change in the plasma glucose concentration compared with time zero.

	% after 15 min	% after 30 min	% after 60 min
Obese (ob/ob) control	-21.7 ± 0.4	-29.5 ± 0.7	-33.9 ± 2.2
Obese (ob/ob) Metformin 120 mg/kg/day	-18.1 ± 1.6	-27.8 ± 1.5	-27.2 ± 2.6
Obese (ob/ob) Metformin 240 mg/kg/day	-19.3 ± 2.4	-23.8 ±4.0	-35.9 ± 4.0

mice highlights the possible existence of differences in the metabolic basis for diabetes in the obese mouse and human type 2 diabetic patients. In the present study however, at the higher dose of metformin (240 mg/kg/day) an improvement in receptor binding was observed. This was due to a proportionally greater increase in the numbers of low affinity receptors. The reduction in the affinity constant $\overline{K}_e$ remains difficult to explain and was not a feature of the action of metforminin the lean mice. Further studies are obviously needed to confirm whether this is an actual physiological effect or an artefact of the analytical method (no effect was observed on the apparent affinity constant) and the small number of mice in the group studied (n=4). It is also possible that the reduced value for $\overline{K}_e$ represents a return to normal receptor status; since the value for $\overline{K}_e$ was raised in the obese mice, possibly to compensate for the observed reduction in receptor number. The increased receptor number observed following metformin therapy negated the requirement for increased receptor affinity, hence $\overline{K}_e$ fell.

The increased receptor number with metformin therapy (240 mg/kg/day) was not reflected in an improvement in the hypoglycaemia induced by a single dose of insulin. This could, however, reflect the inability of the hypoglycaemia test to detect an enhancement of insulin action in the presence of such a severe insulin resistance (Flatt and Bailey, 1981). Moreover, plasma insulin concentrations were reduced following the high dose of metformin, whilst glucose levels were maintained, indicating an improvement in insulin action.

Finally, despite the improved insulim receptor status

in obese mice, following metformin administration (240 mg/kg/day), there appeared to be no major concomitant reduction in insulin resistance. It therefore seems likely that the reduced insulin receptor number observed in obese mice was not the major cause of insulin resistance. Furthermore, obese mice exhibit insulin insensitivity as well as insulin unresponsiveness (Kolterman, 1980), suggesting the presence of both receptor and postreceptor defects (Olefsky, 1981a). The present study confirms the view that the major defect responsible for the insulin resistance of obese mice lies at a postreceptor site (Le Marchand et al., 1978). Moreover, this postreceptor defect may not be the same as that possibly present in human diabetic patients, and thus may not be corrected by metformin therapy (Ciaraldi et al., 1981; Kolterman et al., 1981). In order to pursue the therapeutic mode of action of metformin, studies should be directed toward human type 2 diabetic patients and should attempt to define any postreceptor effects of the drug, bearing in mind the limited physiological significance of increased total insulin receptor number.

4) The effect of acute and chronic metformin treatment on erythrocyte insulin receptor binding in type 2 non insulin dependent diabetic patients

The preceeding studies and a number of reports in the literature indicate that there is now considerable evidence to suggest that the hypoglycaemic effect of metformin therapy in non-insulin dependent (type 2) diabetics is mediated by a potentiation of the action of insulin (Hermann, 1979). Recently it has been reported that metformin increases insulin binding to erythrocytes in a small number of normal subjects (Holle et al., 1981). The

clinical relevance of this result is questionable since normoglycaemia is preserved in non-diabetic subjects. The present study sets out to evaluate the effect of metformin on erythrocyte insulin receptor binding in type 2 NID diabetic patients and any concomitant improvement in their diabetic control.

Method

Obese patients with type 2 diabetes (137.5 ± 10.2 % the ideal body weight, set out in insurance tables) were recruited for the study from the diabetic clinic at Dudley Road Hospital, Birmingham. The patients were carefully selected bearing in mind the following criteria:

a) Patients who were 'diet failed', but who had maintained a constant body weight for 1 month prior to recruitment. Such a choice obviated the effects of fluctuations in weight on insulin receptor status (Gorden et al., 1976).

b) Male or postmenopausal females were chosen, because there is evidence to suggest that insulin binding changes during the stages of the menstrual cycle (De Pirro et al., 1978).

c) Patients must have had diabetes diagnosed for at least 1 year, but no other oral hypoglycaemic therapy or insulin should have been prescribed. Recent use of either of these agents would have made interpretation of the results difficult, since both are known to significantly affect insulin receptor status (Bailey et al., 1983).

Ten patients were initially recruited, but only eight completed the study, five postmenopausal women and three men, average age 61 ± 4.6 years, in whom diabetes had been diagnosed for 4.0 ± 0.8 years. Patients did not show either proteinuria or any biochemical evidence of impaired renal or

hepatic function.

Each patient was asked to follow a fully structured calorie controlled diet for four weeks prior to the study and during the complete course of the study. The diet was designed (by a Dudley Road Hospital dietician) to maintain a constant body weight throughout the study. Each patient served as his/her own control. Tests were performed immediately before treatment; after 1 and 4 weeks of metformin therapy (Glucophage, Rona Laboratories Ltd., 3 x 500mg tablets daily), and 4 weeks after the withdrawal of metformin. Patients presented at 09.00 hours in the clinic after a 12 hour overnight fast. A 24 hour urine collection from the previous day was analysed for glucose (Stevens, 1971). Venous blood was collected for estimation of glycosylated haemoglobin (HbA_1) (Mallia et al., 1981) and plasma concentrations of glucose (Stevens, 1971), insulin (Hales and Randle, 1963) and metformin (Lennard et al., 1978). A 50g oral glucose tolerance test was performed, and blood samples for plasma glucose and insulin were taken at 0 and after 30, 60, 90 and 120 minutes.

Erythrocyte insulin receptor binding was determined by the method of Gambhir et al. (1978) as described in the methods section (page 74). Data were analysed after Scatchard (1949) using a model which assumes two classes of receptors with different but fixed affinities (Kahn, 1975). The apparent receptor affinity, rather than $\overline{K}_e$ or $\overline{K}_f$, was estimated as an indication of insulin receptor affinity (Olefsky and Reaven, 1974) . See page 86 for an explanation of this choice of analysis.

Results

Table 9 summarises diabetic control before, during and

Table 9. Diabetic control before, during and after treatment with metformin (3 x 500 mg daily for 4 weeks)

Treatment	% Ideal body weight	Plasma Metformin µg/ml	Urinary glucose mmol/24h	HbA_1 %
Pretreatment control	137.5 ± 10.2	ND	224.1 ± 71.9	14.0 ± 1.1
Metformin 1 week	137.6 ± 10.2	1.45 ± 0.41	136.1 ± 49.5	12.4 ± 1.4*
Metformin 4 weeks	138.5 ± 10.8	0.94 ± 0.34	77.7 ± 35.6*	12.7 ± 1.0
Metformin withdrawn 4 weeks	137.1 ± 10.3	ND	235.6 ± 95.3	13.8 ± 0.7

	Plasma glucose mmol/l		Plasma insulin mU/l	
	0 min	1	0 min	1
Pretreatment control	11.5 ± 1.0	78.2 ± 5.3	13.6 ± 2.4	125.3 ± 29.3
Metformin 1 week	9.6 ± 0.8***	65.5 ± 4.3***	11.3 ± 2.2	128.6 ± 25.3
Metformin 4 weeks	9.1 ± 0.8**	62.3 ± 4.5***	13.4 ± 1.5	115.3 ± 15.2
Metformin withdrawn 4 weeks	10.8 ± 1.0	76.3 ± 6.8	14.4 ± 1.8	121.4 ± 20.8

[1] sum of plasma glucose or insulin values at 0, 30, 60, 90 and 120 min during 50g oral glucose tolerance tests. Values are means ± SEM , n =8. * P<0.05, **P<0.01, ***P<0.001 compared with pretreatment control using Student's paired t-test. ND= not detectable

after treatment. Body weight was maintained throughout the study. Compliance with metformin therapy, ie. that patients had actually taken the tablets, was confirmed by the measurement of plasma metformin at each time interval. Diabetic control was improved during the 4 weeks of metformin treatment. 24 hour urinary glucose, HbA_1 (reduced but not significantly at 4 weeks) and fasting plasma glucose concentrations were reduced, whilst glucose tolerance was significantly improved. Plasma insulin concentrations were not significantly altered by metformin treatment.

Metformin increased insulin receptor binding in all patients (Table 10). The total number of insulin receptor binding sites was increased by 116% after 1 week and 184% after 4 weeks of metformin treatment. This effect was due almost entirely to an increase in the number of low affinity receptors. There was no alteration in the value for the apparent affinity constant. The effect of metformin on insulin receptor number was still evident 4 weeks after the conclusion of metformin therapy.

Discussion

The present study provides further evidence to suggest that in type 2 NID diabetes the hypoglycaemic effect of metformin is mediated, at least partly, by potentiation of the action of insulin (Hermann, 1979). In addition this study demonstrates that metformin, like sulphonylureas (Beck-Nielsen et al., 1979b; Olefsky and Reaven, 1976b; increases the cellular binding of insulin in type 2 diabetes. In the erythrocyte model, metformin increased the number of low affinity insulin receptors and this observation is consistent with previous data obtained for normal subjects (Holle et al., 1981). As insulin resistance and impaired

Table 10. Erythrocyte insulin receptor binding before, during and after treatment with metformin (3 x 500 mg daily for 4 weeks)

Treatment	Total receptor number per cell	High affinity receptor number per cell	Low affinity receptor number per cell	Apparent affinity constant nM
Pretreatment control	176.1 ± 30.0	36.6 ± 5.4	139.5 ± 28.7	1.08 ± 0.06
Metformin 1 week	381.7 ± 86.0*	38.6 ± 3.7	343.1 ± 70.5*	1.03 ± 0.07
Metformin 4 weeks	500.6 ± 77.9**	46.1 ± 5.4	454.5 ± 79.9**	1.20 ± 0.05
Metformin withdrawn 4 weeks	405.1 ± 61.1**	36.3 ± 5.0	368.7 ± 64.3**	1.18 ± 0.08

Values are means ± SEM , n= 8. *P<0.05, **P<0.01 compared with pretreatment control.

insulin receptor binding are characteristic features of type 2 NID diabetes (Kolterman et al., 1981) this increased insulin receptor binding might contribute to the hypoglycaemic action of metformin. However, additional mechanisms must be considered because impaired insulin binding may be simply an indicator of insulin resistance and not a primary factor in its development. A similar notion was suggested for the obese mouse (page 153).Increasing the number of insulin receptors available for binding does not necessarily improve insulin action. (Bearing in mind the 'spare receptor concept', which suggests that only a fraction of available insulin receptors, some 10-30%, need to be occupied in order to initiate a measured biological effect of insulin [Kono and Barham, 1971]). Support for the 'spare receptor' theory is evident in the receptor binding data produced after withdrawal of metformin therapy, since the number of insulin receptors was still raised 4 weeks after the conclusion of metformin therapy, although plasma glucose and HbA_1 had returned to almost pretreatment levels.

The therapeutic effects of metformin, therefore, are probably not the consequence of raised insulin receptor number, but more probably of some postreceptor action. (The latter cannot of course be investigated in the erythrocyte as glucose metabolism is not controlled by insulin in this cell). This postreceptor activity has yet to be determined in relation to insulin receptor binding, although it is known that metformin improves peripheral glucose uptake and hepatic gluconeogenesis (Hermann, 1979). The prospect of a probable postreceptor action prompted a search for a suitable animal tissue model. Since glucose metabolism in the erythrocyte is not insulin dependent it was decided to

investigate the possible use of an isolated mouse soleus muscle preparation. (Adipocytes were ruled out as the animal tissue model as a minimum of eight mice would be necessary to supply sufficient cells for a receptor binding and post-receptor effect assay, the technique would therefore be very time consuming, expensive, and would strain the obese mouse colony. Hepatocytes were considered, but as the role of insulin in glucose uptake by liver cells is only permissive (Davidson, 1981) the choice of postreceptor effects would be more limited.)

5) The effect of metformin on insulin receptor binding and glycogen synthesis in isolated soleus muscles of streptozotocin diabetic mice

Previous studies have confirmed that metformin improves glycaemic control in type 2 NID diabetic patients, without stimulation of insulin release. This apparent potentiation of the action of insulin might be achieved by either an increase in cellular insulin binding or by the enhancement of postreceptor events.

Studies previously described (page 103), involving normal mice and type 2 NID diabetic patients, have confirmed reports by other authors of increased insulin receptor binding following metformin administration (Holle et al., 1981; Vigneri et al., 1982). However, the clinical study (page 124) raised the possibility that metformin might owe its' hypoglycaemic effect to an influence at the postreceptor level of insulin action, perhaps independent of its effects on the insulin receptor.

The present study sets out to demonstrate a postreceptor action for metformin on insulin action. The object of the study was to produce evidence of a postreceptor effect for

metformin in the absence of an action on insulin binding: a difficult proposition since metformin has been shown to increase receptor binding in all of the models so far studied. However, one model of diabetes remained to be considered, namely chemical or streptozotocin induced diabetes. Streptozotocin induces hypoinsulinaemia by a selective destruction of the β cells of the Islets of Langerhans (Rerup, 1970; Mansfield and Opie, 1968). The consequences of its action on the β cell can be employed as a model of type 1 diabetes mellitus (Rerup, 1970; Kobayashi and Olefsky, 1979). Circulating insulin levels are greatly reduced in this condition, and on this basis one would expect insulin receptor number to be increased since the down regulatory effect of normal insulin levels would be reduced. Evidence in the literature concerning insulin receptor status in either type 1 ID diabetes or streptozotocin diabetes is limited. Ethical considerations make the measurement of receptor status, before insulin treatment, in newly diagnosed type 1 diabetic patients difficult. However, a study by Pedersen et al. (1978) demonstrated an increased insulin binding in type 1 diabetes, that was subsequently reduced by insulin therapy. In addition, Le Marchand and Freychet (1979) have demonstrated raised insulin binding in isolatd soleus muscles of streptozotocin diabetic mice.

The present study described below sets out to demonstrate the effect of streptozotocin diabetes on mouse hepatocyte insulin receptor status and to investigate any subsequent alteration in insulin receptor status following treatment with metformin. A parallel study was also carried out involving treatment with glibenclamide since it is possible that sulphonylureas might also influence postreceptor events

of insulin action. It should be borne in mind that these effects on insulin receptor binding may not account for their therapeutic hypoglycaemic action. In addition insulin hypoglycaemia tests were performed to enable correlations to be made between alterations in receptor status and changes in insulin action.

Methods

Adult male Theiller Original Albino (TO) mice (Bantin and Kingman Ltd., Hull) were used in this study and housed in an air conditioned room at 22°± 2°C with a 12h dark and 12h light lighting schedule. They were fed a standard pellet diet and given free access to water.

10 week old mice were made diabetic with streptozotocin (120 mg/kg) given IP (Vehicle - citrate buffer pH 4.8). After 2 weeks, plasma glucose was determined and only mice with plasma glucose values in excess of 11.00mM were used in the study. The mice were segregated into three groups, with similar mean plasma glucose values. One group received metformin (60 mg/kg/day) and another was given glibenclamide (0.4 mg/kg/day) for 10 weeks. The drugs were administered in the drinking water and control diabetic mice received water only.

Insulin hypoglycaemia test

Insulin hypoglycaemia tests were performed at 10.00h on fed mice one week prior to the insulin binding studies. Blood samples (40µl) were obtained from the cut tail tip immediately before, and 15, 30, 60 and 90 minutes after an intraperitoneal injection of monocomponent porcine insulin (0.06mg/kg). Plasma was separated and assayed for glucose (Stevens, 1971).

Insulin binding studies

Mice were anaesthetised with sodium pentobarbitone (50 mg/kg, IM) and ^{125}I-insulin binding to isolated hepatocytes was measured as previously described (page 72). Binding data were analysed by the method of Scatchard (1949) and interpreted using both the two receptor and the negative cooperativity models (page 76) of insulin receptor binding.

Results

Streptozotocin treatment produced a mild diabetes that was not associated with weight loss often seen in more severe hyperglycaemic states (Le Marchand and Freychet, 1979). Table 11 shows that total insulin and low affinity receptor number is raised in the streptozotocin treated mice compared to normal, age matched, mice both in the absence and presence of oral hypoglycaemic agents. There was no significant increase in receptor binding in the streptozotocin diabetic mice treated with oral hypoglycaemic agents. However, the degree of hypoglycaemia produced by a single dose of insulin was significantly increased in the metformin and glibenclamide treated diabetic mice (Figure 28a and 28b). Receptor affinity was not significantly altered by either streptozotocin induced hyperglycaemia or the drug treatments.

Discussion

The streptozotocin hyperglycaemic mouse appears to provide a model for studying the postreceptor effects of metformin independent of effects at the level of the receptor. Total and low affinity receptor number is raised in the streptozotocin hyperglycaemic mouse to a level that cannot apparently be further increased by metformin or glibenclamide therapy. It is tempting to speculate that there maybe an upper ceiling for hepatocyte receptor number, that is achieved

Table 11. Hepatocyte insulin receptor status in streptozotocin diabetic mice treated with oral hypoglycaemic agents.

Values are mean ± SEM of 5 mice, *P<0.05, **P<0.01 compared with normal age-matched mice.

	Body weight g	Plasma glucose mmol/l	Total receptor number per cell x 10^5	High affinity receptor number per cell x 10^5
Normal age-matched	42.3 ± 1.9	7.0 ± 0.3	3.5 ± 0.2	1.07 ± 0.18
Streptozotocin control	39.9 ± 2.1	20.2 ± 3.5**	6.0 ± 0.3*	1.77 ± 0.62
Streptozotocin metformin	38.0 ± 1.7	17.3 ± 3.5**	6.3 ± 1.1*	1.42 ± 0.17
Streptozotocin glibenclamide	40.1 ± 1.1	16.8 ± 5.4**	5.9 ± 1.0*	1.70 ± 0.19

	Low affinity receptor number per cell x 10^5	Affinity constant $\bar{K}_e$ nM^{-1}	Affinity constant $\bar{K}_f$ nM^{-1}	Apparent affinity constant $\bar{K}$ nM
Normal age-matched	2.50 ± 0.05	0.144 ± 0.005	0.032 ± 0.002	3.06 ± 0.10
Streptozotocin control	4.21 ± 0.38*	0.130 ± 0.007	0.028 ± 0.007	1.51 ± 0.14*
Streptozotocin metformin	4.95 ± 1.20*	0.136 ± 0.003	0.034 ± 0.006	1.82 ± 0.31
Streptozotocin glibenclamide	4.20 ± 0.79*	0.118 ± 0.026	0.027 ± 0.006	1.98 ± 0.24

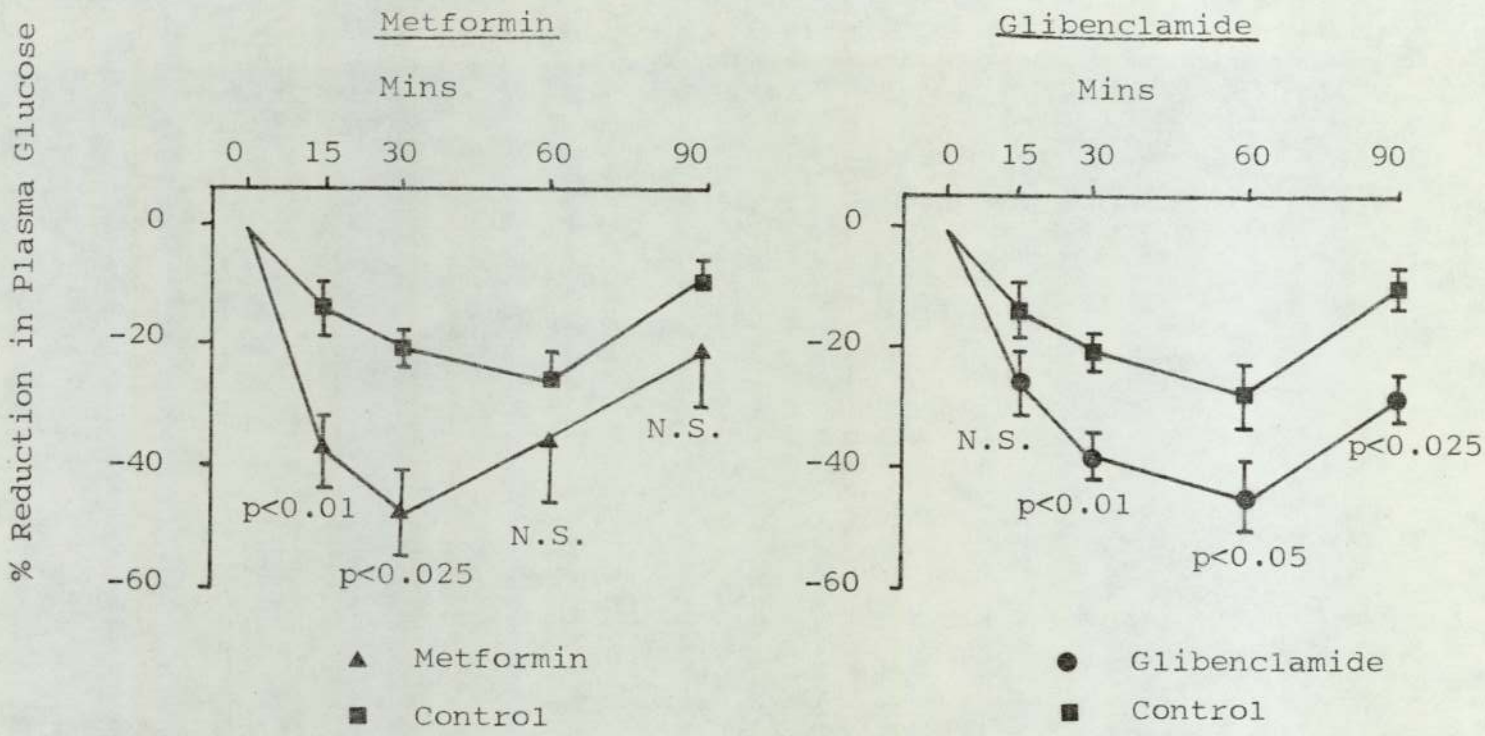


Figure 28a and b. Insulin induced hypoglycaemia tests in metformin (▲) and glibenclamide (●) treated streptozotocin diabetic mice, compared to untreated streptozotocin diabetic mice (■). Insulin (0.06 mg/kg, IP) was administered at 10.00h, one week prior to the insulin binding studies, and blood samples were taken from the tail vein at the time intervals shown in the figures and analysed for plasma glucose.

in streptozotocin diabetes. Such a ceiling could be governed by the receptor synthesis, internalisation and degradation capacities of the receptor bearing cells.

More importantly, there was a potentiated insulin induced hypoglycaemia following treatment with metformin and glibenclamide, that was not reflected by an alteration in receptor status.The action of insulin had therefore been potentiated without an increase in insulin receptor binding, indicating the possible involvement of postreceptor mechanisms. A recent publication by Tiengo et al. (1982) has demonstrated a postreceptor defect in type 2 NID patients that was ameliorated by metformin therapy. Metformin did not, in this case, affect insulin binding but did improve insulin action, providing further evidence for a postreceptor mode of action for this drug. The insulin hypoglycaemia test, whilst giving an indication of an improvement in 'overall' insulin action, does not give any information on the precise location of the sensitive postreceptor event(s). The cellular metabolic consequences of insulin binding are many and vary from tissue to tissue (Denton et al., 1981), but it is more intensive studies on these metabolic effects of insulin that will provide further information on the mode of action of oral hypoglycaemic agents. Such information could not only indicate those sequences in the postreceptor action of insulin that can be influenced by therapeutic agents and thus may be central to the diabetic state, but may lead to the development of more precise hypoglycaemic agents.

5B) Postreceptor studies

To date insulin binding studies in animals have involved the use of isolated hepatocytes as the cell model; its major advantage over other main insulin target tissues - adipocytes

and muscle - is that one animal can provide enough isolated cells for the construction of a comprehensive Scatchard plot. (8 mice are required to produce adequate numbers of adipocytes and at least 15 mice are necessary for the soleus muscle binding assays.) However, if the postreceptor effects of insulin are to be investigated another cell model must be considered since many of the actions of insulin in the hepatocytes are permissive, that is the concentration of glucose is the main regulator of glucose metabolism in the liver (Davidson, 1981). Muscle has been shown to be a major site of glucose utilization and insulin action. As insulin binding studies and several postreceptor assays have already been established (Le Marchand et al., 1978; Le Marchand and Freychet, 1978) for soleus muscle, this model was used in studies concerning the possible postreceptor effects of oral hypoglycaemic agents in preference to the isolated hepatocyte.

Soleus muscle is a slow-twitch skeletal muscle, that is very small and thin, reducing to some extent problems associated with the diffusion of labelled insulin through the muscle. The biological effect chosen to represent a sensitive postreceptor event was ^{14}C-glycogen synthesis (Cuendet et al., 1976). The rate of insulin stimulated ^{14}C-glycogen synthesis was studied at a maximal concentration of insulin, since the previous study (page 131) indicated that receptor binding could not be further improved by metformin therapy and consequently it was assumed to be a postreceptor alteration that was being investigated. Olefsky (1981a) has emphasised that improvement at the postreceptor level of insulin action can only be confirmed by an improvement in insulin resposiveness, at a maximal concentration

of insulin.

The soleus muscle preparation is still relatively expensive in terms of the number of animals required, thus a full Scatchard plot was not possible. Instead binding was studied at six different insulin concentrations, with three muscles at each point and a further three muscles to provide a value for non-specific binding. These data proved sufficient to construct a competition curve, which would show alterations in the level of insulin binding but would not provide sufficient values to estimate receptor number or affinity.

Method

Animals: See page 133.

Protocol

Experiments were initiated on 10 week old streptozotocin treated mice. Plasma glucose levels were monitored to establish the level of hyperglycaemia. Streptozotocin treated mice were assigned to two hyperglycaemic groups, one group receiving metformin(60 mg/kg/day) for 10 weeks. The drug was administered in the drinking water and normal and control streptozotocin treated mice received water alone.

Insulin binding studies

Mice were killed by cervical dislocation at 10.00h on the day of study and soleus muscles were isolated and removed according to the method of Maizels et al. (1969) (page 64). The isolated muscles were weighed, attached to polyethylene holders and insulin binding assays were performed as described in the methods section (page 75). The isolated muscles were incubated with ^{125}I-labelled insulin in the presence of a range of concentrations of

unlabelled insulin, six concentrations were used in all (0.6 - 90nM) exclusive of that required to measure non specific binding (8μM), and this number proved sufficient to produce a competition curve but not a Scatchard plot. The protein content of the muscles was determined (Lowry et al., 1951) and binding data were expressed as the percentage ^{125}I-insulin specifically bound per mg muscle protein.

Studies on the rate of ^{14}C-glycogen synthesis

Soleus muscles were isolated by the method of Maizels et al. (1969), weighed and attached to polyethylene holders. ^{14}C-glycogen synthesis was measured in the absence (basal) or presence of insulin (6nM) as described in the methods section (page 89). The basal value was subtracted from that gained in the presence of insulin, to give the insulin stimulated rate of ^{14}C-glycogen synthesis. Muscle protein content was determined (Lowry et al., 1951) and results were expressed as nmoles ^{14}C-glycogen produced/hour/mg protein.

Results

Data concerning the body weight, plasma glucose concentration, soleus muscle weight and protein content and rates of glycogen synthesis in soleus muscles of normal, streptozotocin treated mice and metformin treated streptozotocin diabetic mice have been summarized in Table 12. The dose of streptozotocin used in this study produced a mild hyperglycaemia which was not accompanied with the weight loss shown by more severe hyperglycaemia (Le Marchand and Freychet, 1979). Soleus muscle weight and protein content did not differ significantly between the three groups of mice (Table 12).

Insulin receptor binding data are illustrated as competition curves in Figure 29. Specific binding of

Table 12. Insulin stimulated glycogen synthesis, body weight, plasma glucose and weight and protein content of soleus muscles from streptozotocin treated mice, with or without metformin treatment. Values for normal mice are also given.

	Body weight (g)	Plasma glucose (mM)	Soleus muscle weight (mg)	Muscle protein content (mg)	Insulin stimulated † glycogen synthesis (nmoles/hr/mg protein)
Normal	40.5 ± 1.1	7.3 ± 0.5	14.5 ± 0.81	1.33 ± 0.22	3.38 ± 0.39
Streptozotocin treated	43.8 ± 1.4	17.5 ± 2.4**	15.4 ± 0.72	1.10 ± 0.06	2.95 ± 0.81
Streptozotocin treated - receiving metformin	42.8 ± 0.9	15.9 ± 2.0**	15.5 ± 0.88	1.09 ± 0.08	9.33 ± 1.90*

All values are mean ± SEM, n= not less than 6. † increment above basal. *P<0.05, **P<0.01 compared with normal mice.

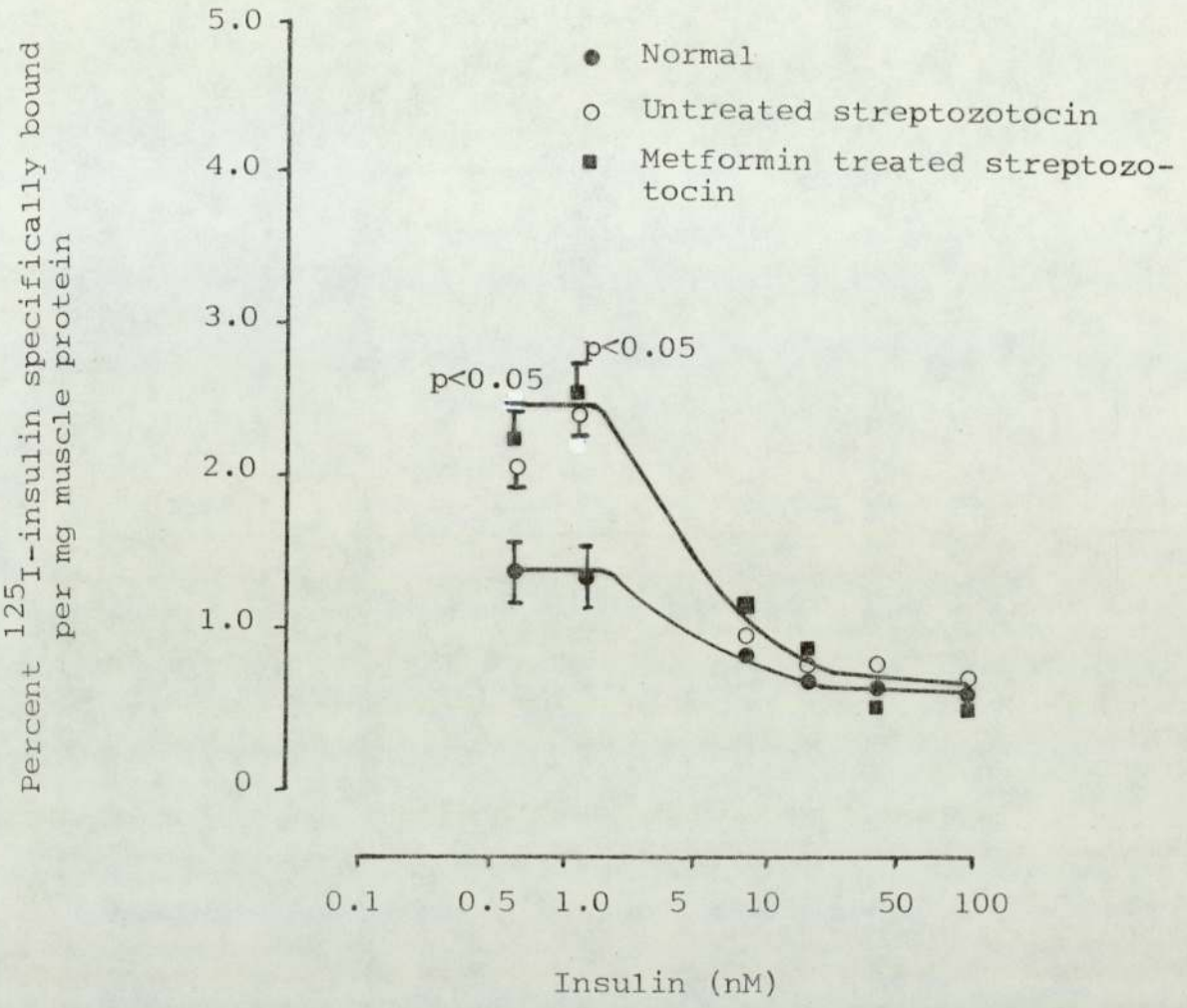


Figure 29. ^{125}I-insulin binding to soleus muscles of normal, untreated streptozotocin and metformin treated streptozotocin treated mice.

^{125}I-insulin was progressively reduced by increasing concentrations of unlabelled insulin, confirming the results of Le Marchand et al. (1978) and confirming the validity of the soleus muscle binding assay. The competition curve for untreated streptozotocin diabetic mice demonstrated an increased insulin binding to soleus muscle at concentrations of native insulin lower than 10nM. At insulin concentrations above 10nM, upto 90nM, there appeared to be no difference in the level of ^{125}I-insulin binding. In the absence of a Scatchard plot, these data can be interpreted as evidence in support of the fact that the increased insulin binding in soleus muscles of streptozotocin diabetic mice is due to an increased receptor affinity rather than increased receptor number. This observation is in agreement with the work of Le Marchand and Freychet (1979). Treatment with metformin did not significantly alter insulin binding to soleus muscles of streptozotocin diabetic mice.

Insulin stimulated glycogen synthesis was not significantly altered in isolated soleus muscles of streptozotocin diabetic mice compared with normal mice, which again supports the observations of Le Marchand and Freychet (1979). However, metformin treatment significantly increased insulin stimulated glycogen synthesis in streptozotocin diabetic mice (Table 12).

Discussion

Work by other investigators (Holle et al., 1980; Vigneri et al., 1982), in addition to the present studies, have shown an increased insulin receptor binding to various cells after metformin treatment. However, an increase in receptor number and affinity should exert only a slight effect on insulin action, because it is thought that the

occupancy of only a small proportion of receptors can elicit a maximal biological response (Kono and Barham, 1971; Olefsky, 1981a).This suggests that potentiation of insulin action at a postreceptor site may be an important component of the hypoglycaemic effect of metformin.

The present studies have shown increased hepatocyte receptor number in streptozotocin diabetic mice, which could not be further increased by metformin therapy. However, metformin potentiated the hypoglycaemic effect of exogenous insulin in the streptozotocin diabetic mice, suggesting a postreceptor level of action for metformin.

This present study also confirms an increase in insulin binding to soleus muscles of streptozotocin diabetic mice, as a result of raised insulin receptor affinity (Le Marchand and Freychet, 1979). As streptozotocin diabetes is associated with hypoinsulinaemia (Kobayashi and Olefsky, 1979), the increased level of insulin binding may reflect a reduction in the down regulatory effect, exerted by normal insulin concentrations (Soman and De Fronzo, 1980). Consistent with the observations in hepatocytes, metformin did not significantly alter insulin binding in soleus muscles of streptozotocin diabetic mice. However, metformin increased the rate of insulin stimulated ^{14}C-glycogen synthesis in these mice. A maximally effective concentration of insulin was used to identify any changes in insulin responsiveness, which is interpreted as an effect at the postreceptor level (Olefsky, 1981a).

Glycogen synthesis is a postreceptor event which can be modulated by changes in the level of glucose transport and/or alterations in the activity of glycogenic and glycogenolytic enzymes. This study cannot determine which step(s)

is affected. However, it has been shown that metformin increases insulin stimulated glucose uptake into the diaphragm muscle of alloxan diabetic rats (Frayn and Adnitt, 1972) and this effect has been considered to be especially important for the hypoglycaemic effect of the drug (Hermann, 1980). The mechanism involved in metformin induced modulation of postreceptor events of insulin action therefore awaits further study, and may in themselves be limited by the uncertainty surrounding the exact mode of action of insulin on target tissues (Denton et al., 1981).

Discussion and conclusions

The present animal and clinical studies with metformin and glibenclamide have, on the whole, confirmed previous studies in the literature of the effects of oral hypoglycaemic agents on insulin receptor status. In particular, both metformin and glibenclamide increased receptor binding in normal mice, metformin had no hypoglycaemic effect in these mice. The increase in binding was due solely to an increase in insulin receptor number. The effect of metformin on insulin binding *in vivo* in normal mice was rapid (within 24h) and reversible and in addition in obese mice the effect exhibited a dose dependency. Insulin binding was increased in insulin sensitive target tissues of streptozotocin diabetic mice, however this was the result of a raised receptor affinity in soleus muscle and an increased receptor number in hepatocytes. These observations may reflect differences in the mode of receptor modulation possible in the two tissues. Receptor binding was reduced in the obese hyperglycaemic (ob/ob) mouse as a result of a decrease in receptor number. This decrease was proportionally greater in the number of high affinity receptors.

The present studies have provided new information concerning insulin receptor status in diabetes and obesity, together with further data on the mode of action of oral hypoglycaemic agents, especially metformin and glibenclamide. Metformin treatment improved the biological effect of insulin in streptozotocin diabetic mice but had little effect in obese hyperglycaemic mice, suggesting that the metabolic lesion responsible for the syndrome in these mice was different from that in streptozotocin treated mice and that metformin could not correct the lesion in the obese mouse. The present studies have also confirmed that increasing receptor number in these mice did not result in an improved insulin action, and that a postreceptor defect was a likely cause of hyperglycaemia in both cases. Metformin did improve diabetic control in obese type 2 diabetic patients, suggesting that the metabolic lesion in these patients was different from that causing hyperglycaemia in the obese hyperglycaemic mouse. Metformin increased insulin receptor number and improved diabetic control in type 2 diabetic patients, however it appears that the two are not causally related: ie. receptor number remained elevated after metformin withdrawal when glycaemia had returned to pretreatment values. Metformin and glibenclamide did not increase hepatocyte receptor number in streptozotocin diabetic mice, but both drugs improved hypoglycaemia induced by exogenous insulin in these mice; suggesting a post-receptor mode of action for these drugs. Metformin did not increase insulin binding to soleus muscles of streptozotocin diabetic mice but did increase the rate of insulin stimulated ^{14}C-glycogen synthesis, thus demonstrating a postreceptor effect for metformin on insulin action.

The present studies further emphasise the doubtful clinical significance of a raised level of insulin receptor binding, as was first suggested by the 'spare receptor' theory of Kono and Barham (1971). Recent studies have shown that it is probably the high affinity receptors that are of physiological importance. Dolais-Kitabgi et al. (1981) have examined insulin binding and amino acid transport in a primary culture of hepatocytes in which receptor loss was induced by trypsin. From their observations they concluded that in hepatocytes, insulin stimulated amino acid transport was mediated through the binding of insulin to its high affinity receptor sites. In addition Olefsky et al. (1978) have presented data on the degradation of insulin by rat adipocytes that is best explained by a model consisting of two functionally distinct receptor classes, in which only the high affinity population participates in the degradation process.

In view of these observations, it may be possible that the reduced number of high affinity receptors seen in obese mice might contribute to the insulin resistance of these animals, in addition to their postreceptor defects postulated by Kolterman et al. (1981). Moreover, if the high affinity receptor is important in mediating the biological action of insulin, the clinical significance of the effect of metformin on low affinity receptor number is confirmed. This observation was further endorsed by the data from the clinical trial and the studies with streptozotocin diabetic mice. In the latter, receptor number was not affected by metformin (or glibenclamide) and yet insulin action was improved. Furthermore, one postreceptor event that was significantly affected by metformin was muscle ^{14}C-glycogen synthesis.

Whilst further studies are obviously required to pinpoint the exact loci of the postreceptor effects of metformin, it would appear that it is in these areas that the site of action of this drug (and probably glibenclamide also) is to be found.

Although alterations in insulin receptor number by metformin may have little clinical importance, further data on the mechanism of its action may give a valuable insight into the way insulin receptors are regulated. Although in the present work no direct studies were carried out on the mechanism by which metformin brought about an alteration in insulin receptor number, certain observations can be made. Firstly, metformin increased the number of low affinity erythrocyte insulin receptors in obese type 2 diabetic patients. As erythrocytes are anucleate the increase in receptor number could not have arisen by de novo receptor synthesis. In addition, the effect of metformin was rapid, occurring within one week in type 2 diabetics and so could not be accounted for by a significant recruitment of new cells into the erythrocyte pool (Dons et al., 1981b).In addition Vigneri et al. (1982) have shown that inhibitors of protein synthesis do not reduce the effect of metformin on insulin binding. As metformin also influences receptor status in vitro (Holle et al., 1981; Vigneri et al., 1982), a direct effect on target tissues is likely, and probably mediated by an increase in the availability of existing insulin receptors. This increased availability could possibly be achieved by either an uncapping of insulin receptors on the plasma membrane, or increased transport of internalised receptors to the plasma membrane or by an alteration in the rate of receptor synthesis and degradation.

These suggestions are only possibilities at present and await confirmation.

In conclusion, evidence has been presented to confirm receptor and postreceptor effects of metformin and glibenclamide on insulin action. The postreceptor action would appear to be the more clinically significant of the two effects and one postreceptor event that has been shown to be enhanced is the rate of glycogen synthesis. The present studies present no contraindications for the use of either drug, and significantly contribute to the body of evidence in favour of prescribing these drugs for the treatment of diabetes mellitus. Both drugs potentiate the biological action of insulin and hence may have a useful role to play in reducing the insulin requirements of type 1 diabetics. Since insulin will down regulate its own receptor a reduction in the dose of insulin required by type 1 diabetics may have a longterm beneficial effect on receptor status. This possibility has already been investigated and a recent report by Gin et al. (1982) has indicated that metformin administration reduces the postprandial insulin requirement of type 1 diabetics.

CHAPTER FOUR

THE EFFECT OF OBESITY AND AGE ON INSULIN RECEPTOR STATUS

The effect of obesity and age on insulin receptor status

Introduction

The association of obesity with type 2 diabetes mellitus is a frequent observation both in man and in animals (Czech et al., 1977; Cahill, 1979). The factor(s) underlying the occurrence of obesity and the development of diabetes remain largely unknown.

Reduced carbohydrate tolerance during aging is well documented (Boyns et al., 1969; Lauvaux and Staquet, 1970; Fedele et al., 1977). This loss of carbohydrate tolerance with age has been attributed to a reduced insulin sensitivity (Hales et al., 1968; Davidson, 1979) and as early as 1939, Himsworth and Kerr noted that young type 1 ID diabetics responded better to insulin therapy than did older diabetics.

Both obesity and aging are therefore associated with a reduced insulin sensitivity, which can lead to the development of diabetes. As described earlier, the action of insulin can be impaired by several mechanisms including raised levels of insulin antagonists, altered insulin receptor status and reduced postreceptor activity. (Whether one or all of these defects are responsible for the insulin resistance seen in obesity and aging is still not clear, but there is an abundance of data in the literature concerning these two states).

Obesity and insulin action

The obese hyperglycaemic (ob/ob) mouse is a frequently used animal model of obesity and type 2 diabetes (Herberg and Coleman, 1977; Bray and York, 1979). The animal presents a variety of abnormalities which appear at various stages in the development of the syndrome, and any prolonged study involving these mice should take into account the

metabolic conditions prevailing at the time of study.

The obese syndrome in the obese hyperglycaemic mouse has been adequately reviewed (Westman, 1968; Bray and York, 1979) and will thus be summarised only briefly. The obese mouse inherits its obesity as an autosomal recessive mutation on chromosome 6 (Coleman, 1978). The strain has been maintained at Aston University for 15 years as inbred stock on the C57BL/6J background. The mouse is characterised by severe obesity, hyperglycaemia and insulin resistance accompanied by moderate hyperphagia, hyperinsulinaemia, hyperglucagonaemia, hypothermia, hypercellularity of adipose tissue and enlarged islets of Langherans (Bray and York, 1979). Obesity per se, from whatever cause, represents an imbalance between energy intake and energy expenditure. Although hyperphagia is observed in almost all syndromes of obesity, the syndrome in obese hyperglycaemic (ob/ob) mice initially develops without hyperphagia (Lin et al., 1977). Milk uptake is normal in the obese mice before weaning (Liu and Yin, 1974) after which food intake is depressed until day 35, at which point hyperphagia is first observed (Liu and Yin, 1974).

A decrease in thermogenesis is the earliest demonstrable malady in these mice, a reduction in core temperature and oxygen consumption is apparent as early as 10-14 days. This property is used to distinguish obese from lean mice at an early age (Kaplan and Leveille, 1974; Trayhurn et al., 1977). Impaired thermogenesis can, however, only partly account for the increase in fat cell size seen after 14-20 days, and it is increased liver and adipose tissue lipogenesis that is the main cause of the increase in body weight seen between days 17-21 (Joosten and Van der Kroon,

1974; Loten et al., 1974; Czech et al., 1977). Obesity is only visibly detectable at 25-28 days (Joosten and Van der Kroon, 1974) but Thurlby and Trayhurn (1978) have demonstrated an increase in body fat at 10-12 days in the Aston (ob/ob) mouse.

At day 17-21 there is a slight increase in serum insulin levels accompanied by hypoglycaemia (Dubuc, 1976). Insulin levels then continue to rise rapidly and insulin resistance gradually appears and is represented by hyperglycaemia in the presence of a raised insulin concentration. The increase in carcass fat, which appears early in the syndrome, is accompanied by a decreased protein deposition which is first evident at 21-28 days and thus occurs before the appearance of overt insulin resistance (Thurlby and Trayhurn, 1978; Liu and Yin, 1974; Bergen et al., 1975). The obese mouse gains weight rapidly from day 35 and hyperinsulinaemia, hyperglycaemia and hyperphagia increase during this dynamic phase of the syndrome, with the concomitant development of progressive glucose intolerance, fasting hyperglycaemia and a resistance to exogenous insulin. With advancing age (25 weeks and over) weight gain slows or stops and a loss of weight in surviving aged obese mice has been reported (Batt, 1978; Flatt and Bailey, 1981). Flatt and Bailey (1981) have noted that at 40 weeks of age plasma insulin concentrations are lowered and insulin resistance is reduced. Additionally, longevity is reduced in the obese mouse and animals that do not shed weight do not live much longer than 40 weeks.

The earliest detectable difference between the obese mouse and its lean littermate is an impairment in thermogenesis in the former. Basal hyperinsulinaemia occurs after this defect has manifested itself and so is not a candidate

for the primary aetiology of obesity. The same is true for the resistance to exogenous insulin which closely follows the appearance of hyperinsulinaemia. The development of the syndrome is not dependent on hyperphagia. The cause of insulin resistance in the obese mouse is not easy to define, but as it is an 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 type 2 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. The first step in the biological action of insulin is its binding to specific receptors in the plasma membrane of target cells, the degree of response to the hormone can be influenced by the concentration and affinity of these insulin receptors. Other, and possibly more physiologically significant, sites of regulation of insulin action could occur distal to the receptor binding event, at postreceptor sites. Several studies have demonstrated reduced receptor concentrations in obese humans and animals (Archer et al., 1973; Olefsky, 1976a;Olefsky and Kobayashi. 1978) but reports concerning any postreceptor defects are not conclusive. Their contrasting results are probably due to tissue differences as exemplified by data from studies concerning the insulin mediated glucose transport system. This system appears to be unaffected by obesity in adipocytes (Freychet et al., 1972; Czech et al., 1978) but is greatly reduced in skeletal muscle (Becker et al.,1978; Crettaz et al., 1980; Czech et al., 1978). Postreceptor abnormalities in insulin action present in obesity may therefore be

heterogeneous in nature. This situation emphasises the need for studies involving all the major target tissues and confirms the folly of extrapolating results gained, with one tissue only, to all tissues.

On the basis that insulin receptor and/or postreceptor defects could account for the insulin resistance of obesity, and also type 2 diabetes, it is of significance to determine which of these defects is the primary agent. To date only one study has related insulin binding with insulin action during the development of the obese hyperglycaemic syndrome (Grundleger et al., 1980). These workers measured the binding of labelled insulin to soleus muscles of lean and obese mice over the period of between 3 to 15 weeks of age. In addition they monitored the effect of insulin on glucose transport and utilization. Their results demonstrated that in soleus muscle a defect in insulin action preceeded a reduction in insulin receptor binding, moreover, the resistance to the action of insulin in this tissue increased over the age range studied.

In order to confirm the work of Grundleger, a study was set up to examine an alternative postreceptor event, the rate of insulin stimulated glycogen synthesis. Also insulin receptor binding was monitored, not in soleus muscle, but rather in liver. Insulin sensitivity was also monitored using an insulin hypoglycaemia test. The age range in this study was extended to cover the lifespan of both lean and obese mice and was designed to provide information on the impact of the aging process on insulin receptor status and insulin action. Data from this study should provide some understanding of the cause of the decreased insulin sensitivity observed in aging obese mice (Flatt and Bailey,

1982) and the reduced insulin resistance that has been observed in older (40 weeks) obese mice (Flatt and Bailey, 1981).

Aging and insulin action - human studies

There is an abundance of evidence in the literature to suggest that there is a loss of insulin sensitivity with age (Davidson, 1979). Both intravenous and oral glucose tolerance tests are impaired with age (Hayner et al., 1965; Boyns et al., 1969; Calloway and Kujak, 1971; O'Sullivan et al., 1971; Smith and Hall, 1973; Palmer and Ensinck, 1975) and this effect cannot be explained by a delayed glucose absorption (Kendall, 1970), physical activity or low carbohydrate diets in the elderly (Seltzer, 1970). Post-prandial glucose concentrations are also raised with age at a rate of approximately 4 mg glucose/100ml/decade (O'Sullivan and Mahan, 1971). Fasting glucose levels are also elevated with age, but this is much smaller, ie. about 1 mg glucose/100ml/decade (Swerdloff et al., 1967; Dudl and Ensinck, 1977).

Several authors have demonstrated an impairment of the acute release phase of insulin (in response to glucose) in the elderly (Metz et al., 1966; Jaffe et al., 1969; Smith and Hall, 1973) but this is not thought to be related to any impairment in glucose disposal since insulin concentrations are similar in young and old at 30 minutes after glucose administration, but the glucose level remains higher in the older age group (Jaffe et al., 1969; Smith and Hall, 1973). Insulin antagonism appears to be the primary cause of the aging effect on carbohydrate metabolism, although direct studies to evaluate such an effect have been few. Insulin hypoglycaemia tests in which glucose is

administered as well as insulin have shown a negative correlation of glucose disappearance rates with age (Himsworth and Kerr, 1939; Dyck and Moorhouse, 1966). Hypoglycaemia tests involving a bolus injection of insulin alone have failed to show such a correlation (Franckson et al., 1966; Calloway and Kujak, 1971). Neither of these methods is a satisfactory measure of insulin sensitivity since both produce supra-physiological concentrations of insulin in the blood.

Recent advances in the assessment of insulin sensitivity have been facilitated by the use of so-called 'clamp techniques'. They all involve the assessment of the tissue response to exogenous insulin at physiological insulin concentrations. Shen and coworkers (1970) used a method that involved the measurement of steady state plasma glucose levels during an infusion of insulin, glucose, propranolol and adrenaline. Propranolol and adrenaline inhibit endogenous insulin secretion. The infusion lasts for 150 minutes and during the final 60 minutes plasma insulin and glucose concentrations reach equilibrium. Insulin levels are similar in each subject and the resulting glucose concentrations are a measure of the effectiveness of the exogenous insulin. A single study by Kimmerling et al. (1977) employing this technique has reported no loss of insulin sensitivity with age. Significantly this experiment has been one of the few not to show an effect of aging on glucose tolerance (Davidson, 1979).

An alternative method of assessing exogenous insulin action is the so-called 'euglycaemic clamp technique'. This involves the administration of a priming dose of insulin over a period of 10 minutes, followed by 110 minutes of

continuous insulin infusion to achieve a steady state concentration that is in the physiological range (usually 100 μU/ml). The additional amount of glucose required to maintain glucose concentrations at basal preinfusion levels is taken as a measure of the effectiveness of the exogenous insulin. Using this technique De Fronzo (1979) has shown impaired insulin sensitivity in aging patients. By employing tritiated glucose De Fronzo demonstrated that hepatic glucose production was not affected in the aging patients, suggesting that muscle and fat may be the significant sites of insulin antagonism during aging. The work of De Fronzo has not gone uncontested, however, Andres and Tobin (1977) have found no effect of age on the effectiveness of either endogenous or exogenous insulin. There is therefore a need for further work in this area, using both euglycaemic and hyperglycaemic clamp techniques to confirm the relative sensitivity to exogenous and endogenous insulin respectively. In addition, in vitro studies using biopsy material may offer the opportunity to obtain direct evidence for an effect of aging on insulin action. There is only limited information in the literature, concerning the effect of age on insulin stimulated glucose oxidation and conversion of glucose to lipid in adipose tissue segments and isolated fat cells. The response to insulin was most pronounced in children under 15 years of age and was reduced in adults over 20 years of age. No difference was observed between adults and older patients ie. over 50 years. Adipose tissue is not the most suitable model for a study of insulin sensitivity on aging since cell size increases with the age of the individual and the age of the cell and so obesity is also involved (Bjorntorp, 1974). On the other hand muscle

appears to be a most suitable tissue and muscle biopsy studies in the future may provide useful information concerning the effect of aging on insulin action. However, for the present, animal experiments must be used to furnish data on the impact of aging on the action of insulin.

Information from animal studies

By and large results from animal studies have tended to confirm the findings of studies involving man. Aged rats exhibit significantly elevated fasting plasma glucose concentrations (Bracho-Romero and Reaven, 1977; Florence and Quarterman, 1972) and impaired tolerance to oral (Bracho-Romero and Reaven, 1977) and intravenous glucose loads (Gommers and Genne, 1975). Insulin secretion is not impaired in old rodents and has been shown to be raised in older rats (Bracho-Romero and Reaven, 1977) and mice (Bailey and Flatt, 1982), suggesting the presence of insulin resistance since plasma glucose concentrations were shown to be either normal or raised. In rats, insulin hypoglycaemia tests with or without a glucose supplement (Gommers and Genne, 1975) have failed to show an effect of age on insulin sensitivity. However, Bailey and Flatt (1982) have shown evidence of an increased resistance to exogenous insulin in mice with age. Their results may reflect a species difference or alternatively may highlight the inability of the insulin hypoglycaemia test to detect slight, but probably significant, changes in insulin sensitivity.

The various clamp techniques have not been used in animals to study the effect of age on insulin sensitivity, but the evidence in the literature, in the form of fasting hyperglycaemia and raised insulin concentrations in older rats and mice, would suggest the presence of insulin

resistance in aged animals. Animal studies do, however, offer the advantage of *in vitro* metabolic studies on tissues which would be either unethical or difficult in man. Such studies can provide more precise data on the effect of age on the insulin responsiveness of selected target tissues, such as liver and muscle. As insulin has only a permissive role in glucose metabolism in the liver (Davidson, 1981) the main tissues chosen for study have been fat and muscle. The majority of data available in the literature concerns the effect of aging on fat cell metabolism, although the few studies with muscle have provided the more accurate information. The fat cell increases in size with age and so the effects of cell size and aging have to be delineated. This can be achieved by sorting and using cells of the same size from young and old animals. Hansen et al. (1974) using this method showed a reduced insulin effectiveness on lipogenesis and lipolysis in rat adipocytes. An alternative approach involves the maintenance of body weight in older animals by dietary restriction. Bracho-Romero andReaven (1977) demonstrated that glucose intolerance and hyper-insulinaemia in old rats is secondary to age and not obesity. Although *in vitro* studies using isolated adipocytes have shown a reduced insulin mediated glucose uptake (Olefsky, 1977), glucose oxidation (Livingstone et al., 1972; Olefsky, 1976 c;Czech, 1976) and lipogenesis (Holm et al., 1977), the results must be viewed with an open mind since dietary manipulation *per se* affects fat cell metabolism (Ogundipe and Bray, 1974).

The cause of the insulin resistance observed in older rats (Bracho-Romero and Reaven, 1977) and mice (Bailey and Flatt, 1982) has therefore not been elucidated at the

cellular level using fat cell studies. Thus the most informative data on the effect of age on insulin-mediated metabolism of glucose has been derived from studies involving muscle, a tissue that is not affected to the same degree by nutritional factors (Vrana et al., 1968; Cohn and Joseph, 1970). Despite the apparent suitability of muscle for the study of aging processes, experimental work in this area has been limited. Work by Davidson & Karjala (1980) where rats were fed hypocaloric diets to maintain body weight, has indicated that the insulin resistance of muscle was due to obesity and not age.

The suitability of the rat for aging studies is thus questionable and it is probably wise to only make comparisons between adult and old animals ie. after most of the weight gain has occurred. Whether the insulin antagonism in the muscle of older rats is caused by obesity or age remains to be established, but Davidson has defined the primary site of insulin antagonism in this tissue. He demonstrated that the insulin stimulated uptake of 2-deoxy-glucose into intact diaphragms from older rats was decreased and levels of glycolytic intermediates were reduced, suggesting that the defect therefore resides at the level of glucose transport and not at some rate-limiting step in the glycolytic pathway (Davidson, 1979).

There is still plenty of scope for further investigations into the impact of the aging process on insulin resistance in older animals. Such studies would usefully encompass measurements of insulin binding to the major target tissues for insulin, since data in this area is limited. Insulin binding has been assessed in a variety of tissues from rats and mice, including liver (Freeman et al., 1973; Soll et al.,

1975; Sorrentino and Florini, 1976), heart plasma membranes (Sorrentino and Florini, 1976) and adipocytes (Olefsky and Reaven, 1975). Insulin binding was shown to be decreased with age in each of these tissues, but on a relative basis was highest in immature animals, the biggest differences in binding occurred between young (4-6 weeks) and adult (3-4 months) animals. As plasma insulin concentrations gradually increase throughout the lifespan of rats (Freeman et al., 1973; Olefsky and Reaven, 1975) and mice (Bailey and Flatt, 1982), the reduction in insulin binding may be due to a down regulatory effect of insulin upon its own receptors. The cause of the raised insulin concentrations in aging animals is not understood, it may be that post-receptor mechanisms are affected prior to an effect at the receptor and the increase in insulin levels is an attempt to combat the reduced effectiveness of endogenous insulin. Although the alternative possibility of a reduced receptor number being the initiator of a raised insulin level cannot be ruled out, the concept of spare-receptors makes this seem somewhat less likely.

The present study, which involves an assessment of insulin receptor status and postreceptor activity throughout the life span of both lean and obese mice, has been designed to shed some light on our understanding of the effect of age and obesity on the biological action of insulin. The work includes an assessment of insulin sensitivity using an insulin hypoglycaemia test and also involves the measurement of the rate of insulin stimulated ^{14}C-glycogen synthesis in muscle. These latter parameters are related to insulin receptor status, which has been monitored in liver and muscle, providing information on the

effect of age and obesity on two major target tissues for insulin.

Protocol

Lean and obese hyperglycaemic mice from the Aston Colony, aged 3 to 60 and 3 to 40 weeks respectively, were used to investigate the effects of obesity on the biological action of insulin. Receptor status was determined in isolated hepatocyte and soleus muscle preparations and the insulin stimulated ^{14}C-glycogen synthesis was estimated in soleus muscle as a measure of postreceptor activity and the biological action of insulin. The soleus muscle was chosen in preference to the hepatocyte for the postreceptor investigations since most of the actions of insulin in the liver are permissive, that is under normal physiological conditions in the liver glucose is the main regulator of its own metabolism (Davidson, 1981). Body weight, plasma insulin and plasma glucose concentrations were measured in each age group. Insulin hypoglycaemia tests were not performed, partly because of their inability to detect slight changes in insulin sensitivity and also because such tests have recently been carried out in this laboratory (Bailey and Flatt, 1982). Data on age related changes in insulin sensitivity as provided by the insulin hypoglycaemia tests are thus already available for comparison.

Materials and methods

Lean (+/+) or (+/ob) and obese (ob/ob) hyperglycaemic mice were used at 3, 5, 10, 20 and 40 weeks of age. Lean mice only were studied at 60 weeks of age since obese mice do not generally live longer than 40 weeks. Mice were fed a standard pellet diet and allowed free access to water. All animals were maintained in an air conditioned room at

$22^{\circ} \pm 2^{\circ}C$ with a 12h dark, 12h light schedule.

Obese mice can be visibly identified at 5 weeks of age onwards, prior to this they appear identical to their lean littermates. Obese mice can, however, be distinguished by a method relying on their reduced ability to maintain body temperature at lower enviromental temperatures (Kaplan and Leveille, 1974). This defect in thermogenesis is detectable as early as 14 days. The anal temperature of the obese animal is taken before and after 1 hour at $4^{\circ}C$. Mice showing a drop in body temperature of $4^{\circ}C$ or more over this period of time were taken to be obese, the lean mice either maintained their body temperature or showed a reduction of around $1^{\circ}C$.

Insulin binding studies

^{125}I-insulin binding was measured in isolated hepatocytes and soleus muscles of lean and obese mice as described in Materials and Methods (page 72). Binding is expressed per square micron of cell surface area for the hepatocyte since previous authors have shown differences to exist in the size of hepatocytes from lean and obese mice (Kahn et al., 1973). Cell diameter was accordingly measured, using a Neubauer haemocytometer and a microscope fitted with an eyepiece graticule. In soleus muscle ^{125}I-insulin binding was measured in the absence (total) or presence of 8μM unlabelled insulin (non-specific). Nonspecific counts were subtracted from total to give the amount of ^{125}I-insulin specifically bound. All results were expressed per mg protein. (Protein was estimated by the micro Lowry technique).

Studies on ^{14}C-glycogen synthesis in lean and obese mouse soleus muscle

Fed mice were killed by cervical dislocation at 10.00h and the soleus muscles were removed by the method of Maizels et al. (1969). Rates of insulin stimulated glycogen synthesis were determined using maximally effective insulin concentrations, on the basis that any differences observed between lean and obese mice could be attributed to post-receptor alterations. Results were expressed as nmoles ^{14}C-glycogen produced per hour per mg protein.

Results

The body weight, plasma insulin and plasma glucose levels of lean and obese mice with age have been summarised in Table 13. Body weight increased with age in both lean and obese mice, upto 60 weeks and 20 weeks respectively. The rate of increase in weight was more rapid in the obese mice and by 10 weeks of age they were significantly heavier than their lean counterparts. In surviving 40 week old obese mice the body weight began to fall slightly.

Plasma glucose concentrations did not significantly alter through the life span of the lean mouse. In obese mice plasma glucose levels increased markedly between 3 and 20 weeks, with a significant fall at 40 weeks.

Plasma insulin concentrations rose steadily from 10 to 60 weeks in the lean mice, whilst in obese mice there was a marked increase from 3 to 10 weeks followed by a decline at 20 and 40 weeks. Body weight was greater in obese mice compared to lean at 10 and 40 weeks, wheras plasma glucose levels were raised in obese mice at 10 and 20 weeks. Plasma insulin levels were greater in obese mice compared to lean between 3 and 40 weeks of age.

Table 13. Body weight, plasma insulin and plasma glucose values of lean and obese mice of various ages.

Age (weeks)	n	OBESE MICE			LEAN MICE		
		Body weight (g)	Plasma insulin (ng/ml)	Plasma glucose (mM)	Body weight (g)	Plasma insulin (ng/ml)	Plasma glucose (mM)
3	6	14.4 ± 1.3	4.3 ± 0.7*	7.4 ± 0.7	13.1 ± 0.4	1.4 ± 0.5	7.9 ± 0.6
5	6	33.4 ± 3.9	11.5 ± 3.3*	8.6 ± 0.8	26.0 ± 1.9	2.0 ± 0.3	7.8 ± 0.3
10	6	63.9 ± 2.5***	32.3 ± 10.0*	10.7 ± 0.7**	34.9 ± 1.2	1.3 ± 0.3	7.2 ± 0.2
20	6	101.5 ± 2.7***	24.0 ± 7.4*	11.6 ± 0.3***	42.2 ± 0.6	2.3 ± 0.5	7.4 ± 0.4
40	6	93.8 ± 1.8***	15.1 ± 4.3*	8.8 ± 0.9	48.4 ± 2.5	2.6 ± 0.5	7.5 ± 0.4
60	6	-	-	-	52.8 ± 2.5	3.2 ± 0.6	7.8 ± 0.4

Values are mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for obese compared to lean mice.

Insulin binding to isolated hepatocytes

Hepatocyte diameter was measured at all ages in lean and obese mice and these values are given in Table 14. Hepatocyte size was significantly increased in obese mice compared to lean at 20 weeks. There was also an age related increase in cell size in the lean mice, with a significant increase in cell size at 60 weeks compared to 20 weeks.

Hepatocyte insulin receptor binding data are summarised in Table 15. Hepatocyte insulin receptor binding was not significantly different in lean and obese mice at 3 and 5 weeks of age, However, receptor binding was significantly reduced in the obese mouse hepatocyte at both 10 and 20 weeks of age compared to lean mice. These alterations in binding were due to a decrease in the number of high affinity receptor sites (per μm^2 cell surface area). Receptor affinity was not significantly altered, except that $\bar{K}_e$ was reduced in the 40 week obese mice compared to age-matched lean mice. Receptor number was significantly elevated at 40 weeks of age compared to 10 and 20 week old obese mice, but values for receptor number at 10, 20 and 40 weeks were all lower than those observed at 3 and 5 weeks of age.

Receptor binding showed a significant age related decline in the lean mice from 5 weeks of age, reaching a nadir at 60 weeks. The alterations in binding were due mainly to changes in receptor number and not affinity, but the low affinity receptors were affected to the greater extent, unlike the changes observed for the obese mouse.

Insulin binding to lean and obese mouse soleus muscle

Soleus muscle weight and protein content have been summarised in Figure 30. Soleus muscles were not signifi-

Table 14. Hepatocyte diameter (μm) in lean and obese mice at different ages.

Age (weeks)	n	Obese mice	Lean mice
3	4	18.0 ± 1.5	18.0 ± 2.0
5	4	24.5 ± 1.5	24.0 ± 1.2
10	4	21.5 ± 2.0	24.0 ± 2.9
20	4	32.0 ± 2.0	24.0 ± 2.0*
40	4	26.0 ± 1.9	26.0 ± 3.0
60	4	-	33.0 ± 2.5 †

Values are mean ± SEM. * $P<0.05$ for obese mice compared to lean mice. † $P<0.05$ for the 60 week compared to the 20 week value in lean mice.

Table 15. Specific ^{125}I-insulin binding to hepatocytes isolated from lean and obese mice at various ages. Data are expressed as receptor sites per μm^2 cell surface area. R_o= receptor number, $\overline{K}_e$ and $\overline{K}_f$ are affinity constants expressed as nM^{-1}.

OBESE MICE

Age (weeks)	n	Total R_o	High Affinity R_o	Low Affinity R_o	$\overline{K}_e$	$\overline{K}_f$
3	4	325 ± 69	140 ± 12	183 ± 75	0.09 ± 0.01	0.012 ± 0.001
5	4	384 ± 79	63 ± 8.5	320 ± 70	0.07 ± 0.008	0.012 ± 0.002
10	4	116 ± 12††	25 ± 1.0†	90 ± 10†	0.10 ± 0.02	0.017 ± 0.0015
20	4	105 ± 20††	15 ± 2.6††	90 ± 21†	0.21 ± 0.03†	0.025 ± 0.007
40	4	221 ± 17	46 ± 5.5	174 ± 17	0.075 ± 0.006	0.016 ± 0.006

Values are mean ± SEM. † $P<0.05$, †† $P<0.01$ for obese mice at 10 and 20 weeks of age compared to 40 weeks of age.

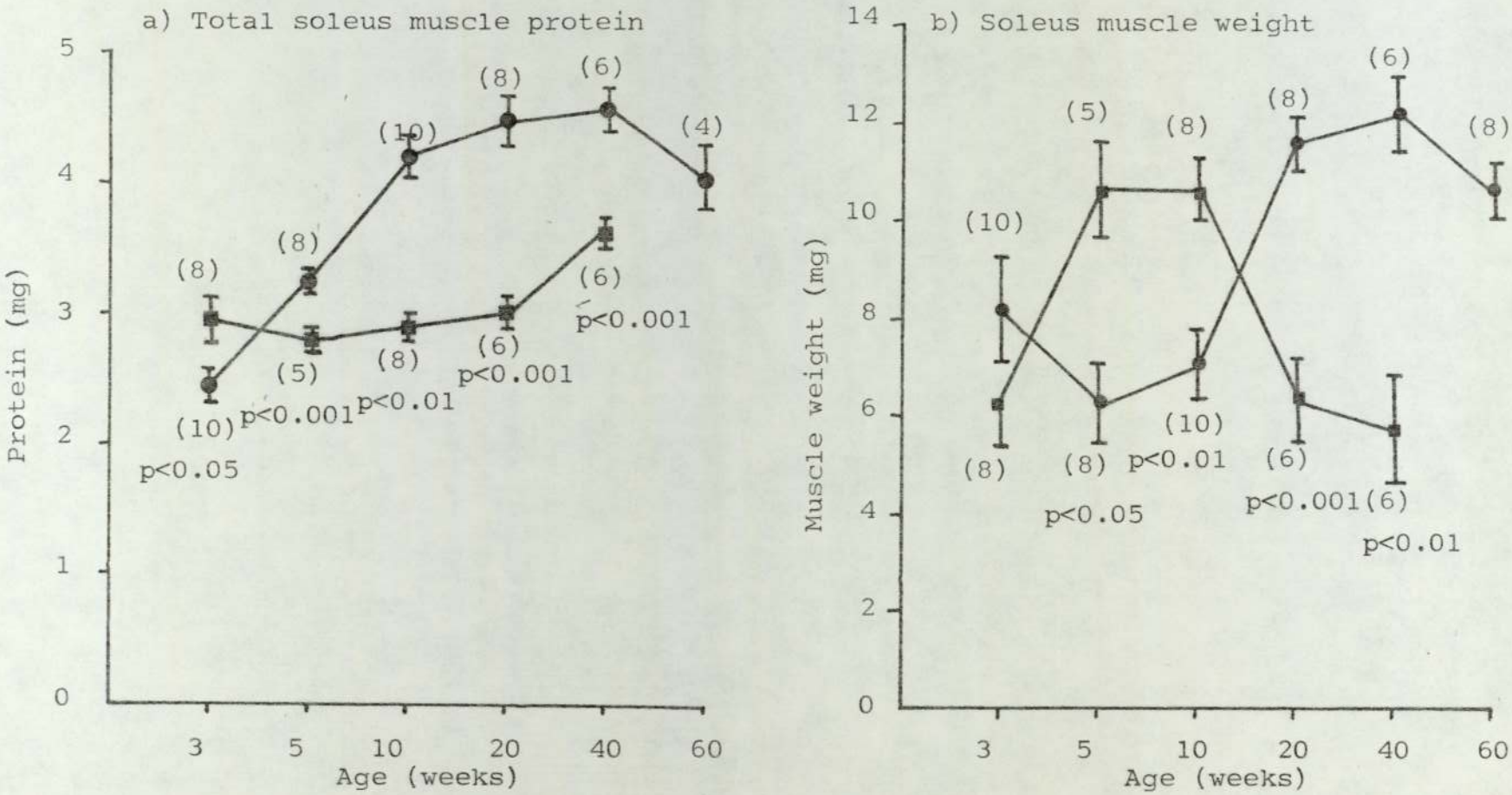
Table 15. (continued)

LEAN MICE

Age (weeks)	n	Total R_o	High Affinity R_o	Low Affinity R_o	$\bar{K}_e$	$\bar{K}_f$
3	4	315 ± 47	91 ± 5.5	223 ± 51	0.13 ± 0.03	0.017 ± 0.0035
5	4	361 ± 24	64 ± 10	296 ± 33	0.08 ± 0.01	0.018 ± 0.004
10	4	258 ± 20**	63 ± 8*	192 ± 40	0.085 ± 0.008	0.012 ± 0.002
20	4	168 ± 11*	50 ± 5.4*	117 ± 11	0.14 ± 0.02	0.032 ± 0.008
40	4	259 ± 14	59 ± 4.2	199 ± 15	0.14 ± 0.02*	0.022 ± 0.004
60	4	100 ± 8 ‡	43 ± 3.7	56 ± 6.5‡	0.17 ± 0.01	0.028 ± 0.004

Values are mean ± SEM. * $P<0.05$, ** $P<0.01$, for obese compared to lean mice. ‡ $P<0.001$ for lean mice at 60 weeks of age compared to 40 weeks of age.

Figure 30. Weight and total protein content of soleus muscles from lean (●) and obese (■) mice of between 3 and 60 weeks of age.



Values are mean ± SEM, numbers in parentheses indicate number of observations

cantly different in lean and obese mice until 10 weeks of age. At 20 and 40 weeks of age muscle weight was reduced in obese mice compared to lean. Muscle protein content was significantly lower from 5 upto 40 weeks of age in the obese mice compared to lean, preceeding the reduction in muscle mass.

Insulin binding to isolated soleus muscle (Table 16) was reduced in the obese mice at 10, 20 and 40 weeks of age, but was not significantly different from lean mice at 3 and 5 weeks. Insulin binding remained fairly constant over the period 3 to 40 weeks in the lean mice, but was significantly reduced at 60 weeks as compared to the 40 week old mice.

Insulin stimulated rate of ^{14}C-glycogen synthesis in isolated soleus muscle from lean and obese mice.

The data for the insulin stimulated rate of ^{14}C-glycogen synthesis in isolated soleus muscles from lean and obese mice has been summarised in Table 17. The rate of insulin stimulated ^{14}C-glycogen synthesis in soleus muscle was significantly lower in obese mice at all ages, from 5 weeks, compared to lean mice. There was no significant decline in the rate of glycogen synthesis in the 60 week old lean mice. There was a dramatic fall in the rate of insulin stimulated glycogen synthesis in 40 week old obese mice, the value was the lowest for all age groups (both lean and obese), almost approaching zero.

Discussion

The present work confirms the age related development of insulin resistance in obese mice first documented by Grundleger et al. (1980). The binding of insulin to both liver and soleus muscle was not significantly reduced in the obese mice until 10 weeks of age (Tables 15 and 16),

Table 16. ^{125}I-insulin binding to isolated soleus muscles of lean and obese mice at different ages. Results are expressed in terms of pg insulin bound/mg muscle protein.

Age (weeks)	n	Obese mice	Lean mice
3	4	7.77 ± 1.66	5.65 ± 1.4
5	5	6.01 ± 0.94	7.40 ± 0.61
10	6	1.78 ± 0.41*	5.58 ± 0.79
20	5	2.31 ± 0.86*	6.29 ± 0.32
40	6	1.83 ± 0.87*	6.21 ± 0.52
60	4	-	5.21 ± 0.40†

Values are mean ± SEM. * $P<0.01$ for obese mice compared with lean mice, † $P<0.05$ for the 60 week value compared to 40 week value in lean mice.

Table 17. Insulin stimulated rate of ^{14}C-glycogen synthesis in isolated soleus muscles of lean and obese mice of different ages. Values for the rate of glycogen synthesis are given as nmoles glycogen/hr/mg muscle protein.

Age (weeks)	n	Obese mice	Lean mice
3	4	0.68 ± 0.16	0.47 ± 0.07
5	4	0.76 ± 0.20*	1.62 ± 0.05
10	4	0.89 ± 0.08*	1.41 ± 0.01
20	4	1.24 ± 0.36*	3.63 ± 1.1
40	4	0.15 ± 0.017**	2.42 ± 0.36
60	4	-	2.68 ± 0.25

Values are mean ± SEM. * $P<0.05$, ** $P<0.01$ for obese compared with lean mouse values.

wheras the rate of insulin stimulated glycogen synthesis was lower in the soleus muscles from obese mice at 5 weeks of age (Table 17). It is probable that differences in binding and glycogen synthesis might have been apparent sooner than 10 and 5 weeks respectively, had these parameters been monitored at weekly intervals (Grundleger et al., 1980). Certainly alterations in insulin action in soleus muscle have been reported at or prior to 5 weeks of age in obese mice (Stauffacher and Renold, 1969; Grundleger et al., 1980). The values for the rate of insulin stimulated glycogen synthesis in lean mice from the (ob/ob) colony are lower than those for Swiss albino mice (Le Marchand et al., 1978) but are in agreement with those reported for the lean mice of the obese hyperglycaemic stock (Grundleger et al., 1980). These differences are in part due to the species variation; such variation within a species does not however seem to occur dependent upon the cause of obesity. In the present study and those of Grundleger et al. (1980) values for the rate of insulin stimulated glycogen synthesis in obese mice are in agreement with each other and those for gold thioglucose obese mice (Le Marchand et al., 1978).

The results of this study suggest that a postreceptor defect of insulin action is a possible primary cause of insulin resistance in the obese hyperglycaemic mouse. The precise location of the defect(s) cannot be determined from this study but might lie at a site close to the receptor binding event. In the case of glycogen synthesis a defect could occur with one or all of the kinases and phosphorylases involved in the regulation of glycogen phosphorylase and glycogen synthase, the rate limiting enzymes in glycogen metabolism. Alternatively obese mice may have a reduced

capacity for the generation of the insulin 'second messenger' following the binding of the hormone to its receptor. The second messenger has not been established for insulin, but there are several possible candidates including a membrane bound peptide that has been reported to be released upon binding and possibly various protein kinases that control the phosphorylation state of key enzymes (Denton et al., 1981). Further work is therefore required to locate and identify the precise postreceptor defect in obese mice, but such a search will be partially hindered by the lack of information concerning the second messenger for insulin action.

Flatt and Bailey (1981) have shown that there is a steady decline in insulin sensitivity from 5 weeks of age in obese mice and more interestingly a marked improvement in insulin sensitivity at 40 weeks of age. This improvement in insulin sensitivity may be the result of changes in either receptor or postreceptor activity. The results of the present study also indicate an improvement of insulin action in the aged obese mice, insulin levels were significantly lower and plasma glucose also fell. The reduction in plasma insulin levels in the 40 week old mice would be expected to bring about an increase in insulin receptor binding. This change would be secondary to and not the primary cause of the change in insulin sensitivity. Hepatocyte insulin binding is increased in the obese mice at 40 weeks but such a change is not seen in soleus muscle from these animals. In addition there was a fall in soleus muscle insulin stimulated glycogen synthesis at 40 weeks. These results may reflect differences in the way the target tissues are affected by the obesity syndrome and further indicate the need for studies involving two or more insulin

target tissues. Alternatively it must be remembered that lean body mass is greatly reduced in the obese mouse (Liu and Yin, 1974) and the failure of these mice to respond to the reduced insulin levels at 40 weeks may reflect an inability of the muscle to accomodate its response to insulin binding. In other words the basal condition of the muscle may already be showing a maximal insulin stimulated rate of glycogen synthesis.

In the present study binding was improved in obese mice in the liver, but bearing in mind the spare receptor concept (Kono and Barham, 1971) changes in receptor number per se are unlikely to cause the improvement in insulin sensitivity seen in the 40 week old obese mice. The change in insulin sensitivity in liver and fat are most likely to be mediated at postreceptor sites.

The aging study in lean mice indicated that there is a steady decline in muscle and liver insulin binding over the period from 5 to 20 weeks of age and is broadly in agreement with the work of previous authors (Goodman and Ruderman, 1979; Grundleger et al., 1980). The present study was extended to cover the complete life span of the animal and not just the juvenile to adult stage. A reduction in liver and muscle insulin binding was demonstrated, in the 60 week old mice compared to the 40 week mice (Tables 15 and 16), there was no significant change between 20 and 40 weeks. The absence of a deterioration in binding between young and more mature rats is in agreement with the literature and this study may be the first to show a decline in receptor status in very old mice. Muscle insulin binding data followed a similar pattern to that of liver throughout, suggesting that modifications to insulin receptor status

due to aging are the same in liver and muscle. Therefore, although insulin resistance is a feature of both obesity and aging, the causes of the resistance to the action of insulin may be at different loci in the two cases.

The insulin hypoglycaemia tests of Bailey and Flatt (1982) show that responsiveness to exogenous insulin is reduced in 60 week old lean mice compared to that of 20 week old mice and these data would tend to confirm a reduced sensitivity in the 60 week old mice since insulin levels are raised with no concomitant reduction in plasma glucose concentrations. As previously stated receptor binding is reduced in both the liver and muscle of these older mice but again this is unlikely to wholly account for the reduced insulin sensitivity as only a limited number of receptors (10-30%) need to be occupied to produce a maximal response to insulin. It is again more probable that a postreceptor defect(s) that is not present in younger mice is present in the old mice producing the resistance to the action of insulin. As the insulin stimulated rate of glycogen synthesis did not decline with age in the lean mice, it would appear that this aspect of carbohydrate metabolism is not adversely affected by the aging process. The reduced insulin binding is the result of an increased down regulation of insulin receptors by the raised plasma insulin levels in the 60 week old mice and although glycogen synthesis is not reduced in aging mice it is quite possible that other post-receptor mechanisms are affected.

The alternative explanation for the insulin resistance of old age could be the presence of raised levels of insulin antagonists in plasma. However, Bailey and Flatt (1982) have reported that although elevated levels of circulating

insulin antagonists may explain the hyperglycaemia in neonatal and immature mice (note the slightly raised plasma glucose in the 3 and 5 week old lean mice (Table 13)), almost all studies, including their own, have shown that unstimulated plasma glucagon concentrations (Dudl and Ensinck, 1977; Codina et al., 1980), plasma growth hormone levels (Bazarre et al., 1976) and plasma glucocorticoid levels (Britton et al., 1975; Tang and Phillips, 1978) are not raised with age.

Yet another explanation for the consequences of the aging process at a cellular level could be that there is a preprogrammed deterioration in cell metabolism such that, as a cell ages, it naturally exhibits a reduction in certain functions eg. cellular enzyme activity. Two groups of workers have provided evidence against this possibility. Hollenberg and Schneider (1979) and Helderman (1980) used cultured rat lymphocytes and fibroblasts from young and aged adult donors and showed no differences in receptor site concentration or affinity. This suggests that there is no genetically programmed aging of insulin receptors or postreceptor events and that any detrimental changes are enviromental in origin.

Clinically related studies have tended to confirm the findings of these animal studies. Pagano et al. (1981) showed reduced receptor binding to adipocytes from aging human donors, receptor binding is greater in the newborn than in children and adults (Thorsson and Hintz, 1977; Neufeld et al., 1978; Kappy and Plotnik, 1979; Herzberg et al., 1980b), indicating a decline in receptor status with age, concomitant with a gradual rise in plasma insulin levels.

The prospect of a postreceptor defect as the primary

cause of insulin resistance in the aged animal is therefore an attractive proposition and future work could usefully be directed towards its identification in target tissues for insulin.

To summarise our present understanding, the development of insulin resistance in the obese syndrome in mice is due primarily to a postreceptor defect(s) in the action of insulin. The precise location of the defect(s) is yet to be confirmed. Muscle and liver did not respond in the same way during the later stages of the obese syndrome. As plasma insulin and glucose levels and body weight fell, insulin resistance was reduced but only the liver showed an improvement in receptor activity. Muscle did not show either an enhanced insulin binding or an improved postreceptor responsiveness. These basic differences may reflect either a true tissue difference or may alternatively indicate a reduced ability of the available muscle to take up glucose, due to the relative reduction in lean body mass.

The contribution of the aging process to the development of insulin resistance has also been considered. In this case liver and soleus muscle responded in a similar manner, both showing an age-related decline in receptor binding. The postreceptor effect investigated ie. insulin stimulated glycogen synthesis, did not, however, show an age-related reduction in responsiveness to insulin and so is probably not involved in the insulin resistance of old age. The raised insulin and plasma glucose levels in older lean mice confirmed the reduced insulin sensitivity of older mice reported by other workers in this laboratory (Bailey and Flatt, 1982). The changes in insulin binding paralleled the increase in plasma insulin concentrations and are almost

certainly caused by increased down regulation of insulin receptors.

Changes in receptor status observed in both obese and aged lean mice were quite different in nature. The reduction in hepatocyte receptor number in obese mice initially concerned the high affinity receptors and throughout the syndrome they were affected to a greater extent than low affinity receptors. However, in the old lean mice the number of low affinity receptors was reduced, with only a slight reduction in high affinity receptors. Despite the 'spare receptor' concept (Kono and Barham, 1971), new evidence suggests that the two groups of receptors (or the two affinity states of the same receptor) have quite different functions. Dolais-Kitabgi (1981) has shown that it is the high affinity receptor that mediates amino acid uptake following the binding of insulin and other workers have shown that it is this very same receptor that is involved in receptor internalisation and insulin degradation (Olefsky et al., 1978). It is therefore possible that the high affinity receptor only is involved in mediating the biological actions of insulin in these target tissues, with the low affinity receptor probably acting as a buffer system, binding insulin at high insulin concentrations but not initiating any postreceptor effects. Such a theory would account for the spare receptor concept, since the high affinity receptor generally forms only a small proportion of the total number of receptors present (approximately 20-25% in the liver). Assuming this to be true, the reduction in high affinity receptor number or affinity in obese mice would assume much greater importance and could explain the insulin resistance of these mice, the biological

effects produced by this class of receptors being gradually reduced as the number of high affinity receptors is reduced. The reduction in low affinity receptor number in old age would not completely explain the insulin resistance in old animals and again a postreceptor defect remains the most likely explanation.

Further studies are thus required to clarify the roles of either the different classes of insulin receptors or receptor states and the influence of changes in receptor status on carbohydrate metabolism. Furthermore, the post-receptor loci for the action of insulin also need further investigation before they can be confirmed as primary causes of insulin resistance in the obese hyperglycaemic syndrome in mice and aged animals.

CHAPTER 5

DISCUSSION

Discussion

The work presented here has investigated several aspects of insulin receptor physiology, namely the effect of aging on insulin receptor binding and selected postreceptor mechanism , the regulation of insulin receptor and post-receptor activity during obesity in mice and type 2 diabetes in man and the therapeutic effects of oral hypoglycaemic agents that may be mediated via an action on insulin receptor activity.

These studies have shown age-related changes in insulin receptor concentration in normal mice, insulin receptor number being highest in young mice (< 10 weeks), with a reduction in receptor number in old mice (60 weeks). In addition, insulin mediated glucose metabolism shows age-related changes; the rate of muscle glycogen synthesis increasing from 3 to 20 weeks and falling slightly at 40 and 60 weeks.The appearance of defects in insulin receptor status and insulin action in obese hyperglycaemic mice have also been monitored, demonstrating that a defect in insulin action is present prior to any alteration in insulin receptor status suggesting that the former may be a primary aetiological factor in the development of the obese syndrome in these mice.

The oral hypoglycaemic agent, metformin, improved insulin receptor status in both lean and obese hyperglycaemic mice as well as in type 2 non insulin dependent diabetic patients. Although this effect was accompanied by an enhanced biological response to insulin in the mice and an improved diabetic control in the type 2, NID patients, studies made after withdrawal of the drug indicated that the effect on insulin receptor binding produced by metformin

was not responsible for the drug's therapeutic effect.

Studies involving streptozotocin diabetic mice treated with metformin confirmed that the drug has a postreceptor action on insulin sensitive tissues and that this effect is more likely to be of therapeutic significance than effects at the receptor level. The oral hypoglycaemic agent glibenclamide appeared to show a similar mode of action to metformin. Namely an increase in insulin receptor number in lean mice and some evidence of a postreceptor action.

Whilst the data concerning the effect of both of these drugs on receptor number and affinity largely confirmed earlier reports in the literature, the data indicating the possibility of a postreceptor action of metformin and glibenclamide are worthy of note.

The investigations presented here have utilised a variety of tissues as receptor models, namely hepatocytes and soleus muscle of mice and human erythrocytes. What is apparent from the results of these studies is that insulin receptors on different insulin receptor bearing cells are not subject to the same regulation, and the extrapolation of data from one cell model to another should be made only with the greatest caution and preferably not at all.

Insulin receptors can be said to be in a dynamic state, both in terms of their turnover and their mobility in the plane of the plasma membrane. As the mechanism of action of insulin is still not fully understood, the interpretation of the data generated from the present work needs to be flexible and can be supported by many 'proposals', 'theories' and 'hypotheses '.

It is well established that a maximal response, by a

target tissue, to insulin can be achieved at only partial occupancy of the total number of insulin receptors (Kono and Barham, 1971). Nichol and colleagues (1981) have shown this situation to be achieved by an insulin concentration of approximately 5 ng/ml for most of the biological actions of insulin. In the light of this knowledge it is reasonable to assume that the increase in insulin receptor number seen in the present studies will have little or no influence on insulin's biological action. Similarly, any alterations in receptor binding affinity would be expected to have little physiological value, especially when one considers the work of Pullen et al. (1976). These workers used insulins of low biological activity from porcupine and chinchilla and showed that whilst the insulins had a biological activity of only 5% that of bovine insulin, they had a receptor binding affinity of 25% compared to bovine insulin. (The reduced biological activity of these insulins is attributed to their inability to form dimers). The notion that the insulin binding function of the receptor is related to its biological effector role in a simple manner is not unexpected if one accepts the 'post-aggregation' model of insulin action proposed by Jeffrey (1982). This theory suggests that after binding to its receptor, an insulin monomer can interact with another similar receptor complex. The formation of these cross-links is accompanied by an enhancement of biological activity and may account for the micro-aggregation of insulin receptors, the process thought to be responsible for transmembrane signalling. The clustering of insulin receptors in the membrane may cause changes in the local receptor enviroment, allowing transport of small molecules, such as glucose, across the plasma

membrane (Schlessinger et al., 1978). Other effects of insulin such as protein synthesis and down regulation, are thought to depend upon the internalization of insulin-receptor complexes (Schlessinger et al., 1978; Kahn, 1979; Blecher, 1979). Such a model emphasises that an increase in insulin receptor binding does not necessarily lead to an increase in the biological action of insulin. More importantly, it is those agents that can effect either insulin dimer aggregation, receptor clustering or the second messenger production stages of the mechanism of insulin action, that could significantly alter insulin bioactivity.

Recent studies that may aid our understanding of insulin receptor physiology have come from the laboratories of Kasuga (Kasuga et al., 1982) and may necessitate a slight revision of the more popular models of insulin action, such as the one proposed by Czech (1981) (Figure 31). This model describes the insulin receptor as a bivalent, disulphide-stabilized oligomeric molecule ($\alpha_2\beta_2$). This model contains a receptor area that is concerned with receptor internalization (region 1) and a further region (region 4) for the interaction with a membrane glycoprotein receptor affinity regulator (GPR). The latter region may play a role in the regulation of the catalytic subunit of adenylate cyclase, which helps to produce the fall in cellular cyclic AMP levels observed in the presence of insulin. Several workers have documented a possible interaction between the insulin receptor and the catalytic subunit of the adenylate cyclase complex (Jakobs and Schultz, 1980; Kahn et al., 1981). The above model proposes that receptor clustering leads to activation of a membrane protease (MP) via phosporylation that liberates a peptide

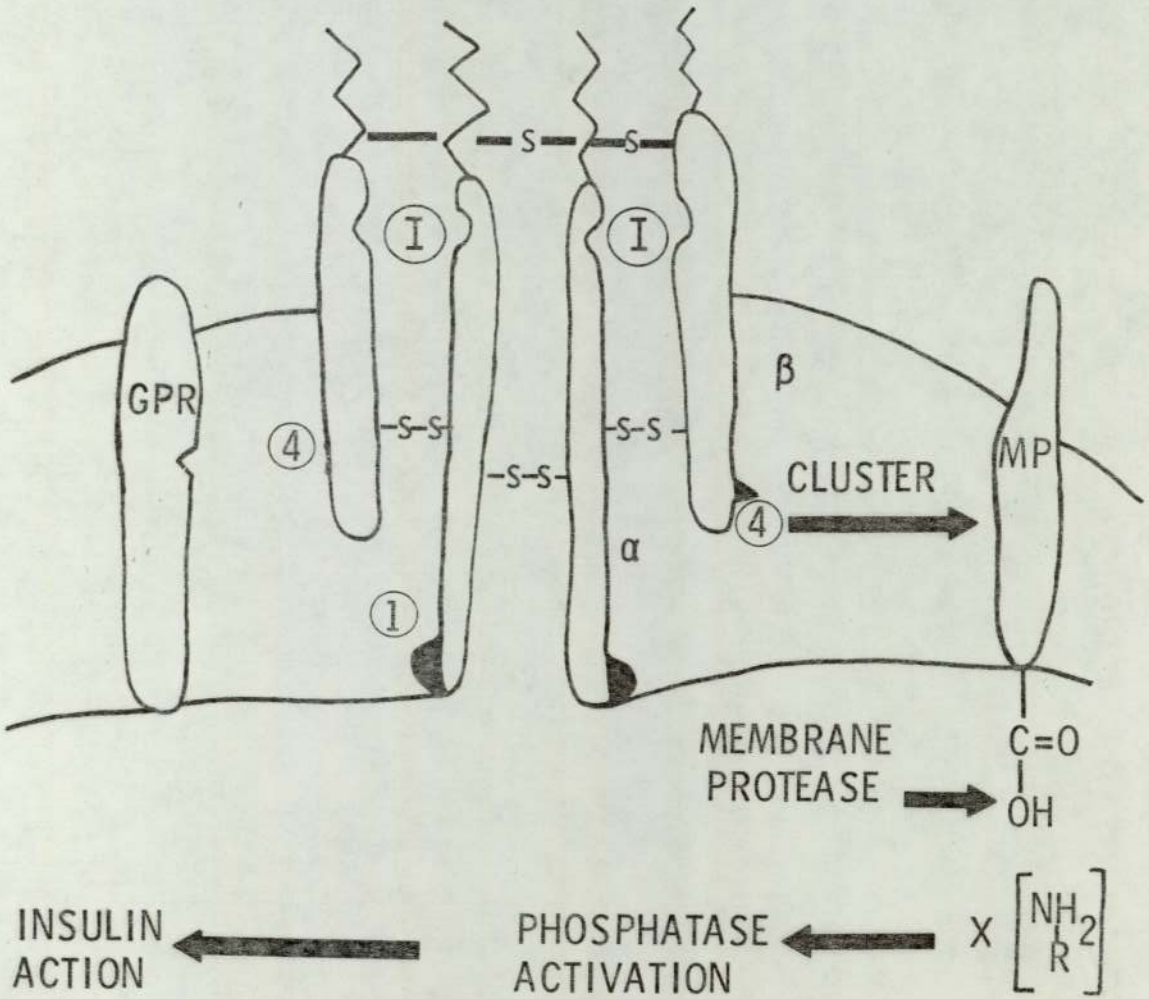


Figure 31. Hypothetical model of insulin action as proposed by Czech(1981). Receptors for insulin (I) are shown as integral membrane glycoproteins (oligosaccharide constituents: ~~) that traverse the plasma membrane. Region 1 is involved in receptor internalisation and Region 4 is shown to interact with a ligand affinity regulator glycoprotein (GPR). MP is the membrane protein substrate of insulin mediated proteolytic activity. The activation mechanism is discussed in the text.

mediator of insulin action (a phosphatase activator). This phosphatase activator is then suggested to affect the phosphorylation state of intracellular enzymes, a mechanism already accepted as a major regulator of enzyme activity, thus leading to either activation or inactivation of these enzymes as a reult of insulin binding.

Control of the phosphorylation and dephosphorylation of membrane proteins and intracellular enzymes is therefore central to the current models of insulin action and it is in this area that the work of Kasuga et al. (1982) will have most impact. This group of workers has shown that the binding of insulin to its receptor leads to phosphorylation (of tyrosine residues) of the receptor itself. It is therefore possible that the insulin receptor is a tyrosine protein kinase, whose activity is controlled by phosphorylation and dephosphorylation induced by the binding of insulin. Czech's model of insulin action will now have to accomodate a role for the phosphorylation of the receptor in the process of transmembrane signalling. It may well be that the activated (phosphorylated) form of the receptor then controls the phosphorylation of the membrane protease, resulting in the release of the phosphatase activator. Alternatively the phosphorylated form of the receptor may form dimer aggregations more easily than the dephosphorylated form and thus enhance the biological activity of insulin.

On this basis it becomes easier to consider membrane localised insulin receptors as oligomeric proteins with regulatory mechanisms that operate as multienzyme systems, having mobility in the plasma membrane and capable of exhibiting an allosteric ligand binding nature, as proposed by O'Brien (1979).

At this point it may be useful to propose several possible mechanisms by which the effects on insulin action, produced by the various conditions and agents considered in the present studies eg. obesity, type 2 diabetes mellitus, oral hypoglycaemic agents and age, may be mediated. It is possible that the defect in insulin action seen early (5 weeks) in the obese hyperglycaemic mouse, may have been the result of an inability of insulin receptors to form dimers and aggregate following binding, thus reducing transmembrane signalling and insulin bioactivity. Alternatively the membrane composition of target cells in these mice may be altered, such that local changes in the plasma membrane, that would normally follow insulin binding, are not initiated thus leading to a reduction in the transport of small molecules such as glucose. An inability of the insulin receptor to combine with the GPR unit is also a possibility, thus influencing actions of insulin that are mediated via changes in adenylate cyclase activity. As phosphorylation reactions are so important in mediating the action of insulin, a defect at one of these stages would have an effect on insulin bioactivity. Phosphorylation of the receptor may not occur on binding of the insulin molecule or alternatively the signal produced by the binding of insulin may not be transmitted to either the membrane protease or other substrates for the protein kinase, due to conformational restrictions. The number of receptors per unit surface area has also been shown to be reduced in obese hyperglycaemic mice. This can be corrected by starvation and appears to be related to circulating insulin levels, therefore down regulatory mechanisms are probably responsible for this observation. As mentioned earlier it

is the internalised hormone-receptor complexes that are thought to be involved in down regulatory mechanisms controlling receptor number.

The amelioration of defects in insulin action by metformin and glibenclamide might have been produced by a variety of routes. One could envisage that these drugs might aid conformational realignment of receptors, leading to an increase in cross-linking of hormone-receptor complexes and thus to an increase in the biological activity of insulin. As the chronic effect of these drugs is not mediated via an increase in insulin secretion, the increased receptor number observed after the administration of these drugs cannot be explained by an effect on down regulatory mechanisms. These agents may therefore affect receptor turnover by slowing the internalization process, possibly by sterically hindering the movement of insulin receptor complexes into coated pits. Feedback mechanisms operating distal to the receptor binding event (Caro and Amatruda, 1980a) may then lead to an increase in receptor synthesis which would accomodate the reduced entry of insulin receptor complexes into the recycling processes.

Metformin is not metabolised by the liver and so effects mediated via entry into the cell seem unlikely, the main mechanism of action would more probably be mediated via modulation of the membranal components of insulin action, namely transmembrane signalling.

Aging produced two major effects, namely a reduction in both receptor number and insulin action in older mice and a higher receptor number in young mice. The latter observation occurs despite the presence of normal levels of insulin in young mice, therefore overriding down regulatory mechanisms.

It may be that the control of receptor turnover by circulating levels of insulin is not fully developed in young mice. The decline in receptor number in older mice coincides with raised insulin levels and so is probably mediated via the down regulatory receptor internalisation process. The enhanced insulin bioactivity in younger mice (note the low insulin/glucose ratio in 3 week old lean mice) may be a consequence of an increased receptor concentration per unit cell surface area, receptors in closer proximity may aid post-dimer aggregation and so amplify the insulin 'signal'. Insulin action may be modulated at sites beyond membrane interaction, at the level of peptide mediator release and the phosphorylation of enzymes.

The data from the present study does not allow the precise identification of the postreceptor mechanisms affected, but supplements data in the literature indicating the significant role of postreceptor alterations in the regulation of insulin action.

It is clear that 'the road ahead' for studies on insulin receptors and the control of insulin action lies in the investigation of postreceptor stages of insulin action. In the case of oral hypoglycaemic agents it would be useful to determine whether these drugs actually enter target cells and whether they affect the degree of receptor cross-linking. The latter phenomenon could be monitored in obese hyperglycaemic and aged mice, together with studies of insulin receptor phosphorylation, peptide mediator release, adenylate cyclase and cAMP phosphodiesterase activity and the phosphorylation and dephosphorylation of intracellular enzymes.

It would be of value to carry out studies on as many insulin target tissues as possible since metabolic regula-

tion may be different in each cell type. Moreover, the present work included one clinical study, future endeavour should be directed towards the location of defects in insulin action and their possible correction in man. Animal studies, whilst forming the basis of our present knowledge of the action of insulin and the irregularities occurring in disease states, can only be used as a background to investigation in man.

In conclusion the present studies have indicated that whilst receptor binding may initiate the sequence of events leading to the biological actions of insulin, the regulation of these actions is more likely to be mediated at steps beyond the binding of insulin to its receptor. Physiological processes and external agents having an effect on one or more of these postreceptor events, rather than simply altering the level of receptor binding, would thus be capable of modulating the action of insulin.

APPENDIX

Glycogen determination

The method used for the determination of glycogen in protein hydrolysates was that of Lo et al. (1970) devised for use with small tissue samples.

Tissues samples were hydrolysed in 1M sodium hydroxide (100°C for 10 minutes), aliquots were removed for protein estimation and the tubes were allowed to cool. 1 mg carrier glycogen (rabbit liver glycogen, 2 mg/ml water) was added to each tube followed by 2 ml of a 66% ethanol solution to precipitate the glycogen. Tubes were mixed and left on ice for 1 hour. At the end of this time the samples were centrifuged (1000g for 10 minutes at 20°C). The supernatant was decanted and the pellet washed once with 2 ml of 66% ethanol (100g, 10 minutes). The supernatant was discarded and the glycogen pellet was dissolved in 0.4 ml of water. The glycogen solution was then mixed with 2 ml of Protosol, transferred to a scintillation vial containing 9 ml of scintillant (Econofluor, NEN) and counted for radioactivity. The counts represented the amount of ^{14}C-glucose converted to ^{14}C-glycogen during the incubation period. As the specific activity of the ^{14}C-glucose was known, the nmoles of ^{14}C-glycogen in the tissue sample could be calculated.

Protein estimation

The procedure used for this estimation is the colorimetric method of Lowry et al. (1951). The intensity of the colour that is produced during the reaction serves as a measure of the amount of protein present. Even though the relation of colour to protein concentration is not quite linear, the protein present in the samples may be estimated by comparison with a standard curve.

Materials

Solution A - 2% Na_2CO_3 in 0.1N NaOH, containing 0.02% sodium potassium tartrate.

Solution B - 0.5% $CuSO_4.5H_2O$

Solution C - prepared as required by mixing the two solutions A and B in the proportion 50 parts of A to 1 part of B.

Folin-Ciocalteaus' reagent was purchased from BDH Ltd; bovine serum albumin (Fraction V) was used as the reference standard. A stock solution of 1 mg/ml in water was diluted appropriately, to give a range of protein concentrations (Figure A1).

A spectrophotometer was used to measure the colour developed by reading absorption of light at 750 nM in a final volume of 2.1 ml using a 3 ml glass cuvette.

Method

Protein samples to be tested were thawed and 20µl aliquots were removed for protein estimation. The samples were diluted 1 in 50 with distilled water. 1 ml of solution C was then added to each tube, including the standard tubes which contained 1 ml of the appropriate albumin solution. The tubes were mixed well on a whirlimixer and allowed to stand at room temperature for 10 minutes. 100µl aliquots of dilute Folin-Ciocalteaus' reagent (1:1) were then added very rapidly into all tubes and mixed immediately, to obtain maximum colour. After 30 minutes the colour developed in each tube was read using a spectrophotometer set at 750 nM. From the readings thus obtained, a standard curve was plotted as shown in Fig.A1. The graph was found to be reproducible and the amount of protein in each sample was calculated from the graph.

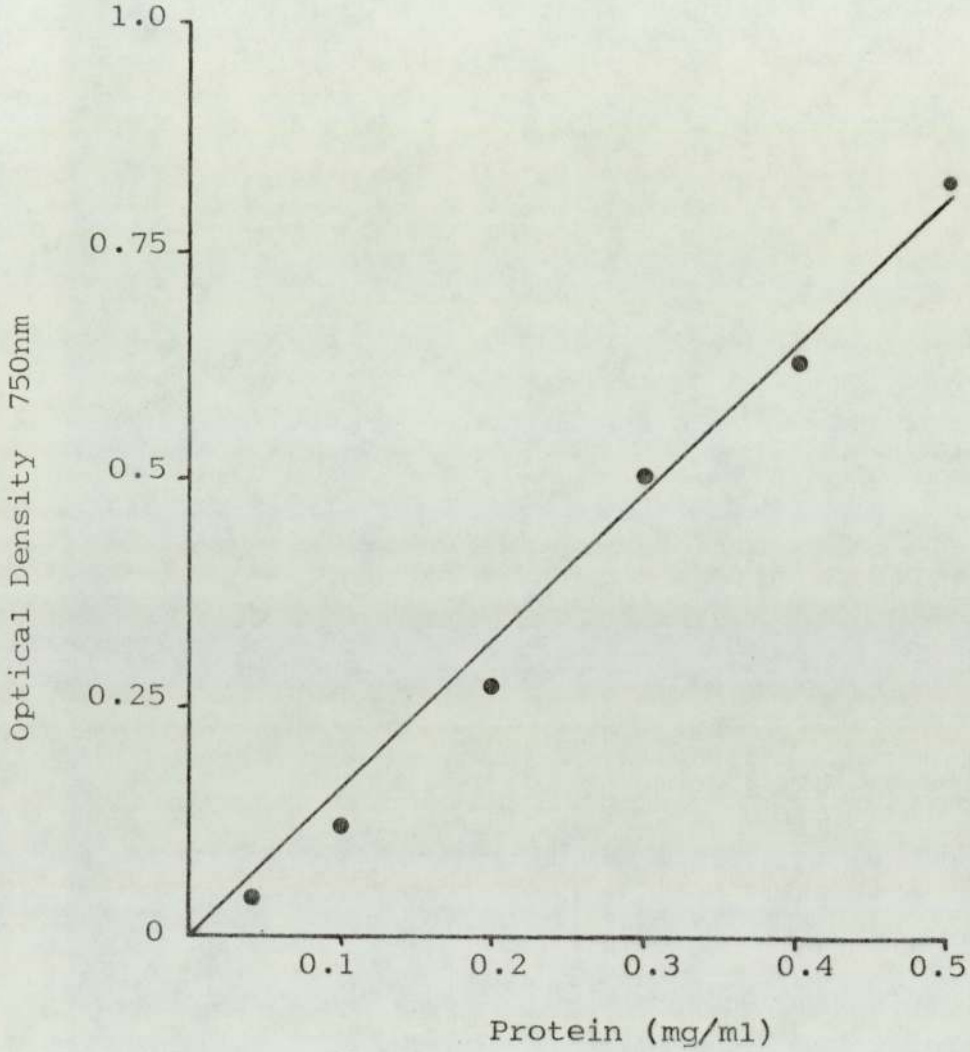


Figure A1. The protein standard curve produced using the method of Lowry et al. (1951), with bovine serum albumin (0.05-0.5 mg/ml) as the standard protein.

Insulin radioimmunoassay

RIA is based on the principle of isotopic dilution which is dependent on competition between the labelled and unlabelled antigen (Ag) for binding sites on specific antibodies (Ab) (Berson and Yalow, 1957). Increasing amounts of unlabelled Ag in the sample produce a proportional decrease in the binding of labelled Ag to the Ab (Yalow and Berson, 1960). Thus the level of radioactivity associated with the Ab-Ag complex is related to the concentration of unlabelled Ag in the original sample. The latter is obtained by comparing the bound radioactivity with that produced by standard solutions containing known amounts of unlabelled Ag. The preparation of Ab and labelled hormone and the selection of assay design parameters have been extensively discussed elsewhere. (Ekins and Newman, 1970; Flatt, 1977).

The double-antibody RIA method employs a second Ab to separate the Ab-bound and free insulin in the reaction mixture, the technique is relatively simple, quick and sensitive. A further advantage is that commercially available antisera can be obtained and that the labelled insulin produced for the binding assays can be used. The pre-precipitation procedure described by Hales and Randle (1963) was selected since it produces rapid results and involves easy separation of the bound and free labelled Ag by centrifugation. In this technique the first Ab (an anti-insulin Ab) raised to porcine insulin in guinea-pigs and the second Ab (induced in rabbits against the γ-globulin of the guinea-pig) was used to precipitate the primary Ab-Ag complex.

Reagents

Antiserum. The double-Ab (anti-insulin, anti-guinea-pig γ-globulin serum) was stored at 4°C and reconstituted in assay buffer on the day of the assay and used at the dilution recommended by the supplier, usually about 1:16,000. At this dilution approximately 40% of a selected dose of labelled insulin at the lower end of the physiological range (0.25ng) is bound.

Tracer. Labelled insulin was iodinated as described in the methods section (page 48) by the chloramine-T method (Freychet et al., 1971) and purified by gel-filtration. The preparation was stored at -20°C and had a specific activity of 200-250 μCi/μg.

Standards. Freeze-dried mouse insulin (Novo) containing 1 mg human albumin was diluted in double-distilled deionised water to give a stock solution (100 μg/ml) which was divided into 120μl aliquots. From each aliquot, a working solution (1 μg/ml) was prepared in assay buffer and divided into 200μl aliquots. The solutions were stored at -20°C in tightly capped tubes. A series of standard dilutions (0.5-10 ng/ml) was prepared from the working solution for each assay. Thus freezing and thawing of the standard was minimised. This regime was based on the general recommendation of Bangham and Cotes (1974).

Procedure

The RIA incubations were performed at 4°C and all additions were made using a Gilson micropipette equipped with disposable plastic tips (Anachem Ltd., Luton). Reactants were mixed without frothing using a variable speed rotomixer (Hoak and Tucker Ltd., London).

The protocol used for the assay of insulin is shown in

Figure A2. Each assay was conducted on the unknown samples and triplicate sets of standard insulin solutions (0.5-10 ng/ml), zero, blank and total counts. Briefly the procedure was performed as follows. The initial reactants (standard or unknown plus antisera) were mixed and incubated at 4°C for 24 hours. Labelled insulin was added, the reactants mixed and incubated at 4°C for 6 hours. Following the second incubation, the reactants were diluted in buffer, mixed and then the free and Ab-bound insulin separated by centrifugation at 1500g (Mistral Coolspin centrifuge, MSE Scientific Instruments, Surrey) for 30 minutes. The supernatant, containing the free labelled insulin, was decanted and the final drops of fluid at the tip of the tubes were aspirated with a pasteur pipette connected to a vacuum pump. The radioactivity in the precipitate Ab-Ag complex was counted for one minute using a well-type crystal scintillation counter. A standard curve, using the logarithm of the concentration against the bound radioactive count, was prepared and the insulin in the unknown samples was interpolated from this graph (Fig. A3).

The criteria for a successful assay were:-

a) good agreement between replicates of standards, zero, blank and total counts. Replicates differing by more than 200 counts/minute were discarded.

b) a zero count that corresponds to a theoretical percentage of the total count. At the dilution used, 40% of the total label will be bound. The zero count evaluates the amount of radioactivity of the insulin-Ab complex in the absence of unlabelled insulin.

c) a blank count less than 5% of the total count. Non-specific binding can be assessed by assaying blank tubes

Figure A2. Protocol for insulin radioimmunoassay

	Buffer	Mouse insulin standard	Unknown plasma sample	Binding agent	Labelled insulin (I^{125})	Buffer
Total count	-	-	-	-	50µl	-
Blank	100µl	-	-	-	50µl	500µl
Zero count	50µl	-	-	50µl	50µl	500µl
STANDARDS						
0.5 ng/ml	-	50µl	-	50µl	50µl	500µl
1.0 ng/ml	-	50µl	-	50µl	50µl	500µl
2.0 ng/ml	-	50µl	-	50µl	50µl	500µl
5.0 ng/ml	-	50µl	-	50µl	50µl	500µl
10 ng/ml	-	50µl	-	50µl	50µl	500µl
UNKNOWN SAMPLES						
1	-	-	50µl	50µl	50µl	500µl
2	-	-	50µl	50µl	50µl	500µl
etc.						

After addition of the binding reagent to the insulin standards or unknown plasma samples, the reactants were mixed and incubated for 6 hours at 4°C. After addition of label the tube contents were mixed and incubated for 24 hours at 4°C. Finally, after the second addition of buffer, the radio-immunoassay reagents were mixed, centrifuged, decanted (except total counts) and counted for radioactivity.

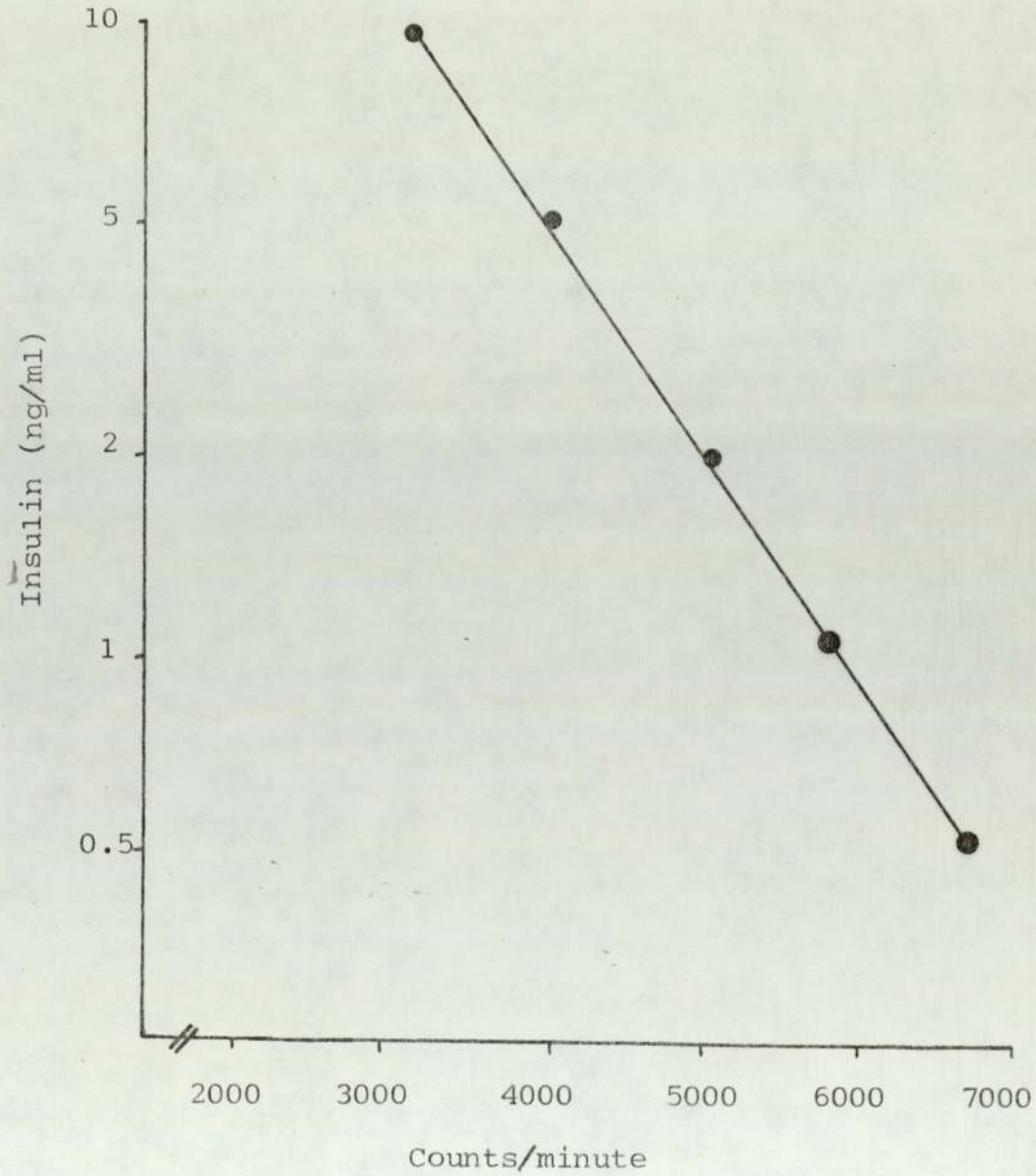


Figure A3. The standard curve for insulin produced by a double-antibody radioimmunoassay (Hales and Randle, 1963), standard insulin solutions ranged from 0.5 to 10 ng/ml.

which contain only buffer and label. An acceptable limit for the percentage of label bound is 5% or less of the total count added. Good agreement between replicates indicates a constant error. The blank count also evaluates the working procedure of the antibody precipitate.

The sensitivity of the assay was 0.18 ng/ml and the intra-assay coefficient of variation was 3.58%.

Statisitics

Student's t test

Formulae used:

Mean of first sample $M_1 = \frac{x}{N_1}$, where N_1 is the number of values in the first sample.

Variance $s_1^2 = \frac{N_1 \Sigma x^2 - (\Sigma x)^2}{N_1 (N_1 - 1)}$

M_2, N_2, s_2^2 are calculated similarly for the second sample.

Estimate of standard deviation, σ :

$$\sigma = \sqrt{\frac{(N_1 - 1)s_1^2 + (N_2 - 1)s_2^2}{N_1 + N_2 - 2}}$$

Estimate of standard error, SEM:

$$SEM = \frac{\sigma}{N}$$

$$t = \frac{M_1 - M_2}{\sigma \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

The corresponding 'P' values were obtained from t test tables and differences were taken to be significant if $P < 0.05$.

Paired t test

Used to test the differences between means that are made up of paired observations, in the present work only the clinical metformin data could be tested by the paired t test.

Formulae used:

$$t = \frac{\sum D/n}{\sqrt{\frac{\sum D^2}{n-1} - \frac{(\sum D)^2}{n(n-1)}}{n}}$$

Where, n = number of pairs

D = (x - y) for each pair, where x and y are paired observations

Best straight line by the Least Squares method

Using the following notation:

$x = X$, $y = Y$, $x^2 = Q$, $y^2 = S$ and $xy = W$, with N as the number of pairs;

Then,

$$\text{Slope} = \frac{W - XY/N}{Q - X^2/N} \qquad \text{Intercept} = \frac{XW - YQ}{X^2 - NQ}$$

or by substitution, $$\text{Intercept} = \frac{Y - X(\text{slope})}{N}$$

$$\text{Correlation coefficient} = \frac{W - XY/N}{\sqrt{(S - \frac{Y^2}{N})(Q - \frac{X^2}{N})}}$$

All calculations were performed on a programmable calculator, Olivetti Programma 101.

Insulin receptor calculation

Calculations on the computer (ICL 1904S) were done by means of a macro (set of instructions) called Recepcao.

This uses a program called Receptors K, written in Algol-68R, which performs the calculations and prints out as shown by the attached flow chart (Figure A4); see also the lineprinter output. It also feeds data to a second program called Recegrafica, written in Algol-60, which prepares the graphical output of the Scatchard plots produced by the program (Figures A5a and A5b).

The reason for two programs and two languages is that having written the first one (in Algol-68R) it was found that the graphplotter did not, at that time, have any capability for accepting instructions in Algol-68R. The easiest thing was thus to write a supplementary program in Algol-60, which the graphplotter would accept. Since then, Algol-68R has become available on the graphplotter, but there is even so a limit on the maximum length of graph it will produce, which restricts it to 3 A4 graphs side by side, that is 6 blocks of data (since Recegrafica puts the graphs in pairs, one above the other). Figures A6a and A6b are listings of ReceptorsK and Recegrafica respectively, and Figure A6c is a sample run of ReceptorsK.

Figure A4. Flowchart for receptors calculation

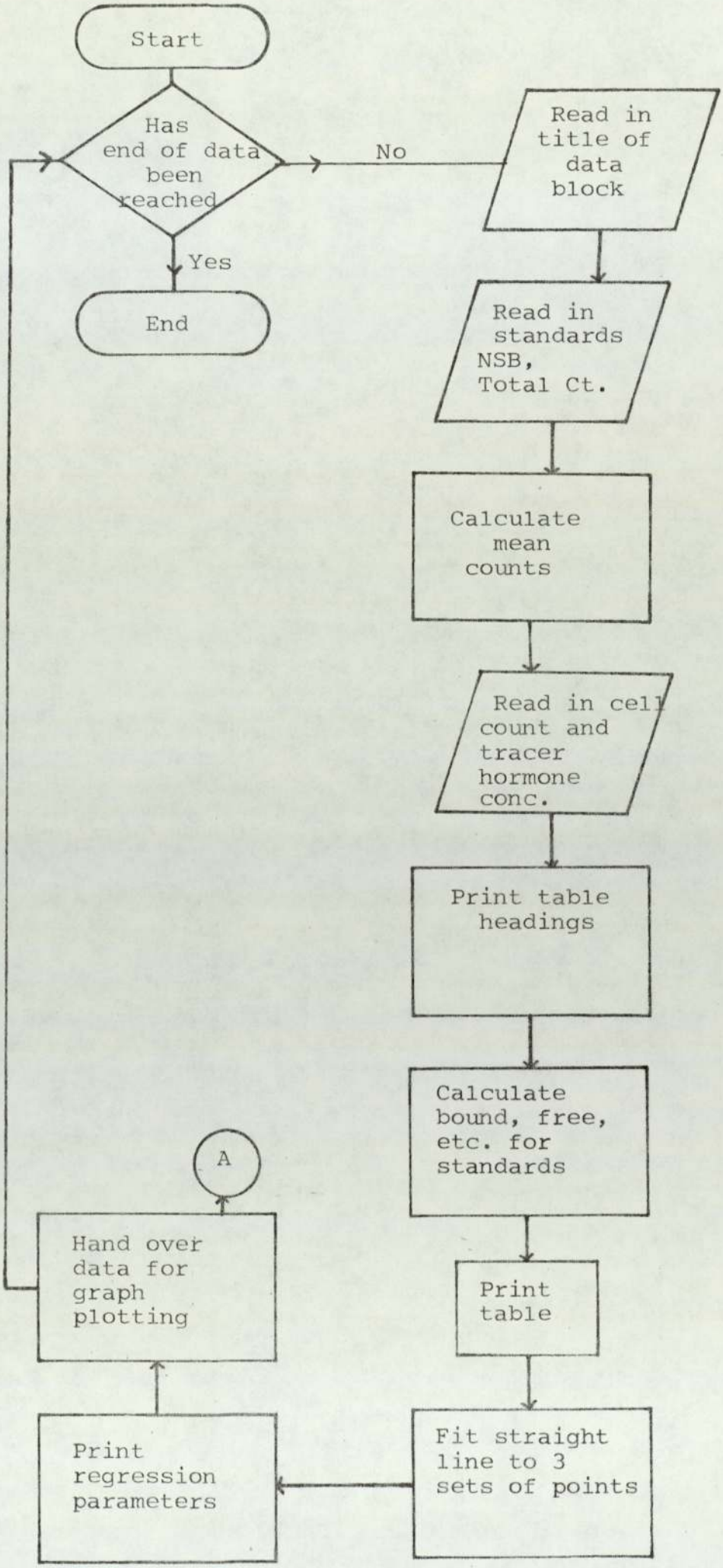
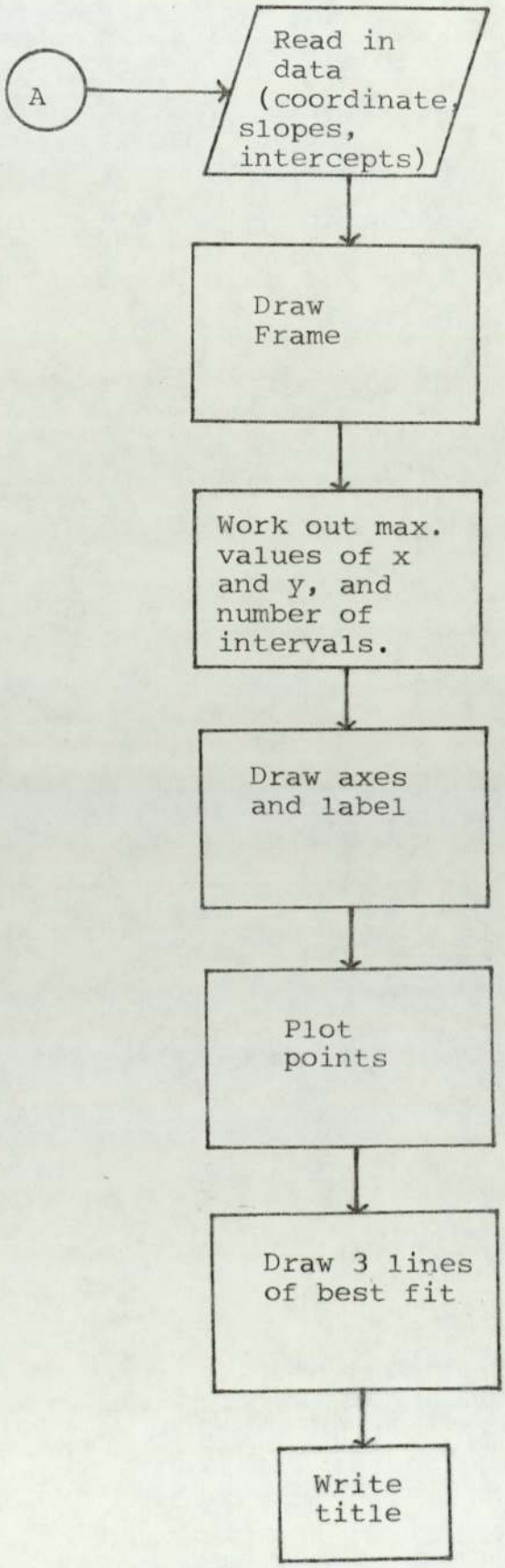


Figure A4. (cont.)
Graph plotting for each block of data.



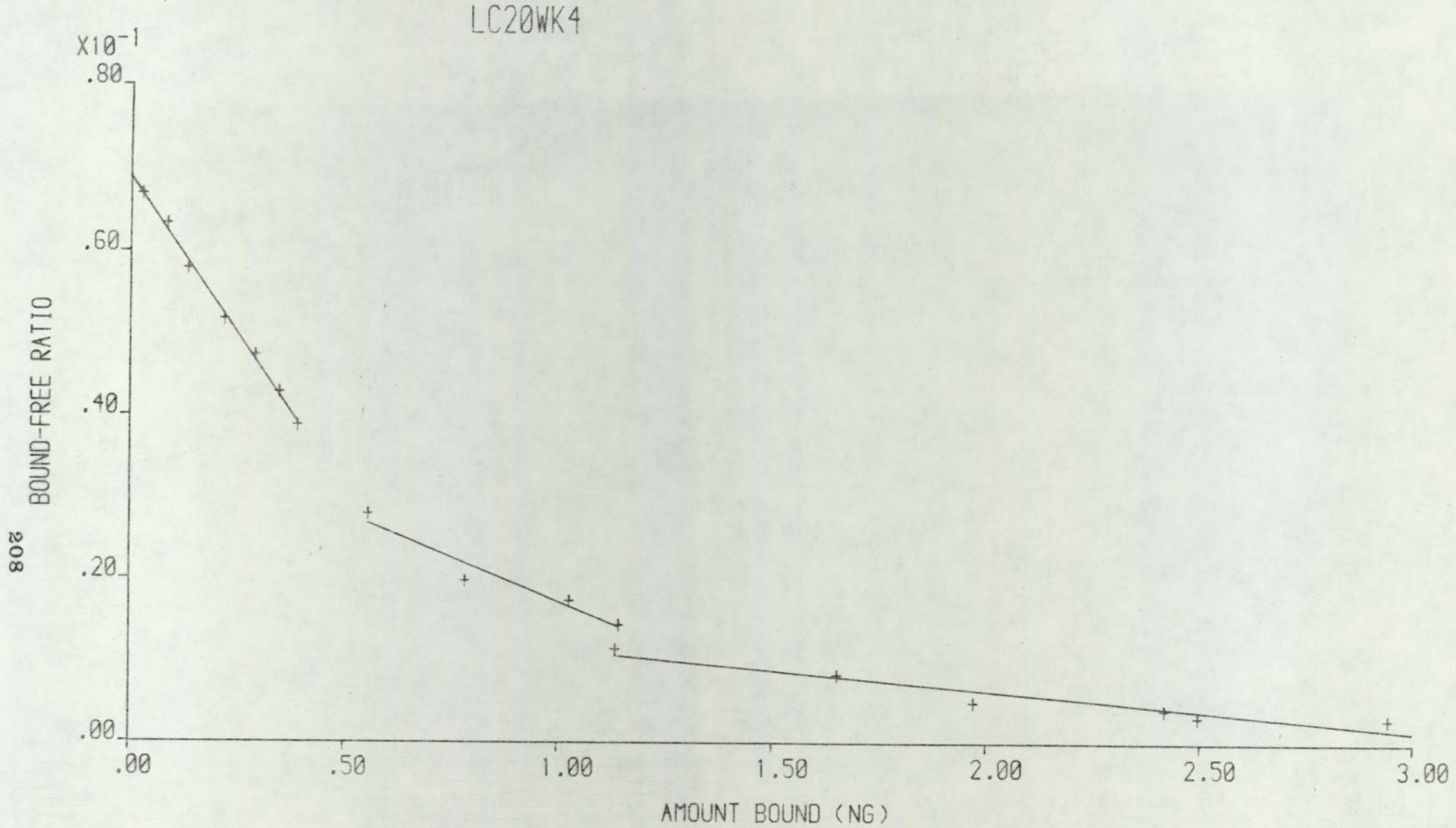


Figure A5a. Computer printout of the Scatchard plot produced by insulin binding to hepatocytes from a 20 week old lean mouse.

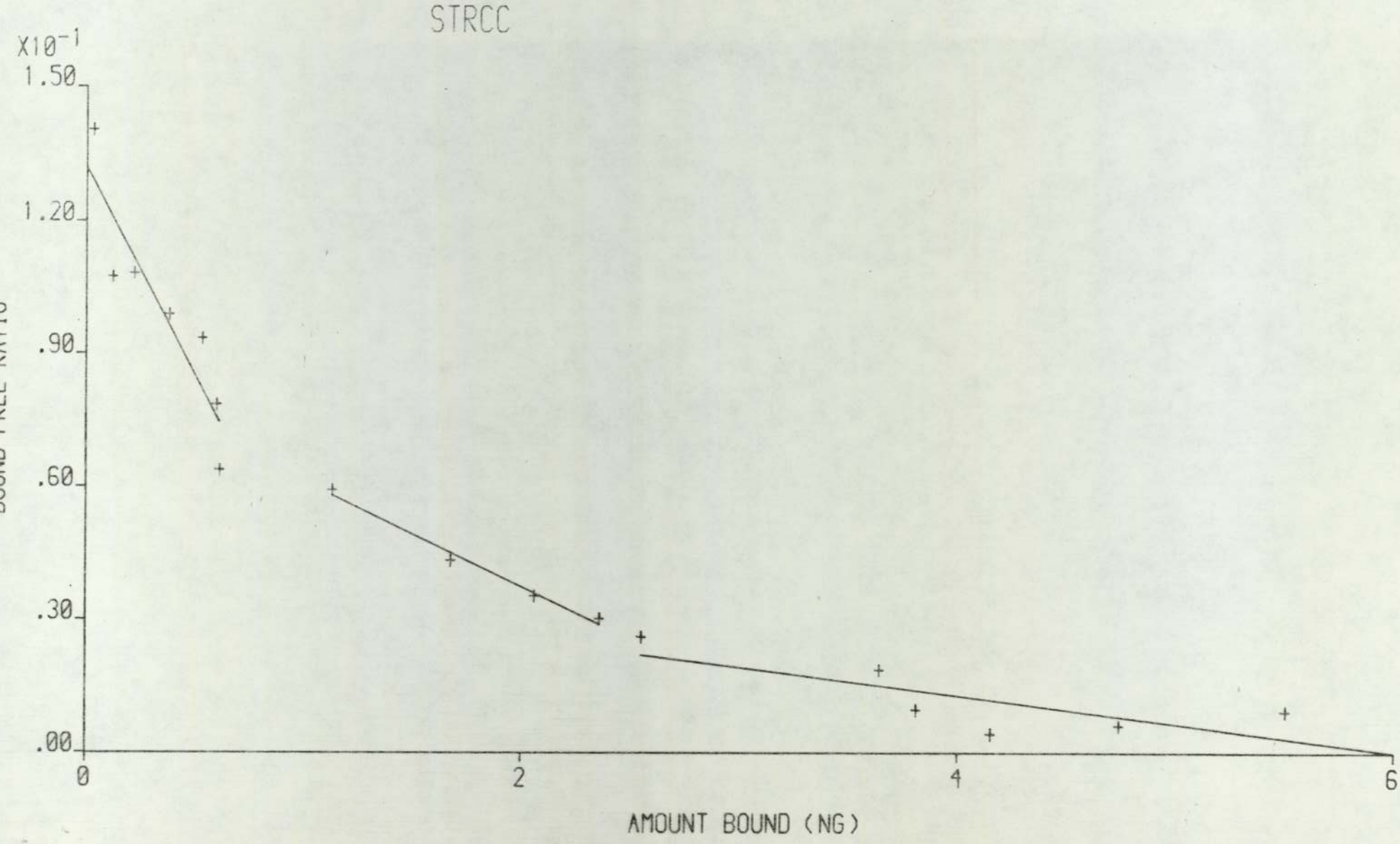


Figure A5b. Computer printout of the Scatchard plot for insulin binding to hepatocytes from a streptozotocin diabetic mouse.

FigureA6a.

Listing of computer program RECEPTORSK

```
Listing of : SBS 7006.RECEPTORSK(11/)   Produced on 9Aug82

at 15.26.59

G8.65G AT ASTON  IN ':SBS7006.M418WED' on 17Nov82 at

10-53.41 using U15

DOCUMENT    RECEPTORSK


BINDING
'BEGIN'
'REAL' CELLCT, TOTALCT, TRAH, FREE;
[1:18]'REAL' COUNT;  [1:3]'REAL' C, M;  [1:17]'REAL' HCONC;
'INT' K:=1, N;  'STRING' S;

'PROC' CALC = ('INT' B) 'REAL':
       'BEGIN' 'REAL' R:=0;   'INT' DATA;
       'TO' B 'DO''BEGIN' READ(DATA); R:=R + DATA 'END';
       R:=(R*100)/(B*N);
       R      'END';

'PROC' PRINTVAL1 = ('REAL' C) 'VOID':
       'BEGIN' OUTF(STANDOUT, S<5.2>2XS, C)  'END';

'PROC' PRINTVAL2 = ('REAL' C) 'VOID':
       'BEGIN' OUTF(STANDOUT, S<2.5>2XS, C)  'END';

'PROC' REGRESSION = ('INT' FIRST, LAST) 'VOID':
       'BEGIN' 'REAL' SX:=0.0, SXQ:=0.0, SXY:=0.0, SY:=0.0,
       SYQ:=0.0, XB:=0.0;
       FREE:=0.0;  N:=N+!;
       'FOR' I 'FROM' FIRST 'TO' LAST 'DO''BEGIN'
             SX:=SX + HCONC[I];  SXQ:=SXQ + HCONC[I]+2;
             SY:=SY + COUNT[I];  SXY:=SXY + HCONC[I]* COUNT[I];
             SYQ:=SYQ + COUNT[I]+2; FREE:=FREE + 1.0  'END';
       XB:=SX/FREE;  SY:=SY/FREE;
       M[N]:=(SXY*(SX*SY)) / (SXQ*(SX*XB));
       PRINT((NEWLINE,NEWLINE,"POINTS ", FIRST, " -",LAST,
             ":                SLOPE M =")));    PRINTVAL2(M[N]);
       C[N]:=SY-(M[N]*XB);  PRINT("  Y-INTERCEPT C =");
       PRINTVAL2(C[N]);  PRINT((NEWLINE, "       CORRELATION
       COEFFICIENT R =));
       PRINTVAL2((SXY/FREE - XB*SY) / SQRT((SXQ/FREE -XB+2)*
       (SYQ/FREE - SY+2))); FREE:=XB-(SY/M[N]); 'IF M[N] <0.0
       'THEN' PRINT("    X-INTERCEPT ="); 'IF' FREE < 100.0
       'THEN' PRINTVAL2(FREE) 'ELSE' PRINTVAL1(FREE) 'FI'
       'FI'    'END';

SIGN'OF'NUMBERSTYLE:=29;  INT'OF'NUMBERSTYLE:=2; SPACES'OF'
NUMBERSTYLE:=0;
START:   READ((S, N)); 'IF' N < 0 'THEN' K:=N;  'GOTO' DEPOT
'FI';
'FOR' I 'TO' 16 'DO' COUNT[I]:=CALC(3);
'FOR' I 'FROM' 17 'TO' 18 'DO' COUNT[I]:=CALC(2);
'C'1-16 ARE TRIPLICATE STANDARDS, 17-18 ARE DUPLICATE 1000
AND N.S. BINDING 'C'
```

```
TOTAL CT:=CALC(1);  READ((CELLCT, TRAH)); CELLCT:=CELLCT * 0.8;
PRINT((K, NEWLINE, S, NEWLINE, NEWLINE, "       TOTAL COUNT =
"));
PRINTVAL1(TOTALCT);
PRINT(("COUNTS/MINUTE", NEWLINE, CELL COUNT(4/5 OF VALUE
INPUT) ="));
PRINTVAL2(CELLCT);
PRINT(("MILLION CELLS/ML", NEWLINE,"      TRACER HORMONE ="));
PRINTVAL2(TRAH);
PRINT(("NG/ML", NEWLINE,"        NON-SPECIFIC BINDING ="));
PRINTVAL1(COUNT[18]);
PRINT(("COUNTS/MINUTE",  NEWLINE, NEWLINE,
" MEAN    HORMONE   APPROX.   FRACTION   AMOUNT  AMOUNT  B/F",
    NEWLINE,
" COUNT   CONC.   CTS.BOUND   BOUND     BOUND     FREE  RATIO",
    NEWLINE,
"(COUNT/MIN)  (NG/ML)   (CPM/M.CELLS)   (NG/ML) (NG/ML)",
    NEWLINE));
'FOR' I 'FROM' 0 'TO' 2 'DO''FOR' J 'TO' 5'DO' HCONC[I*5+J+2]:
     2*J*(10 I);
HCONC[1]:=0;  HCONC[2]:=1;
'FOR' I 'TO' 17 'DO''BEGIN'
     PRINT(NEWLINE);  PRINTVAL1(COUNT[I] );  'C' MEAN COUNT 'C'
     HCONC[I]: HCONC[I] + TRAH;  COUNT[I]:=(COUNT[I] -
        COUNT[18]) / CELLCT;
     PRINTVAL1(HCONC[I] );  PRINTVAL1(COUNT[I]);
        'C' HORMONE CONC, APPROX.COUNTS BOUND 'C'
     COUNT[I]:=COUNT[I]/TOTALCT;  PRINTVAL2(COUNT[I]); 'C'
        FRAC BOUND 'C'
     COUNT[I]:=COUNT[I] * HCONC[I]; FREE: HCONC[I] - COUNT[I];
     PRINTVAL2(COUNT[I]);  PRINTVAL1(FREE); 'C' BOUND ( X),
       FREE 'C'
     HCONC[I]:=COUNT[I];  COUNT[I]:=COUNT[I]/FREE;
       PRINTVAL2(COUNT[I])
     'C' BOUND/FREE RATIO (=Y) 'C'  'END';
PRINT((NEWLINE, NEWLINE, "NOTE: APPROX.COUNTS BOUND CALCULATED
     ON 4/5 OF VALUE OF CELL COUNT AS INPUT"));  N:=0;
REGRESSION(1, 7);  REGRESSION(12, 17);
PRINT((NEWLINE, NEWLINE, "*"));
'FOR' I 'TO' 17 'DO''BEGIN'
     PRINT(NEWLINE);  PRINTVAL2(HCONC[I]); PRINTVAL2(COUNT[I])
     'END'
'FOR' I 'TO' 3 'DO''BEGIN'
     PRINT(NEWLINE);  PRINTVAL2(M[I]);  PRINTVAL2(C[I])  'END';
PRINT((SPACE, S, "        @@@", NEWLINE));  READ(NEWLINE);
K:=K+1;  'IF' K<17 'THEN''GOTO' START 'FI';
DEPOT:  PRINT(K)     'END'
'FINISH'
```

Figure A6b.

Sample listing of graph plotting file, RECEGRAFICA

```
'BEGIN' 'INTEGER''PROCEDURE' INTS (MAX); 'REAL' MAX;
      'BEGIN' 'INTEGERS' NI, S; I:=0;
      STAR:    'IF' MAX < 3.0 'THEN''BEGIN' MAX:=MAX*10;
      I:=I+1;
            'GOTO' STAR     'END';
      'IF' MAX > 30.0 'THEN''BEGIN' MAX:=MAX/10; I:=-1  'END';
      'IF' MAX > 15 'THEN' S:=5 'ELSE''IF' MAX > 10 'THEN'
      S:=3 'ELSE''IF' MAX > 5 'THEN' S:=2 'ELSE S:=1;
      MAX:=MAX/S;
      NI:=ENTIER(MAX); 'IF' (MAX - NI) > 0.0 'THEN'
      NI:=NI+1;     MAX:=NI*S / (10+I);   INTS:=NI
      'END' OF INTS;

'PROCEDURE' READTRAP(P); 'PROCEDURE' P; 'EXTERNAL';
'PROCEDURE' T; 'BEGIN' WRITETEXT ('(''('2C')'DATA%ERROR%
FOUND')');
   'GOTO' DEPOT   'END';
'INTEGER''ARRAY' W[1:20]; 'REAL''ARRAY' X, Y [1:17], M,
C[1:3];
'INTEGER' I, K;    'REAL' XM, YM;

OPENGINOGP; SOFCHA; DEVPAP (2500.0, 340.0, 1); READTRAP(T);
START: K:=READ; 'IF' K<0 'THEN''GOTO' DEPOT;
'IF' K=17 'THEN''BEGIN' WRITETEXT('('CAPACITY%OF%GRAPHPLOTTER%
    REACHED%-%NO%MORE%DATA%PROCESSED')'); 'GOTO'DEPOT 'END';
COPYTEXT('('*')'); NEWLINE(4); XM:=YM:=0;
'FOR' I:=1 'STEP' 1 'UNTIL' 17 'DO''BEGIN' X[I]:=READ;
    Y[I]:=READ;
    'IF' X[I] > XM 'THEN' XM:=X[I];
    'IF' Y[I] > YM 'THEN' YM:=Y[I]   'END';
'FOR' I:=1 'STEP' 1 'UNTIL' 3 'DO''BEGIN' M[I]:=READ;
    C[I]:=READ          'END';
INSTRARR('('@@@')',W);
LINTO2 (0.0, 160.0); MOVTO2 (297.0, 160.0); LINTO2
    (297.0, 0.0);
SHIFT2 (35.0, 15.0);   CHASIZ (2.5, 3.5);
AXIPOS (0, 0.0, 0.0, 240.0, 1); AXISCA (3, INTS(XM), 0.0, XM, 1);
AXIPOS (0. 0.0, 0.0, 125.0, 2); AXISCA (3, INTS(YM), 0.0, YM, 2);
AXIDRA (1, 1, 1);   AXIDRA (-1, -1, 2);
MOVTO2 (100.0, -15.0);     CHASTR ('('AMOUNT%BOUND%(NG)*.')');
MOVTO2 (-15.0, 45.0);      CHAANG (90.0);
CHASTR ('('BOUND-FREE%RATIO*.')');
GRASYM (X, Y, 17, 3, 0);
GRAMOV (0.0, C[1]);     GRALIN (X[7], M[1]-X[7] + C[1]);
GRAMOV (X[8], M[2]-X[8] + C[2]);
GRALIN (X[11], M[2]*X[11] + C[2]);
GRAMOV (X[12], M[3]*X[12] + C[3]); M[1]:=M[3]-XM + C[3]
'IF' M[1] < 0 'THEN' GRALIN (-C[3]/M[3], 0) 'ELSE'
    GRALIN (XM, M[1]);
MOVTO2 (60.0, 135.0); CHASIZ (3.0, 5.0); CHAANG (0);
CHAARR (W, 20, 4);
'IF' K/2 - K'/'2 = 0 'THEN' SHIFT2 (265.0, -180.0) 'ELSE'
    SHIFT2 (-35.0, 150.0);
MOVTO2 (0.0, 0.0);       'GOTO' START;
DEPOT:    DEVEND       'END'
```

FigureA6c.

Sample run of receptor calculation program - RECEPTORSK

```
STARTED :SBS7006,M418JANB, 9AUG82 12.40.35 TYPE: BACK 10;
STREAM B ; PRIORITY
12.40.35  RJ M418JANB, RECEPCAO(5), PARAM(DATA JAN-B),
JD(JT300, MZ40K)
12.40.36 JOB IS NOW FULLY STARTED
12.40.36  RECEPCAO        DATA JAN-B
12.40.36  ER REC2
ERROR IN PARAMETER 1 IN ER : ENTRANT REC2 DOES NOT EXIST
12.40.36  CE !
12.40.36  IN !, T////
12.40.37  CE !
12.40.37  CY  JAN-B, !
12.40.38  CY !1, !(APPEND)
12.40.39  UAALGOL68 LOAD RECEKBIN, LINES 1500, DATA !,
*LPO REC2, PMD, EXIT
ENTERING :MACROS.UAALGOL68(52/)
12.40.39       IF MOP, (RP AB,CM,JL) ELSE (TA AB,CM,JL)
12.40.39  TA AB,CM,JL
12.40.51  0.02 CORE GIVEN 12352
0.22 :DELETED : OK
12.42.21 FREE *TRO ,136 TRANSFERS
12.42.21 FREE *LPO ,869 TRANSFERS
12.42.21 0.22 DELETED,CLOCKED 0.19
UAALGOL68R: NORMAL EXIT
12.42.24  UAALGOL LOAD RECEGRABIN, DATA REC2, *GP GINO, PMD
EXIT
ENTERING :MACROS.UAALGOL(164/)
12.42.25          TA AB,CM,JL
12.42.53 0.25 CORE GIVEN 25216
0.25 :HALTED : 93
DISPLAY: UAALGOL: GRAPHICAL OUTPUT FILE IS UACALN149737
DISPLAY : GINO-F MK 2.6+
ALLOT       *CRO     N
0.26: MONITOR
ALLOT       *LPO     N
0.27: MONITOR
12.45.15  FREE *CRO ,869 TRANSFERS
12.45.15  FREE *LPO ,632 TRANSFERS
1.11 :HALTED : AH
DISPLAY: UAALGOL: NORMAL EXIT
12.45.17  FREE *FH7 ,387 TRANSFERS
12.45.17  1.12 DELETED,CLOCKED 0.42
1.12 :DELETED
12.45.19  ****
12.45.19 1.12 FINISHED : 2 LISTFILES
12.45.19 JOBTIME USED 73 ; MAXIMUM CORE USED 25216
12.45.19 JOB UNITS 160
```

Publications arising from this thesis

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