CHARACTERISTICS OF INSULIN RECEPTORS IN A HUMAN LYMPHOBLASTOID CELL LINE (Raji)

by

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CONTENTS

Page N°.

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PREFACE

The experimental work described in this thesis was carried out in the Department of Chemical Pathology, University of Natal Medical School, Durban.

These studies represent original work by the author and have not been submitted to any other university. Where use was made of the work of others, it has been duly acknowledged in the text.

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INTRODUCTION

The notion that insulin binds to a specific site on the cell membrane was first proposed many years ago. However, experimental proof of a membrane bound insulin receptor did not come until the early 1970s when biologically active radiolabelled insulin was used in direct binding studies (Cuatrecasas, 1971).

Recent advances in understanding the mechanism of insulin action are the result of studies on the structure and function of the insulin receptor. The membrane receptor would appear to have two functions: firstly, it must bind insulin and secondly, it must couple insulin binding to insulin action. Defects in either of these receptor functions will result in an impaired response to insulin, or insulin resistance (Taylor, 1985).

Insulin resistance is a common disorder in a number of disease states in man. For example, non-insulin-dependent diabetes mellitus and obesity are associated with mild insulin resistance (Bar *et al.*, 1976). There are also a number of relatively rare syndromes of extreme insulin resistance in which there is either impaired receptor function, or an immunological defect resulting in the development of auto-antibodies against the insulin receptor (Taylor *et al.*, 1985). Studies on insulin receptor defects associated with these disease states have led to progress in understanding the molecular mechanisms of insulin action.

Ideally when investigating these disease states one should study insulin action on classical target cells such as adipocytes, hepato-

cytes or muscle. However, it is now well established that the kinetics of insulin binding to its membrane receptor is similar in all human tissue whether or not it is a target for insulin action. This has led to a great deal of research on the more accessible human tissues such as monocytes, erythrocytes, cultured fibroblasts and Epstein-Barr virus (EBV) transformed B-lymphocytes. The most convenient tissue to study is EBV transformed B-lymphocytes, as these cells can be taken from individual patients and grown in culture in large quantities, which facilitates biochemical studies. Despite these advantages, it is important to establish that this virus-induced receptor is a true insulin receptor and not an artifact of viral transformation.

Studies on B-lymphocyte proliferation have shown that the insulin receptor appears on the cell membrane during the proliferative phase of B-cell activation. However, this is a transient event and once the cell reaches maturation the insulin receptor is no longer evident (Marchalonis & Galbraith, 1987). The insulin receptor has also been demonstrated in a number of cultured human lymphoblastoid cell lines (Gavin *et al.*, 1983; Maegawa *et al.*, 1983). It seems, therefore, that the insulin receptor is normally expressed by blast cells.

The purpose of this study was to investigate insulin binding characteristics on a human lymphoblastoid cell line with B-cell characteristics which was originally derived from a patient with Burkitt's lymphoma. These cells, which are known as Raji cells, are unusual in that they carry multiple copies of the EBV genome in their DNA. For this reason they provide a useful model system for studying the

insulin receptor in EBV transformed lymphocytes. In addition, studies on the mechanism of insulin action in these cells should give some insight into the function of the insulin receptor during B-cell proliferation.

In this study four major characteristics of insulin binding to insulin receptors on Raji cells are described.

Firstly, on the basis of kinetic studies a model for insulin-receptor interaction was established.

Secondly, processing of insulin and the receptor was investigated to determine whether the receptor is functional.

A third aspect was elucidation of the receptor structure and the insulin binding site.

Finally, the cross-reaction between insulin and type I IGF receptors was studied, and the cellular response mediated by the insulin receptor and growth factor receptor was determined.

LIST OF ABBREVIATIONS

Å	angstrom
α-AIB	α-aminoisobutyric acid
ATP	adenosine 5'-triphosphate
B_T	bound hormone
B/F	ratio of bound and free hormone
B	total bound hormone
BCGF	B cell growth factor
BCDF	B cell differentiation factor
BL	Burkitt's lymphoma
BSA	bovine serum albumin
°C	degree Celsius
Ca^{++}	calcium ion
$CaCl_2$	calcium chloride
Ci	Curie
cm	centimetre
CO_2	carbon dioxide
cpm	counts per minute
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DSS	disuccinimidyl suberate
DTT	dithiothreitol
EBSS	Earle's balanced salt solution
EBV	Epstein-Barr virus
EDTA	ethylenedinitrilotetracetic acid
FCS	foetal calf serum
g	gram
g	gravity, acceleration due to
GIT	glutathione-insulin dehydrogenase
H	hormone
HBB	hepes binding buffer
HEPES	(N-2-hydroxyethyl piperazine-N'-2 ethane sulphonic acid)
hr	hour
ID_{50}	the concentration of unlabelled insulin which displaces half-maximal bound insulin
IDE	insulin degrading enzyme

IGF	insulin-like growth factor
IMDM	Iscove's modified Dulbecco's medium
k_1	association rate constant
k_2	dissociation rate constant
kD	kilodalton
$\bar{K}$	average affinity
K_a	association constant or equilibrium constant
K_d	dissociation constant
K_e	empty sites conformation
K_f	filled sites conformation
K_M	Michaelis-Menton constant
KCl	potassium chloride
mA	milliamp
MAAS	mild acetic acid solution
Mg^{++}	magnesium ions
$MgSO_4$	magnesium sulphate
min	minute
mmol	millimole
mol	mole
MWt	molecular weight
N	Avagrado's number
$NaCH_3COO.3H_2O$	sodium acetate
NaCl	sodium chloride
Na_2HPO_4	di sodium phosphate
NaH_2PO_4	sodium dihydrogen phosphate
ng	nanogram
nmol	nanomole
NSB	non-specific binding
NSILA	non-suppressible insulin-like activity
p	probability
pA_h	negative logarithm of the concentration (molar) of antagonist which depresses the maximum to one half
PBS	phosphate buffered saline
pH	negative log of hydrogen ion concentration
R	receptor
Rc	receptor number per cell
RH	receptor-hormone complex

R_T	total receptor concentration
s	second
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFM	serum free medium
t	time
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl) aminomethane
µCi	micro Curie
µg	microgram
µℓ	microlitre
$\bar{Y}$	fractional saturation
$\bar{Y}_e$	threshold of site-site interactions
$\bar{Y}_f$	fractional occupancy at which site-site interactions are maximal
V_e	elution volume
V_o	void volume
V_t	total volume

CHAPTER 1

A LITERATURE REVIEW OF INSULIN RECEPTORS

"Regardless of whether or not intact cellular morphology is an obligatory requirement for insulin action, the first step in the action of insulin in cellular systems must be its entrance into, or attachment to, some morphological element of the intact cell."

-STADIE et al. 1949

1.1 HISTORICAL REVIEW

Evidence that insulin acts on tissue membranes became available in the late 1940s and early 1950s.

In the first of these experiments Stadie *et al.* (1949), on the basis of studies on insulin action on rat hemidiaphragms, suggested that insulin became bound to tissue membrane. Shortly afterwards work by Levine *et al.* (1950) on galactose transport in dogs and rats, suggested a rapid membrane action of insulin. However, for several reasons these studies were slow to develop. Firstly, because of the low concentration of circulating hormone, the amount bound to the cell was low and, therefore, difficult to measure. Secondly, measuring the uptake of an unlabelled hormone by following its disappearance from the incubation medium was complicated by the difficulty in distinguishing hormone binding from hormone degradation. These difficulties prompted the use of radioactively-labelled hormone.

In 1953 Stadie *et al.* introduced the use of [^{35}S]insulin sulphate and ^{131}I-insulin to directly study the interaction of insulin with rat diaphragm. These studies however, were criticised by Newerly and Berson (1957) who pointed out the lack of "specificity" of ^{131}I-insulin binding to diaphragm and the altered biological activity of the labelled hormone. Direct binding studies using radiolabelled insulin thus received little attention for nearly two decades.

However, in the late 1960s and early 1970s several indirect studies produced evidence that insulin has its primary action at the cell surface. Crofford (1968) demonstrated that the biological effect of insulin on isolated fat cells could be rapidly reversed with anti-insulin serum, suggesting insulin interaction with the cell surface.

Another investigation showed the lack of insulin response following trypsin treatment of isolated fat cells. Trypsin digestion was carried out without loss of cellular integrity, without altering the glucose transport system itself and, without impairing cellular response to other hormones (Fain & Loken, 1969; Kono, 1969 a, b). The evidence from these studies suggested that trypsin destroyed a protein membrane component necessary for insulin action.

A further indirect approach was provided by Cuatrecasas (1969). He reported that insulin coupled to insoluble agarose (Sepharose) particles was biologically active. Because of the relatively large size of these hormone-agarose complexes, it was concluded that the hormone could not enter the cell and, therefore, the metabolic effects were a result of hormone-complex interaction with the cell membrane.

Although at the time these studies were considered to be definitive proof of hormone interaction with the cell membrane, it was later discovered that the hormone could be released from the complex during incubation; the interpretation placed upon these results was therefore, in doubt (Butcher *et al.*, 1973; Davidson *et al.*, 1973; Katzen & Vlahakes, 1973).

The most conclusive evidence for insulin-membrane interactions was provided by electron microscopic studies using a ferritin-insulin conjugate (Jarrett *et al.*, 1974). In these experiments it was observed that the conjugate bound specifically and irregularly to only one side of the fat cell membrane.

Visualisation of the insulin-ferritin conjugate bound to the membrane provided qualitative proof of insulin interaction with a membrane "receptor." However, a necessary step in characterising any receptor is a study of the binding kinetics of the ligand to the receptor. Such studies provide not only qualitative data, but the potent dimension of quantitative information is added. Manifestly, such knowledge greatly enhances understanding of the dynamic process of function.

In the early 1970s the introduction of a biologically active monoiodo-insulin, which is radiolabelled on the A14 tyrosine residue and has high affinity binding to liver membranes, led to explosive development in direct insulin binding studies (Freychet *et al.*, 1971).

1.2 METHODOLOGY

The basic methodology employed for binding studies entails incubating ^{125}I-labelled hormone with the receptor preparation and then separation of receptor bound hormone from the medium containing free hormone, either by centrifugation or filtration.

1.2.1 ^{125}I-Insulin

Ideally, the iodinated hormone should retain the binding characteristics and the biological activity of the native hormone. Freychet *et al.* (1971) used the mild lactoperoxidase method to produce monoiodoinsulin. A further refinement of this ^{125}I-insulin was the separation of insulin labelled on tyrosine in position A14 from the A19 substituent (Gliemann *et al.*, 1979). The ^{125}I-(TYR-A14) insulin was shown to have the same biological potency and affinity for the receptor as native insulin. ^{125}I-(TYR-A19) insulin, by contrast, was only half as potent, because the tyrosine in position A19 is believed to be in the binding region of the hormone (Pullen *et al.*, 1976). Most insulin binding studies since 1980 have used the ^{125}I-(TYR-A14) insulin, which can be separated by ion exchange chromatography.

1.2.2 Receptor Preparations

The tissue preparations which have been used in binding studies include intact cells, particulate fractions of cells and "solubilised" cell membranes. There are both advantages and disadvantages to be considered when using such preparations.

1.2.2.1 Intact Cells

Isolated intact cell preparations from a variety of different tissues have been used in hormone-receptor studies (Table 1). The isolated cells used in these studies have been obtained from the classical insulin target tissue such as liver, fat and muscle and, from more accessible tissue which is not considered a major target for insulin. Non-target tissues such as monocytes, erythroctyes and cultured lymphocytes possess insulin receptors similar to those found on the target tissues (Archer *et al.*, 1973a,b; Gambhir *et al.*, 1978; Gavin *et al.*, 1973). These metabolically active isolated intact cells can be used to correlate hormone binding and biological activity, although hormone degradation may be higher. The use of enzymes such as collagenase, trypsin and hyaluronidase to isolate fat cells introduces a variable which can influence the receptor number and affinity and hence requires rigid control. Normal circulating lymphocytes do not have insulin receptors, however, *in vitro* transformation of these cells with mitogens induces large numbers of insulin receptors (Krug *et al.*, 1972). These cells become transformed and can be maintained in culture indefinitely. Cells in culture can be readily isolated for biochemical investigations(Taylor *et al.*, 1982).

1.2.2.2 Particulate Cell Fractions

Hormone-receptor interactions have been studied on particulate cell fractions from a number of different tissues and have varied from crude homogenates to purified plasma membranes (Table 1). These fractions are useful for biological studies and are stable on storage. However, it is important to assess plasma membrane yield and possible contamination with other organelles (Kahn, 1976).

Table 1. Isolated Cells for Insulin Receptor Studies

TISSUE	METHOD OF ISOLATION	REFERENCE
Adipocytes	Enzymatic Digestion	Freychet *et al.*, 1971 Cuatrecasas, 1971
Liver	Enzymatic Digestion	Kahn *et al.*, 1974
	Cultured	Cuatrecasas, 1971
	Mechanical Disruption	Olefsky *et al.*, 1974
Fibroblasts	Cultured	Gavin *et al.*, 1972
Chondrocytes	Enzymatic Digestion	Hintz *et al.*, 1972
Muscle	Mechanical Disruption	Freychet & Forgue, 1972
Placenta	Mechanical Disruption	Clark *et al.*, 1978
Lymphocytes		
cultured	Cultured	Gavin *et al.*, 1973
peripheral	Isopycnic Centrifugation	Archer *et al.*, 1973
thymic	Mechanical Disruption	Goldfine *et al.*, 1972
Monocytes	Isopycnic Centrifugation	Bar *et al.*, 1976
Erythrocytes	Isopycnic Centrifugation	Gambhir *et al.*, 1978
Endothelial cells	Culture	Jialal *et al.*, 1984

1.2.2.3 Solubilised Cell Fractions

Solubilised receptors have been prepared from particulate fractions for hormone-receptor studies. There are several criteria used to determine whether a receptor is solubilised. These are: failure to sediment during ultracentrifugation at 100 000 *g*, passage through suitably-sized millipore filters or retardation during gel filtration (Kahn, 1976). Neutral detergents such as Triton X-100 have been useful for solubilising receptors (Cuatrecasas, 1972). Solubilised receptors have been used in structural studies and in characterising the chemical composition of the receptor. However, the disruption of the membrane phospholipids may result in decreased affinity of the receptors for insulin and the number of receptors recovered will obviously be affected. A further problem is that the labelled hormone may associate with micelles of the detergent and form a complex which on gel filtration, may be mistaken for the hormone-receptor complex (Giorgio *et al.*, 1974).

1.3 BINDING CHARACTERISTICS OF INSULIN RECEPTORS

In section 1.2.2.1 it was mentioned that insulin receptors have been found in various preparations from nine different tissues. However, not all these tissues are responsive to insulin and although insulin binds to the receptor, there is no biological effect. Despite the lack of metabolic effects in some of these tissues, several binding characteristics are common to all cells. These include specificity, kinetics of binding, temperature dependency and the effect of pH and specific ions.

1.3.1 Specificity

It is generally accepted that the word "receptor" refers to a molecule or molecular complex which is capable of specifically recognising a particular ligand, and interacting with it in such a way that a biological response is effected.

An important property of the insulin receptor is its ability to recognise and interact with a single hormone, insulin. In some cases, various insulin analogues and peptides with insulin-like activity will bind to the receptor, but their affinity for the receptor is directly proportional to the biological effect they exert. For instance porcine, bovine and human insulin have the greatest affinity for receptors on liver membranes and cause maximal glucose oxidation in fat cells. Proinsulin and desoctapeptide have a lower affinity for liver membrane receptors and a proportionally lower effect on glucose oxidation in fat cells (Freychet *et al.*, 1971).

1.3.2 Insulin-like Growth Factors (IGFs)

A more complicated relationship exists between insulin and the insulin-like growth factors (IGFs). Olez *et al.* (1972) described a serum protein which lacked insulin immunoreactivity, but had weak insulin-like effects on glucose utilisation and lipid metabolism, and was more potent than insulin in stimulating cell growth. This protein was called "non-suppressible insulin-like activity" (NSILA). It was later demonstrated that NSILA bound to the insulin receptor with a low affinity and its metabolic effects were mediated via the insulin

receptor. Likewise, insulin bound to the NSILA receptor with low affinity and the growth effects of insulin were mediated through this receptor (Megyesi *et al.*, 1974). The NSILA serum protein was identified by Rinderknecht & Humbel (1978a) as two distinct proteins which were called the insulin-like growth factors I and II (IGF-I and IGF-II).

The structures of IGF-I and IGF-II have been elucidated (Rinderknecht & Humbel 1978a,b). They are homologous and are structurally similar to insulin (Fig. 1.1). An interesting distinction between the IGFs and insulin is the retention of the connecting-peptide (C-peptide) by the former, whereas this structural peptide is removed in the conversion of proinsulin to insulin. As a result, insulin consists of two polypeptides linked by disulphide bonds, while the IGFs are single chains (Froesch *et al.*, 1985).

It has also been established that two subtypes of receptors for IGF exist. The type I receptor has a similar structure to the insulin receptor but, has a greater affinity for IGF-I than IGF-II and a weaker affinity for insulin. The type II receptor has a different structure from the other two receptors and it does not bind insulin. Both IGF-I and IGF-II, however, bind with low affinity to the insulin receptor (Rechler & Nissley, 1985).

Recent studies have shown that the type 1-IGF receptors and insulin receptors are homologous structures (Massagué & Czech, 1982). In addition they have similar antigenic determinants (Kull *et al* ., 1983)

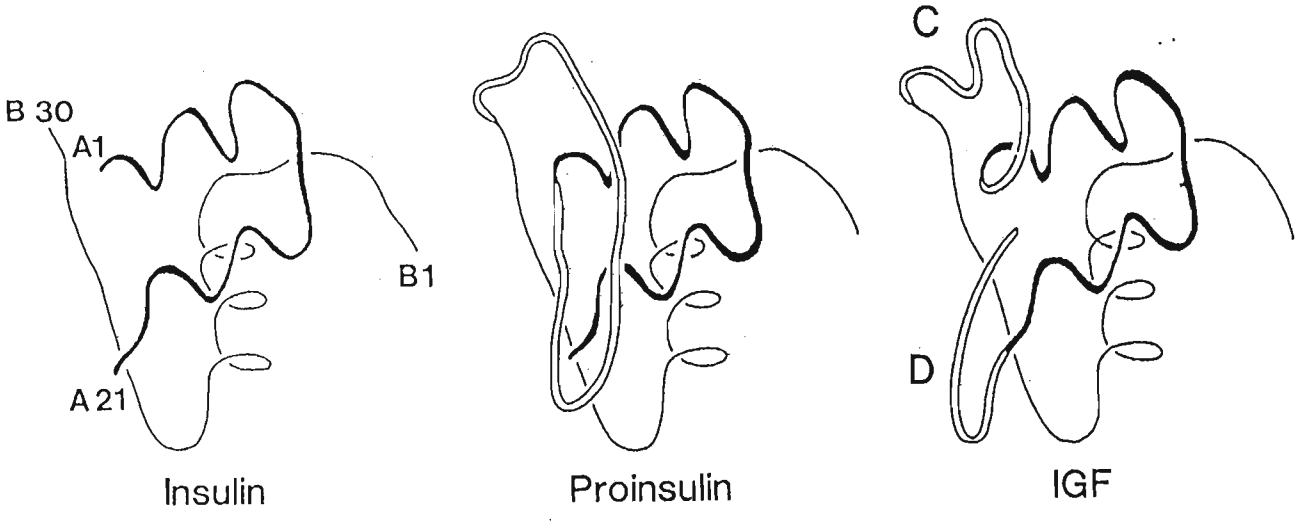


Fig. 1.1 **Schematic representation of the three-dimensional structure of insulin** based on the x-ray analysis of Rhombohedral Porcine 2-Zn insulin crystals and proposed conformations based on model building for proinsulin and IGF-I showing the close structural homology. C, C-Domain; D, D-Domain. (Reproduced from ZAPF *et al* , 1981).

and both receptors are downregulated by IGFs and insulin (Rosenfeld *et al.*, 1982).

Initially it was proposed that growth factor receptors mediated cell proliferation and insulin receptors mediated acute metabolic effects. However, there is an increasing number of important exceptions to this simple rule (Czech, 1982). For example, in the rat hepatoma H35 cell line insulin stimulates cell growth via the insulin receptor, whereas both classes of peptides stimulate glucose transport and metabolism via IGF receptors in human skin fibroblasts (Knight *et al.*, 1981; Koontz & Iwahashi, 1981).

It should be mentioned that although insulin-like growth factors are present in serum in great excess (1 µg/mℓ), they are bound to specific carrier proteins. In this form they do not appear to bind readily to the insulin or IGF receptor (Zapf, 1979).

1.4 KINETICS OF BINDING

The purpose of kinetic analysis is to obtain quantitative data on the nature of insulin binding to the receptor at cellular and molecular levels. These data are useful in describing receptor-binding models. Such studies are generally carried out under steady state conditions in which the law of mass action applies. However, before steady state binding experiments can be undertaken, it is important to establish the optimal conditions for insulin binding to the receptor.

1.4.1 Receptor Binding Assay

In section 1.2 the basic requirements for the insulin binding assay have been detailed. Given these requirements, the principle of a competitive binding assay is to incubate an insulin preparation with physiological concentrations of labelled insulin in the presence of increasing concentrations of unlabelled insulin, under equilibrium conditions. This is followed by measuring the decrease in bound labelled insulin (Fig. 1.2). The concentrations of unlabelled insulin employed range from "tracer" levels (0,2 ng/mℓ) at which only a small number (1%) of receptors are occupied, to very high levels (10^5 ng/mℓ). The percentage of bound ^{125}I-insulin decreases as the total insulin concentration increases and all data are corrected for non-specific or nonsaturable binding (Cuatrecasas, 1971).

In all systems, hormone binding consists of at least two processes, one of which is saturable or "specific" and the other of which is non-saturable or "non-specific." In the physiological range, most binding occurs to the specific binding sites which, by definition, are finite in number. The non-specific binding is generally defined as the amount of insulin bound in the presence of 10^3 - 10^5 ng/mℓ unlabelled insulin (Kahn, 1975).

1.4.1.1 pH and Specific Ions

Insulin binding studies should be carried out under physiological conditions, because extremes of pH, temperature and ionic composition in the assay may give aberrant results.

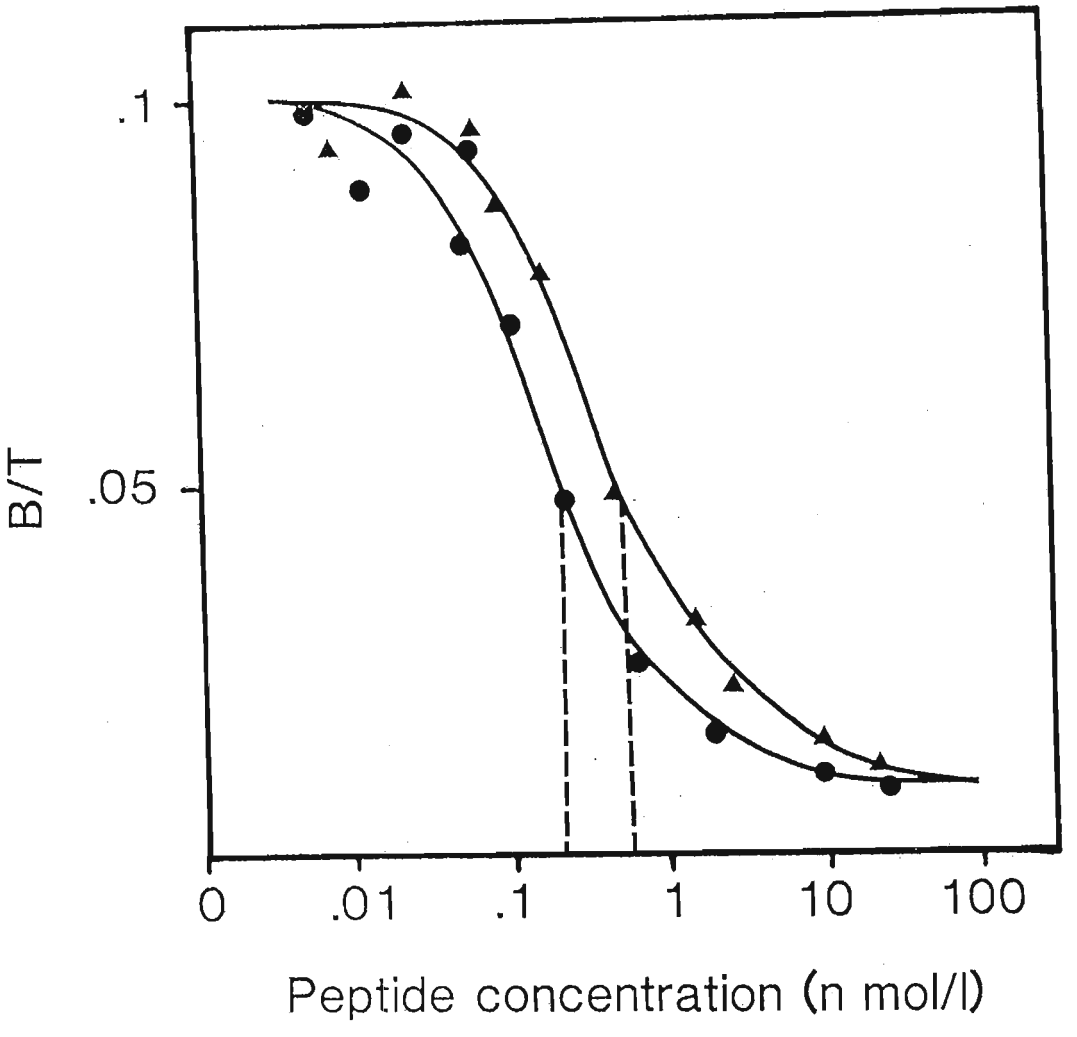


Fig. 1.2 **Displacement or competition curves** for homolologous (▲—▲) (chemically identical) and heterologous (●—●) (chemically different) competitors. ID_{50}'s are indicated with the vertical dashed lines. The important characteristics of the curve include upper and lower plateau levels, ID_{50} location and slope factor (reproduced from Munson and Rodbard, 1983).

For instance, it is now well established that most tissues have a distinct pH optimum, between pH 7,6 and 8,0, at which insulin binds to its receptor (Gavin *et al.*, 1973).

The influence of specific ions such as Ca^{++} and Mg^{++} is not as consistent. For example, in cultured human lymphocytes increasing concentrations of Ca^{++} have no effect on binding (Gavin *et al.*, 1973), whereas in isolated fat cells increased binding has been reported in the presence of high Ca^{++} concentrations (Desai *et al.*, 1978).

However, increased binding on fat cell plasma membranes was observed at high ionic strength (Cuatrecasas, 1971). This could account for the high binding reported in the presence of increased Ca^{++} concentrations for fat cell binding studies.

From these data it appears that insulin binding is not dependent on Ca^{++} and Mg^{++} as specific co-factors, although a high ionic strength does marginally increase binding.

1.4.1.2 Temperature Dependency

Both the rate and steady state level of insulin binding are dependent on temperature. However, the amount of insulin bound at higher temperatures is consistently lower, because the rate of dissociation of insulin bound to receptor is accelerated to a greater extent than the association rate under these conditions, this leads to an apparent overall decrease in the affinity (Ginsberg, 1977).

Higher temperatures also lead to an increase in the internalisation

and degradation of insulin and the receptor, which results in an apparent decrease in insulin binding to the receptor.

1.4.2 Kinetic Analysis

Binding of radiolabelled insulin to insulin receptors can be described by the law of mass action. According to the law of mass action the hormone-receptor interaction can be expressed by the equation

$$[R] + [H] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RH]$$

where R is receptor, H is hormone and RH is the hormone-receptor complex, k_1 is the rate constant of association and k_2 is the rate constant of dissociation.

Once optimal steady state binding conditions have been established and the insulin binding assay has been carried out, the data can be analysed by plotting the ratio of bound and free hormone (B/F) versus bound (B) (Scatchard, 1949). This graphical representation of insulin binding enables the calculation of the equilibrium constant (K) and the number of receptors (R) present in the preparation. The mathematical formulation of the Scatchard plot will be described in Chapter 3.

Scatchard plots of insulin binding in adipocytes, liver membranes and human lymphocytes are all typically curvilinear with upward concavity (Gammeltoft, 1984). Initially this was interpreted as indicating two types of binding sites: a high affinity, low capacity site and a low affinity, high capacity site. Later, De Meyts *et al.* (1973) provided

direct evidence for site-site interactions between insulin receptors and suggested a model for negative co-operativity. Based on their data the model proposes that insulin binding sites are homogeneous, but the binding affinity decreases with increasing occupation of the sites by insulin. The affinity of the sites is thus not represented by a fixed binding constant: the model predicts that it varies as a function of receptor occupancy (De Meyts & Roth, 1975).

A graphical representation of negative co-operativity called the average affinity profile, was described. The mathematical derivation of this plot will be discussed in more detail in Chapter 3.

Although the model for negatively co-operative site-site interactions does explain the curvilinear Scatchard plot, some workers disagree with the experimental evidence and have shown that the dissociation rate is independent of receptor occupancy (Pollet *et al.*, 1977). More recent evidence using a double probe labelling (DPL) technique, has shown that each insulin receptor binds two insulin molecules. The first molecule is bound with a high affinity ($K_d \sim 6 \times 10^{-10}$ mol/ℓ) and the second molecule of insulin is bound with a much lower affinity ($K_d \sim 8 \times 10^{-8}$ mol/ℓ). The presence of two distinct binding sites with different affinities on a single receptor unit is consistent with the model for heterogenous binding sites. However, the presence of site-site interactions cannot be excluded and it has been suggested that insulin binding to the receptor is a combination of both heterogeneous binding sites and negative co-operativity (Pang & Schafer, 1984).

Recent evidence that most cell types possess both insulin receptors, which bind insulin with a high affinity, and type I IGF receptors, which bind insulin with a lower affinity favours the model for heterogeneous binding sites (Rechler & Nissley, 1985).

On the other hand, studies using a variety of monoclonal antibodies have shown that the insulin receptor can exist in different conformational states which are stabilised by ligand induced site-site interactions (Gu *et al.*, 1988). These data support the negative co-operativity model.

1.5 INSULIN RECEPTOR STRUCTURE

1.5.1 Physicochemical Properties

Early indirect experiments suggested that the insulin receptor is protein in nature. Trypsin treatment decreased the receptor numbers in plasma membranes and in solubilised preparations of receptor obtained from human placentae, adipocytes, and human lymphocytes (Clark *et al.*, 1978; Kono & Barham, 1971; Gavin *et al* ., 1973). Treatment of cells with neuraminidase and plant lectins indicated that specific carbohydrate moeities are components of the receptor (Cuatrecasas & Illiano, 1971).

Solubilised receptors have been characterised as asymmetric proteins with a Stokes radius of 70-72 Å (Cuatrecasas, 1972; Gavin *et al.*, 1972). The insulin receptor complex has a sedimentation coefficient of

11S, suggesting an indicated molecular weight of about 300 kD (Pollet *et al.*, 1981).

1.5.2 Subunit Structure of the Insulin Receptor

A variety of techniques has been used to establish the subunit structure of the insulin receptor. It is now well established that the receptor consists of two subunits denoted α and β.

Using affinity chromatography the α subunit was isolated and identified by sodium dodecyl sulphate (SDS)-gel electrophoresis of solubilised receptor (Jacobs *et al.*,1980). It has an apparent molecular weight of 125-135 kD. These results have been confirmed using affinity labelling, photo-affinity labelling and immunoprecipitation of the receptor with anti-receptor antibodies (Yip *et al.*, 1978; Pilch & Czech, 1979; Kasuga *et al.*, 1981b).

This subunit is the dominant labelled species observed when the receptor is affinity labelled by ^{125}I-insulin using the bifunctional chemical crosslinker disuccinimidyl suberate (DSS), a finding which suggests that it probably contains the insulin binding site. Furthermore, competitive inhibition with unlabelled insulin decreases the amount of ^{125}I-insulin crosslinked to the α subunit which shows that affinity labelling of this subunit is specific for insulin (Pilch & Czech, 1980). The α subunit has been identified in receptors prepared from cultured human lymphocytes, monocytes, muscle, rat liver membranes, adipocytes and endothelial cells (Czech, 1985).

The β subunit is also a glycoprotein and has an indicated molecular weight of 95 kD. It has been isolated by affinity chromatography (Jacobs *et al.*, 1980) and is the lesser species observed in SDS gels after crosslinking the insulin-receptor complex with DSS. This finding suggests that the β subunit is not normally active in binding insulin (Czech *et al.*, 1981).

The α and β-subunits have been shown to be present in equal quantities within the receptor complex (Fujita-Yamaguchi, 1984). In fact, there is increasing evidence that the α and β-subunits are synthesised in the cell from a single polypeptide precursor which is first glycosylated and, thereafter, undergoes proteolytic cleavage before insertion into the cell membrane (Hedo & Gorden, 1985). The mature membrane receptor is a heterotetrameric disulphide-linked complex consisting of two α and β-subunits, with an apparent molecular weight of 300-350 kD (Fig. 1.3) (Czech *et al.*, 1981; Jacobs & Cuatrecasas, 1981). From the proposed receptor model it appears that each α-subunit has the ability to bind a single hormone molecule which suggests that the receptor is bivalent.

1.6 *RECEPTOR FUNCTIONS*

Understanding of the protein chemistry of the insulin receptor is based on an assumption that insulin receptor functions are directly related to the insulin receptor structure. For instance, specific structures within the receptor complex must participate in such processes as biological signal transduction, ligand-induced receptor internalisation, downregulation and receptor recycling.

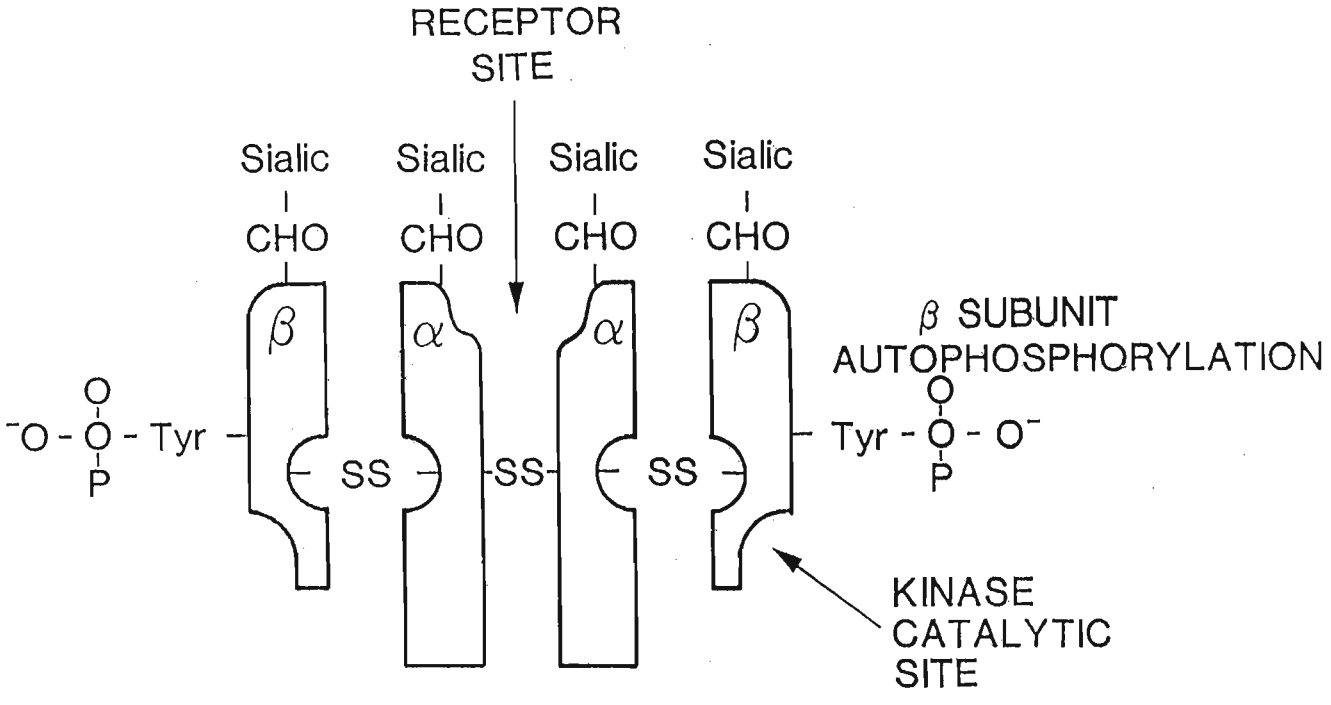


Fig. 1.3 **A schematic model of the insulin receptor structure.** The receptor unit is composed of two α subunits and two β subunits which are linked by disulphide bonds. The α subunit is found on the cell surface, whereas the β subunit forms part of the inner membrane. Insulin binds to receptor sites on the α subunit which induces auto phosphorylation of the β subunit. Protein kinase activity is associated with the β subunit. (Adapted from Czech, 1985).

1.6.1 Tyrosine Kinase Activity

An important breakthrough in the elucidation of insulin receptor function was the finding that, in the presence of ATP, insulin induces tyrosine phosphorylation of the receptor's β subunit. Once phosphorylated, the β subunit will, in turn, phosphorylate tyrosine moeities on intracellular proteins. However, at present no physiologically relevant endogenous substrate has been identified (Stadtmauer & Rosen, 1983).

There is some doubt as to whether these phosphorylations play a role in mediating the metabolic actions of insulin. The reason for this controversy is that the antireceptor antibodies, which mimic insulin action, vary with respect to their ability to stimulate the receptor-associated protein kinase activity (Taylor, 1985).

1.6.2 Intracellular Processing of Insulin

Considerable evidence now indicates that after insulin has bound to the receptor on the plasma membrane, the insulin-receptor complex is internalised by the process of endocytosis. This process is ligand-induced and the main functional consequences appear to be removal of the hormone from the external environment with subsequent hormone degradation and receptor regulation.

1.6.2.1 Internalisation of Insulin

Based on electron microscopy studies, the sequence of binding and internalisation appears to be an ordered pattern: the hormone can

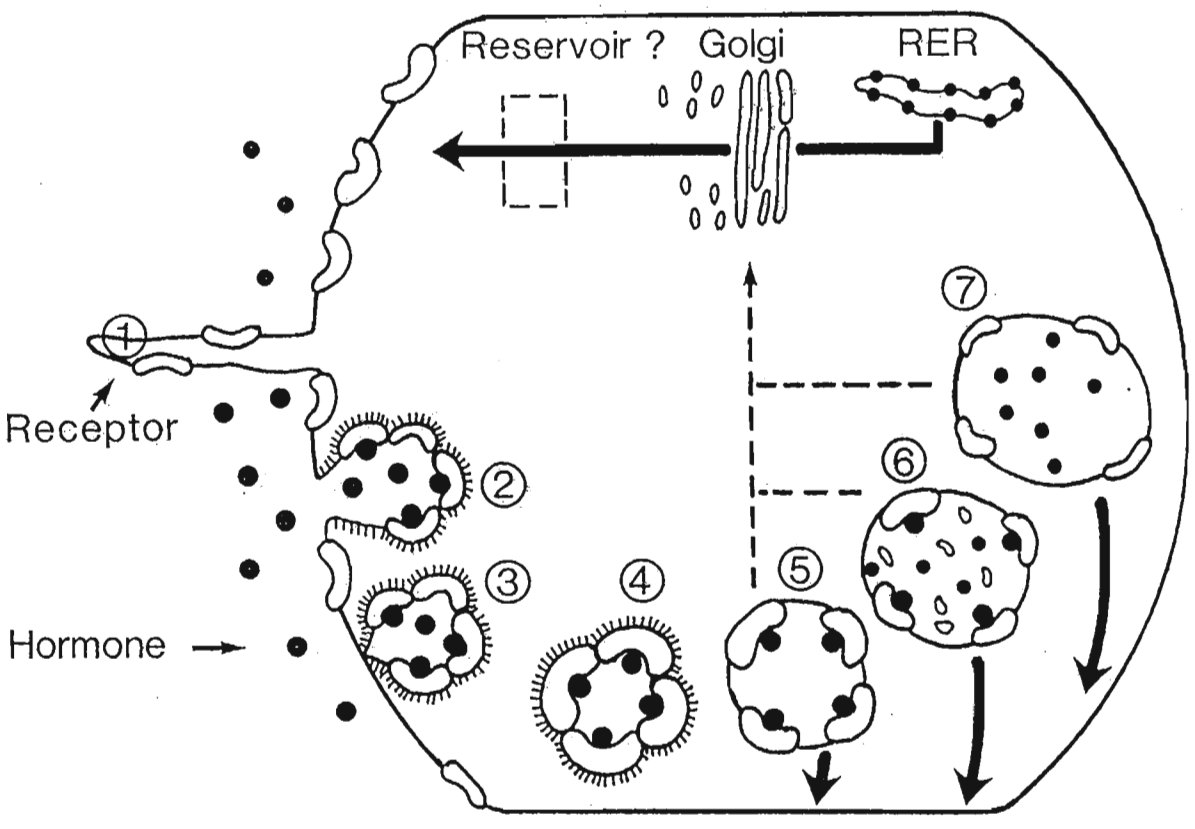


Fig. 1.4 **Schematic drawing of receptor mediated endocytosis.** The pathway includes steps (1) the microvilli, (2) coated pits, (3) fusion of the coated pit, (4) coated vesicle, (5) non-coated vesicle or endosome, (6) multivesicular body, (7) lysosome. The solid arrows in steps 4-6 represent points at which membrane receptors can recycle back to the plasma membrane. The dashed arrow indicates that recycling could also occur by way of *de novo* receptor synthesis. The reservoir indicates the possibility of a pre-formed pool of intracellular receptors. (Reproduced from Carpentier *et al* , 1986).

either bind initially to receptors on the microvilli and then move into the coated pits by lateral mobility (Schlessinger *et al.*, 1980) or the ligand binds directly to receptors in the coated pits (Fan *et al.*, 1982) (Fig. 1.4). The neck of the coated pit then fuses and a coated vesicle is formed which has a short half-life (Carpentier *et al.*, 1986). By some unknown mechanism the coat is shed, and the labelled ligand is next associated with a larger clear vesicle called an endosome (Fan *et al.*, 1983). The presence of a proton pump in its membrane allows the acidification of the vesicle's internal milieu (Tycko & Maxfield, 1982). This acidification step is regarded as important in the processing of the insulin-receptor complex because decreased pH promotes dissociation of the ligand from the receptor. Further processing of insulin was visualised in a multivesicular body composed of small vesicles thought to be the result of endosomal fusion (Carpentier *et al.*, 1986) The final processing of ligand seems to take place in the lysosomes (Fan *et al.*, 1983).

A number of polypeptide hormones including insulin, epidermal growth factor and growth hormone are degraded primarily in the lysosome by acidic proteases (Carpentier *et al.*, 1986).

Biochemical studies using a mild acid treatment to remove membrane bound labelled ligand with subsequent measurement of intracellular label, have confirmed the morphological demonstration of ligand internalisation (Haigler *et al.*, 1980). These studies have demonstrated ligand internalisation in adipocytes (Kahn & Baird, 1978), endothelial cells (Jialal *et al.*, 1984) and mouse melanoma cells (Shimizu *et al.*, 1981).

The fate of the insulin receptor following internalisation is not as straightforward as insulin. The receptor may be degraded, recycled or both situations may apply (Carpentier *et al*., 1986).

1.6.2.2 Insulin Degradation

As mentioned in section 1.6.2.1, insulin is internalised and degraded in the lysosomes by acidic proteases. The degradation products rapidly leave the cell, so at any given time only a fraction of cell-associated insulin is in this compartment.

Biochemical studies have clearly demonstrated the role of the lysosome in the degradation of internalised insulin. For example, chloroquine, a lysosomotropic agent, inactivates the intralysosomal acidic proteases by increasing lysosomal pH (Marshall & Olefsky, 1979). Treatment of cells with chloroquine before carrying out binding studies depresses insulin degradation resulting in an increase of cell-associated intact ^{125}I-insulin.

Two specific enzymes with the ability to degrade insulin have been isolated from target tissues for insulin action and have been hypothesised to function in insulin degradation *in vivo* (Duckworth & Kitabchi 1981). Insulin degrading enzyme (IDE) which is found mainly in the cytosol fraction has also been isolated from media pre-incubated with IM-9 cultured lymphocytes (Roth *et al*., 1985) and has been demonstrated on the cell surface of IM-9 lymphocytes, rat hepatoma cells and primary cultures of rat hepatocytes (Yokono *et al*., 1982). The second enzyme, glutathione-insulin transhydrogenase (GIT) is located prima-

rily in the microsomal fraction of the cell (Varandani, 1978) and was not detected on the plasma membrane of the IM-9 lymphocytes (Yokono *et al.*, 1982).

Receptor-mediated lysosomal degradation of insulin has been demonstrated in rat fat cells, human monocytes, human adipocytes and human H4 hepatoma cells (Gliemann & Sonne, 1978; Beck-Nielsen & Pedersen, 1979; Pederson *et al.*, 1981; Hofmann *et al.*, 1980). However, this degradative pathway for insulin was absent in both human IM-9 lymphocytes (Sonne & Gliemann, 1980) and human cultured lymphocytes RPMI-1788 cell line (Maegawa *et al.*, 1983). In cultured endothelial cells ^{125}I-insulin is rapidly internalised with minimal degradation and it has been suggested that this process serves to transport insulin from the intravascular space to the sub-endothelial tissues (Jialal *et al.*, 1984).

The function of this receptor-mediated degradation has been viewed in several ways. Firstly, the receptor binding and subsequent internalisation of insulin is an efficient mechanism for removing insulin from the circulation. Secondly, the removal of insulin from the cell surface and its subsequent degradation could terminate insulin action (Gliemann *et al.*, 1975). Thirdly, it has been suggested that the degradation fragments may be involved in the biological action of insulin ie. a second messenger system (Kikuchi *et al.*, 1980). Fourthly, the internalisation of the insulin receptor complex and subsequent degradation of insulin may play an important role in the ligand-induced downregulation of receptors and cellular desensitisation to insulin (Kosmakos & Roth, 1980; Krupp & Lane, 1981).

1.6.3 Downregulation

An important consequence of insulin-receptor endocytosis is ligand induced loss of membrane receptors, commonly known as "downregulation" (Gavin *et al.*, 1974).

Clearly, this phenomenon plays an important role in modulating cell surface receptor numbers. It has been suggested that insulin induces downregulation by increasing receptor degradation rather than inhibiting receptor biosynthesis (Kosmakos & Roth, 1980; Kasuga *et al.*, 1981a). At present, no linear relationship between downregulation and degradation of the receptor has been established, probably because of the added complication of measuring receptor recycling. For instance, Carpentier *et al.* (1984) have demonstrated that individual cell lines have different rates of insulin receptor internalisation which, in turn, determine the rate of receptor recycling.

Thus the regulation of cell surface receptors is not only determined by the hormone-induced receptor degradation, but is also a function of the cellular rate of internalisation and receptor recycling.

Downregulation of the insulin receptor is associated with a number of disease states in man in which chronic hyperinsulinaemia and insulin resistance are present.

1.7 INSULIN RECEPTORS ON NON-TARGET CELLS IN DISEASE STATES

One of the major problems involved in studies of insulin binding and insulin action is that target tissues are not usually available for routine clinical investigations. However, as mentioned in section 1.2.2.1, non-target tissues such as monocytes, erythrocytes and transformed lymphocytes have insulin receptors which are similar to those found on target cells. These findings have led to a great deal of research on insulin receptor binding to non-target cells in normal and disease states. There are advantages and disadvantages associated with the different cell types.

1.7.1 Monocytes

Circulating monocytes isolated from peripheral blood have been used to study insulin binding and insulin receptor functions in patients with insulin resistance (Bar *et al.*, 1976; Grunberger *et al.*, 1983).

Although these cells are relatively simple to isolate, large volumes of blood must be drawn to obtain enought cells for binding studies.

1.7.2 Erythrocytes

Erythrocytes have been used for receptor binding studies in normal and disease states (Dons *et al.*, 1981a).

The major advantage of these cells is the small quantity of blood required for binding studies.

However, circulating erythrocytes do not have nuclei and they lack the enzymes necessary for *de novo* protein synthesis. As a result the number of insulin receptors on the cell surface decreases with erythrocyte age (Dons *et al.*, 1981b). Therefore, when interpreting insulin binding data on erythrocytes the mean age of circulating erythrocytes should be taken into account.

A further disadvantage of this cell type is the presence of type I IGF receptors (Polychronakos *et al.*, 1983). If the number of high affinity insulin binding sites is decreased, insulin binding would reflect binding to the type I IGF receptor which has a lower affinity for insulin.

Kinetic analysis of decreased insulin binding, in cell types which possess type I IGF receptors, should include competitive binding studies between ^{125}I-insulin and unlabelled IGF-I to determine the degree of cross-reaction.

1.7.3 Cultured Fibroblasts

It is relatively simple to establish human skin fibroblasts in culture. These cell lines are grown under standard conditions and any changes in insulin binding or insulin action would reflect defects which are intrinsic to the cell line. There are two major disadvantages in using fibroblasts for receptor studies. Firstly, biochemical studies generally require large numbers of cells. As human fibroblasts have a finite lifetime in culture it is difficult to grow sufficient quantities of cells for these studies. Secondly, cultured skin fibroblasts have large numbers of type I IGF receptors (Rechler & Podskalny, 1976).

In section 1.3.2, it was mentioned that both insulin and IGF-I stimulate glucose transport via the type I IGF receptors in human skin fibroblasts. These observations further complicate the interpretation of insulin binding and insulin action on this cell type.

1.7.4 Epstein-Barr Virus Transformed B-Lymphocytes

As previously mentioned in section 1.2.2.1, normal circulating lymphocytes do not possess cell surface insulin receptors. Transformation of B-lymphocytes *in vitro* with Epstein-Barr virus (EBV) induces significant numbers of insulin receptors. These cells can then be grown in culture in large quantities which enables detailed biochemical studies.

In addition, EBV transformed lymphocytes do not have detectable numbers of type I IGF receptors (Taylor *et al.*, 1982).

Although there is a good correlation between genetic defects observed in EBV lymphocytes and peripheral monocytes (Taylor *et al.*, 1981), there is a possibility that incorporation of the EBV genome into the lymphocytic DNA could affect gene expression. Recent studies using insulin receptor complementary DNA suggest that expression of the insulin receptor gene occurs normally in EBV transformed lymphocytes in normal and insulin-resistant patients (Yoshimasa *et al.*, 1988; Kadowaki *et al.*, 1988).

A number of receptor functions including downregulation and phosphorylation have been demonstrated in these cell lines (Wagman *et al.*, 1983; Whittaker *et al.*, 1985). However, intracellular processing of insulin

and the biological effects of insulin on these cell lines have not been reported.

1.8 INSULIN RECEPTORS ON LYMPHOCYTES

1.8.1 The Role of Insulin Receptors During Normal Lymphocyte Activation

Both B-lymphocytes (bone marrow derived) and T-lymphocytes (thymus derived) perform an essential role in the immune response. The lymphocyte response to antigen stimulation is an ordered sequence of events. After interaction with the antigen, metabolic activation occurs, then cell proliferation and finally cell differentiation (Fig. 1.5). There is a significant increase in the number of cell surface insulin receptors during the second stage of this response. It has recently been suggested that these receptors play a "supportive" role by influencing cell metabolism (Marchalonis & Galbraith, 1987). The third stage of B-lymphocyte activation is triggered by a B-cell growth factor (BCGF) of T-cell origin. In order to stimulate cell proliferation this hormone-like lymphocyte-active peptide must bind to a BCGF receptor on the B-lymphocyte. Although recent studies support the conceptual identification of BCGF, the molecular identity of this factor and its receptor have not been established (Zola, 1985).

An interesting finding is that normal B-lymphocytes transformed by Epstein-Barr virus and B-lymphocyte cell lines derived from Burkitt's

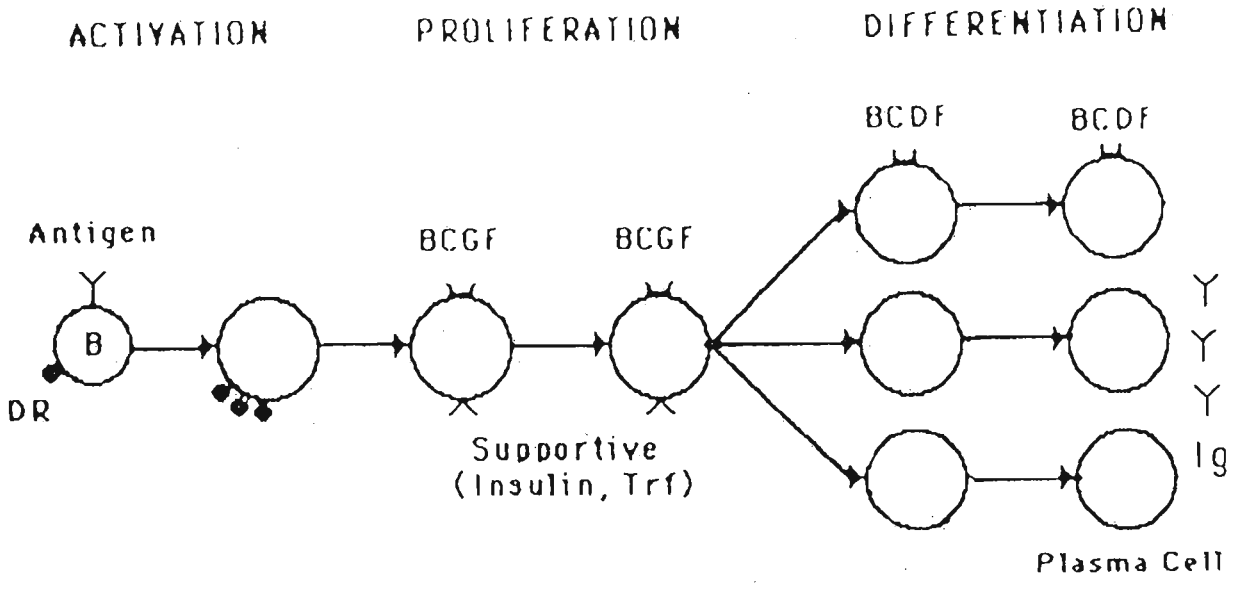


Fig. 1.5 **Conceptual model of normal B cell response.** After interactions with antigen, B cells proceed under optimal conditions through an orderly sequence of metabolic activation, proliferation (clonal expansion), and differentiation (acquisition of effector function). The progress of B cells to proliferation depends on the expression of both B cell growth factor (BCGF) and B cell differentiation factor (BCDF) receptors. Interaction of BCGF with its receptor "triggers" the proliferative phase. The insulin receptor seems to facilitate proliferation and is therefore a "supportive" receptor (reproduced from Marchalonis and Galbraith, 1987).

Lymphoma produce an autostimulatory factor which is similar to BCGF (Gordon *et al.*, 1985).

1.8.2 The Raji Cell Line

The "Raji" cell line was first established in culture by Pulvertaft (1964) from tissue which was taken from a Nigerian patient with Burkitt's Lymphoma (BL). It is now well established that most BL cell lines, including the Rajis, harbour several copies of the EBV genome (Klein & Klein, 1985). In addition, the Rajis produce an autostimulatory factor resulting in an autocrine loop (Gordon *et al.*, 1985).

If the Raji cells display insulin receptors which are similar to those on target cells, then the former cell line could be used as a model for studying the kinetics, structure and function of insulin receptors induced by EB virus.

The aim of this thesis is to show that Raji cells express a true insulin receptor which is not an artifact of viral transformation. The characteristics of the insulin receptor will be analysed as follows:

Firstly, it will be established that Raji cells have a specific insulin receptor which binds insulin with a high affinity and has similar binding characteristics to receptors on target cells. In order to determine the most accurate receptor model for insulin-receptor interaction, the kinetics of insulin binding are studied in detail.

Secondly, to show that the insulin receptor on Raji cells is functional, endocytosis of the insulin-receptor complex and subsequent processing of insulin are investigated.

Thirdly, the molecular structure of the high affinity insulin binding site is elucidated.

Finally, cross-reaction between insulin and IGF-I is studied to determine whether Raji cells display type I IGF receptors. In addition, the biological response of Raji cells to both peptides is correlated with binding studies to determine which receptor is mediating a particular response.

CHAPTER 2

BINDING CHARACTERISTICS OF INSULIN RECEPTORS IN RAJI CELLS

Optimal conditions are assumed to be definable for the process by which any result is achieved.
-INGLE, 1968

2.1 INTRODUCTION

As mentioned in section 1.8, the Raji cells provide a useful model system for studying insulin binding to human lymphocytes which have been transformed by the Epstein-Barr virus (EBV). If these studies are to be extended to disease states, it is important to establish whether this virally induced insulin receptor exhibits the same binding characteristics as the receptor on classical target cells.

The principle of the insulin binding assay was previously discussed in section 1.4. In this chapter ^{125}I-insulin has been used to directly study insulin binding to the membrane receptor on Raji cells. Other important considerations in characterising insulin binding to the receptor include the specificity of binding, steady state binding, temperature dependency, and the effect of reaction conditions such as pH, buffers and specific ions.

2.2 MEASUREMENT OF ^{125}I-INSULIN BINDING TO RAJI CELLS

2.2.1 Reagents

Reagents used in these studies were commercially available grades of the highest purity from various manufacturers: labelled insulin was human A14 - ^{125}I-monoiodo insulin (specific activity 200-300 μCi/μg) supplied by Amersham (UK); unlabelled insulin was human monocomponent insulin supplied by Novo Industries (Denmark). HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) was obtained from Sigma Chemicals Company (USA). Tris (hydroxymethyl) methylamine was supplied by BDH Chemicals Ltd., (England). Manufacturers of specific chemicals are listed in the relevant sections. Distilled water was used at all times.

^{125}I-Radioactivity was counted on a Berthold LB 2100 multi-crystal gamma counter.

2.2.2 Raji Cell Line

The origin of the Raji cell line was discussed earlier in section 1.8.2.

2.2.2.1 Morphology

Morphologically the cells are slightly different from normal lymphocytes. They are larger than small lymphocytes and are markedly granular (Fig. 2.1). In culture the cells adhere to each other in clumps which are readily dispersed with gentle agitation. The cells do not stick to glass.

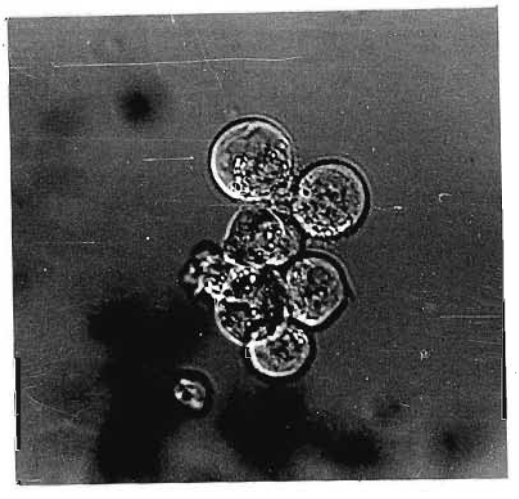


Fig. 2.1 **Raji cells.**
The Raji cells are slighty larger than normal lymphocytes. Their cytoplasm is granular and they appear to grow in clumps. (Magnification x 800).

2.2.2.2 Maintenance of Cells in Culture

The Raji cells were grown in 80 cm^2 flasks (Nunc, Denmark) in RPMI 1640 medium (Flow Laboratories, UK) containing 10% foetal calf serum (Gibco, UK) and supplemented with penicillin (100 U/mℓ),streptomycin (100 µg/mℓ) and fungizone (0,25 µg/ mℓ) (Gibco, UK). Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air with a relative humidity of 85% and fed every 3-4 days; a 1:3 split with medium was carried out every 8-10 days.

2.2.3 Insulin Binding Assay

2.2.3.1 Composition of the Binding Assay Buffer

HBB (Hepes binding buffer) pH 8,0

100	mmol/ℓ	HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
120	mmol/ℓ	Sodium chloride (NaCl)
1,2	mmol/ℓ	Magnesium sulphate ($MgSO_4$)
2,5	mmol/ℓ	Potassium chloride (KCl)
10	mmol/ℓ	Glucose
1	mmol/ℓ	Ethylenediaminetetra-acetic acid disodium salt (EDTA)
10	mmol/ℓ	Calcium chloride ($CaCl_2$)
15	mmol/ℓ	Sodium acetate ($NaCH_3COO.\ 3H_2O$)
1%	w/v	Bovine serum albumin (BSA).

2.2.3.2 Procedure

The Raji cells were grown until stationary growth phase was reached ($1\text{-}2 \times 10^6$ cells/mℓ). Cells were not "fed" for 3-4 days prior to the insulin binding assay to prevent possible downregulation of the

receptor by insulin in the foetal calf serum (FCS) (Gibco FCS insulin = 8,65 ± 1,2 μU/mℓ). A schematic representation of the procedure followed is given in Figure 2.2. Cells were harvested by spinning at 200 x *g* in a bench top centrifuge for 5 min at room temperature; the packed cells were then resuspended in HBB and recentrifuged. The cells were washed three times in the HBB and resuspended in the same buffer at a concentration of 1-2 x 10^7 cells/mℓ. The incubation mixture for the binding assay consisted of 400 μℓ of cells in HBB, 50 μℓ ^{125}I-insulin (final concentration of about 0,2 ng/ml) and 50 μℓ of unlabelled insulin (final concentration of about 10 ng/mℓ). The incubation mixture was maintained at 15°C for a period of 120 min unless otherwise stated. After incubation 200 μℓ portions of suspended cells were removed and layered onto 200 μℓ of ice cold HBB in Beckman microfuge tubes. These were then spun in a Beckman microfuge (8740 *g*) at 4°C for 2 min. The supernatant was removed and the cell pellet was counted in the Berthold multi-head gamma counter. Total counts per minute (cpm) were obtained by pooling the remaining 100 μℓ of the 500 μℓ incubation mixture and counting the radioactivity in 200 μℓ portions. The binding studies were carried out according to the method of Gavin *et al.* (1973).

2.2.4 Data Analysis

Data were expressed as maximum % specific binding. Non-specific binding (NSB) was defined as the amount of ^{125}I-insulin bound to the cell pellet in the presence of 10^5 ng/mℓ unlabelled insulin. The NSB was subtracted from total binding (B_T) to give specific binding (Sp.B). ^{125}I-Insulin specifically bound was then expressed as a

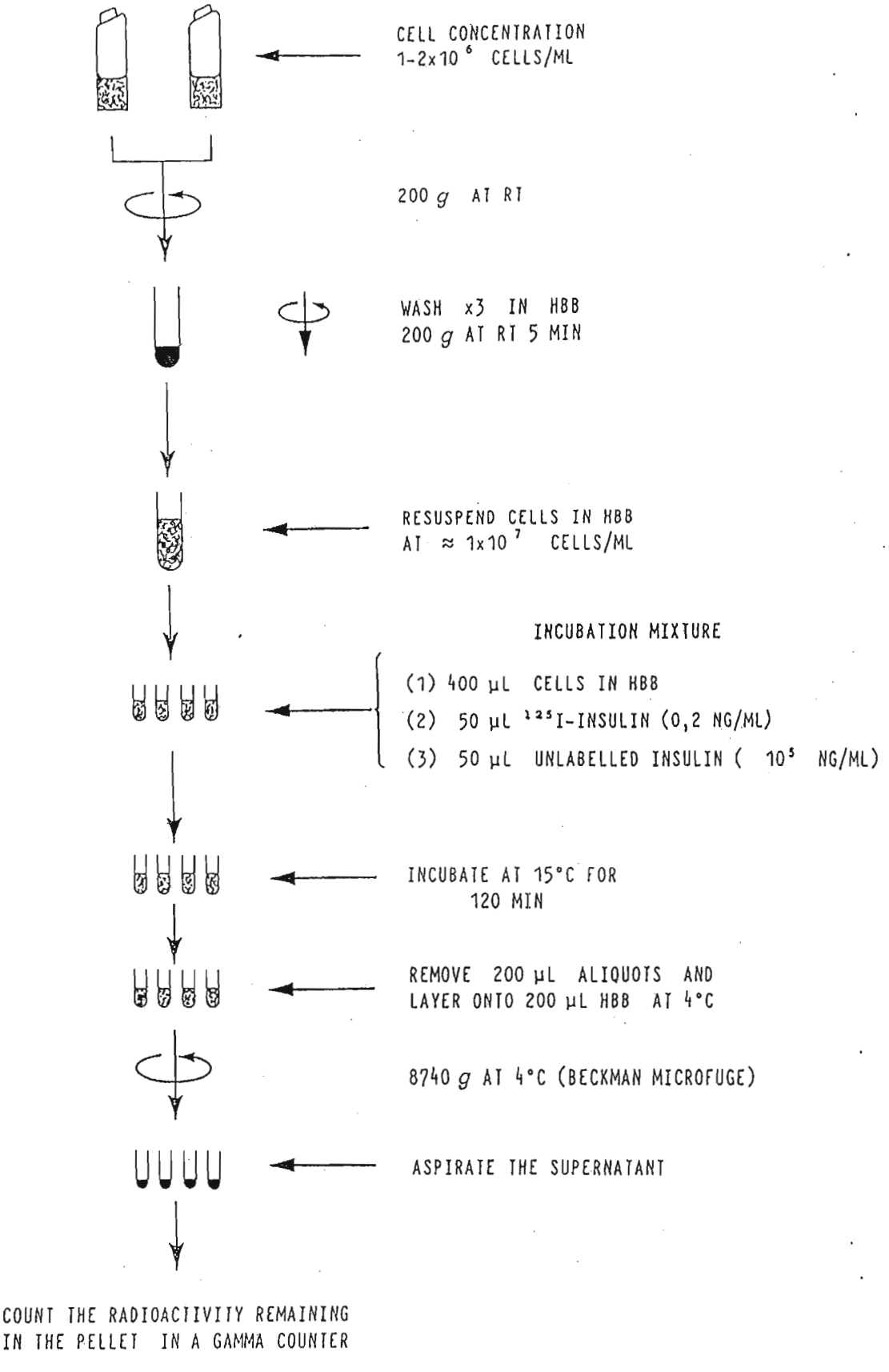


Fig. 2.2 **A schematic outline of the insulin binding assay.**

function of cell concentration which was usually 10^7 cells/mℓ. Finally, the data were divided by the total amount of radioactivity in 200 μℓ portions of incubation mixture and expressed as percentage ^{125}I-insulin specifically bound (% Sp.B). Calculation of % Sp.B. per 10^7 cells/mℓ is detailed below.

Calculation

B_T - NSB = Sp.B. .....(1)

$$\text{Sp.B.} \times \frac{1 \times 10^7 \text{ cells/m}\ell}{? \quad \text{cells/m}\ell} = \text{Sp.B. per } 10^7 \text{ cells/m}\ell \quad ...(2)$$

$$\frac{\text{Sp.B.}}{\text{TOTAL COUNTS.}} \times 100 = \text{\% Sp.B. per } 10^7 \text{ cells/m}\ell$$

2.2.5 Measurement of the Affinity Using ID_{50}

Although Scatchard plot (equation 7, section 3.2) is the most widely used method for measuring the affinity of the insulin receptor, the ID_{50} can be used as a rough index of affinity. The ID_{50} is defined as the concentration of unlabelled insulin required for displacement of one-half the maximum of ^{125}I-insulin specifically bound and it is calculated from the competition curve (Grunberger *et al.*, 1983). This parameter originates from the Michaelis-Menten constant (K_M) (Lehninger, 1975). A similar measurement was termed the pA_h and was used to determine the concentration of drug antagonist which depresses the agonist effect by one-half (Schild, 1957).

2.3 *DETERMINATION OF OPTIMAL CONDITIONS FOR INSULIN BINDING TO RAJI CELLS*

Prior to kinetic studies of insulin binding, the optimal reaction conditions for insulin-receptor binding were determined.

2.3.1 Measurement of Cell Growth

Cells were split (1:10) to give a final cell density of about 2×10^5 cells/mℓ. The cell suspension was distributed between three 25 cm^2 flasks (Nunc) and incubated under normal conditions as described in section 2.2.2.2. Cells were counted at regular 24 hr intervals for 6 days and a graph of mean cell number versus time (days) was plotted.

2.3.2 Competitive Binding Assay

A competitive binding assay was carried out by incubating a fixed amount of ^{125}I-insulin (0,2 ng/mℓ) with the receptor preparation (Raji cells) in the presence of increasing concentrations of unlabelled insulin. The concentration of unlabelled insulin in the incubation mixture ranged from 0 ng/mℓ to 10^5 ng/mℓ. Incubation was carried out as previously described in section 2.2.3.2.

In this assay the percent of bound ^{125}I-insulin decreased as the total insulin concentration increased and all data were corrected for non-specific binding (NSB) by subtracting the percent bound at 10^5 ng/mℓ from the percent bound at all other concentrations. The reason for this correction is that in the presence of a large excess of unlabelled hormone the specific activity of labelled hormone is

diluted so greatly, that statistically no labelled molecules should bind to specific receptors. Data were expressed as a competition curve by plotting percent bound versus total insulin concentration (ng/mℓ).

2.3.3 Binding Buffer

Initially Gavin *et al.* (1973) used a Tris buffer (TBB) for their insulin binding studies on IM-9 lymphocytes. This buffer contained 25 mmol Tris (hydroxymethyl) methylamine, 120 mmol NaCl, 1,2 mmol $MgSO_4$ 2,5 mmol KCl, 10 mmol glucose, 1 mmol EDTA and 1% BSA at pH 7,6. Later studies on monocytes showed improved binding when HBB was used instead of TBB (Bar *et al.*, 1976; Beck-Nielsen & Pederson, 1979).

To establish the optimal reaction buffer for insulin binding to Raji cells, the insulin binding assay was carried out in both TBB and HBB at pH 7,8. The binding assay was carried out according to the procedure outlined in section 2.2.3.2, in the presence of increasing concentrations of unlabelled insulin. The % specific bound was calculated as stated in section 2.2.4.

2.3.4 Determination of the Ideal pH for Insulin Binding

The specific binding of ^{125}I-insulin to the membrane receptor is dependent on the pH of the binding buffer. HBB was made up with a range of pH 6-9 and Raji cells were washed 3 times in HBB of the appropriate pH and then resuspended in HBB with the same pH. Cells were then incubated with ^{125}I-insulin (0,2 ng/mℓ) as previously de-

scribed (section 2.2.3.2), in the presence or absence of 10^5 ng/mℓ unlabelled insulin to establish maximum specific binding; % insulin specifically bound was plotted as a function of the pH.

2.3.5 The Effect of Ca^{++} and Mg^{++} on Insulin Binding

To determine whether Ca^{++} or Mg^{++} ions are necessary for the binding interaction, cells were washed 3 times as previously detailed in section 2.2.3.2 in HBB pH 8,0 containing no $CaCl_2$ or $MgSO_4$. The cells were then resuspended in this buffer and graded concentrations of $CaCl_2$ and $MgSO_4$ (0-10 mmol/ℓ) were added to portions of these cells and incubated with ^{125}I-insulin in the presence or absence of 10^5 ng/mℓ unlabelled insulin. The % insulin specifically bound was plotted as a function of molarity of $MgSO_4$ and $CaCl_2$.

2.3.6 Time and Temperature Dependency of Insulin Binding

Both the rate and the steady state level of insulin binding to its receptor depend on the temperature of the incubation. Optimum temperature and time required to reach steady state binding were determined by incubation of ^{125}I-insulin with cells as described in section 2.2.3.2 at 4°C, 15°C and 37°C for various time periods. Cells were incubated with ^{125}I-insulin in the presence or absence of 10^5 ng/mℓ unlabelled insulin to determine NSB. The % insulin specifically bound at each temperature was calculated as previously described (section 2.2.4) and a graph of % Sp.B. as a function of time was plotted.

2.3.7 Specificity of Insulin Binding

The receptor specificity for insulin was determined by displacement of bound ^{125}I-insulin by a number of hormones with possible structural homology. These hormones included porcine proinsulin, porcine glucagon and porcine C-peptide which were supplied by NOVO Research Institute, Denmark.

Cells were prepared as outlined in section 2.2.3.2 and then incubated with ^{125}I-insulin (0,2 ng/mℓ) and increasing concentrations (0-10^5ng/ mℓ) of C-peptide, glucagon, proinsulin and insulin. The incubation mixture was maintained at 15°C for 120 min. Results were expressed as a competition curve as described in section 2.3.2.

2.4 RESULTS

2.4.1 Raji Cell Growth Curve

Figure 2.3 represents the growth rate curve for Raji cells in culture. After splitting, the number of cells doubled within 36 hours, thereafter the doubling time was 24 hours. The logarithmic phase of growth continued for 36 hours until the stationary growth phase was reached 96-108 hours after splitting. Unless otherwise stated, cells were used at the beginning of the stationary phase.

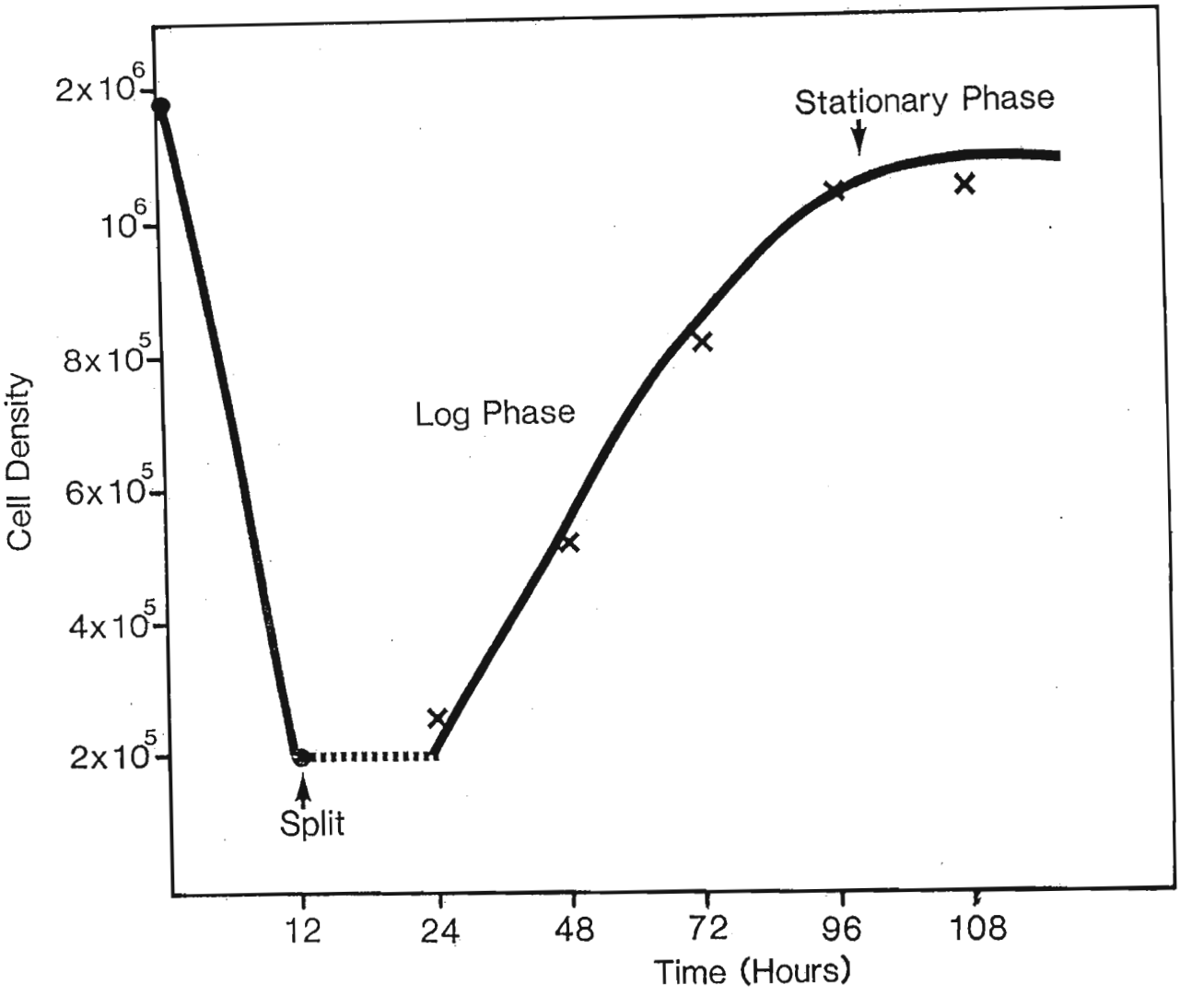


Fig. 2.3 **Measurement of the Raji cell's growth rate** was determined by counting triplicate cell samples in a haemocytometer at specific time intervals.

2.4.2 Competition Curve

Displacement of bound ^{125}I-insulin by increasing concentrations of unlabelled insulin is shown in figure 2.4. Maximum specific binding of insulin to Raji cells was 7,7 ± 1,3% per 10^7 cells and non-specific binding was always less than 25% of total binding. The concentration of insulin required to displace 50% of bound hormone (ID_{50}) was 3,2 ng/mℓ ($5,2 \times 10^{-10}$ mol/ℓ) which indicates high affinity binding.

2.4.3 Optimal Binding Buffer

Maximum specific binding was higher in the presence of Hepes buffer as compared to Tris buffer (Figure 2.5). In addition, the ID_{50}s for insulin binding in Hepes buffer and Tris buffer were 3,3 ng/mℓ and 9 ng/mℓ, respectively. This indicates higher affinity binding in Hepes buffer as a lower concentration of insulin is capable of displacing 50% of the bound ^{125}I-insulin.

2.4.4 Optimum pH

Figure 2.6 shows the effect of pH on insulin binding to the Raji cells. There is a definite peak in specific insulin binding at pH 8,0, whereas this pH had no significant effect on non-specific binding.

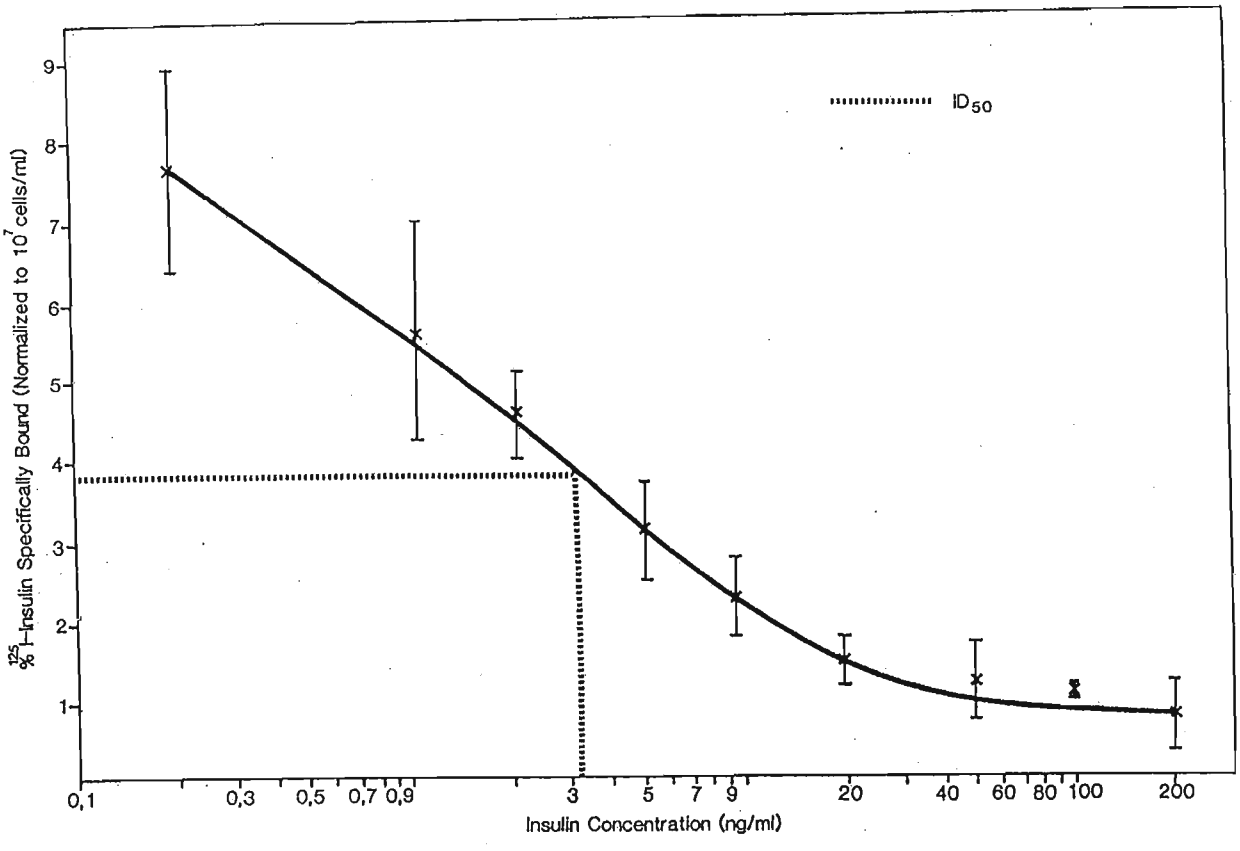


Fig. 2.4 **The competition curve between ^{125}I-insulin (0,2ng/ml) and increasing concentrations of unlabelled insulin.** Non-specific binding has been subtracted from each point. Individual points represent the mean and one standard deviation (S.D.) from two separate experiments. The ID_{50} is about 3,3 ng/ml insulin.

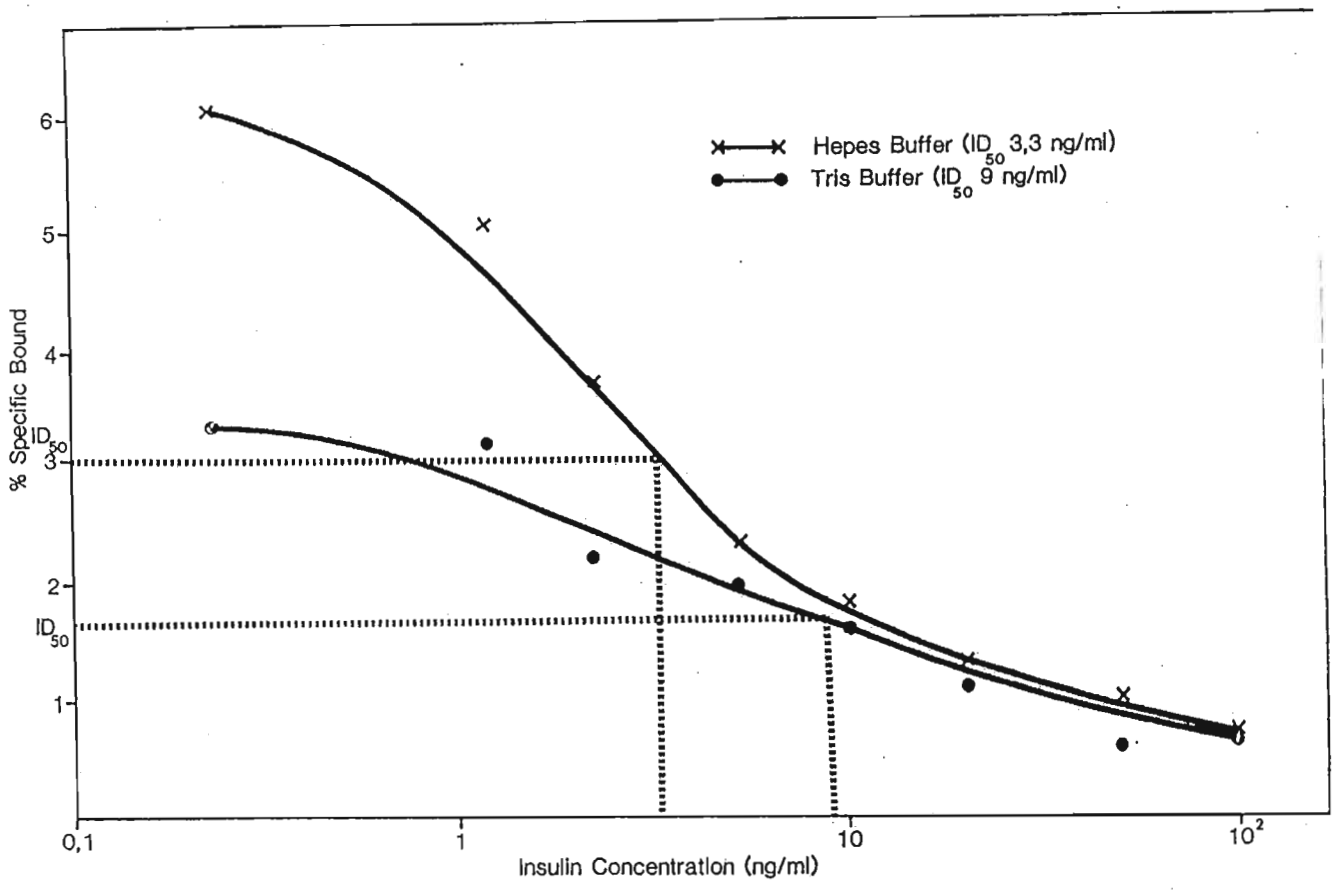


Fig. 2.5 **Insulin binding to Raji cells in Hepes Buffer or Tris Buffer.** Each point represents the mean of duplicate determinations. The ID_{50} for hepes buffer is 3,3 ng/ml and Tris Buffer is 9 ng/ml.

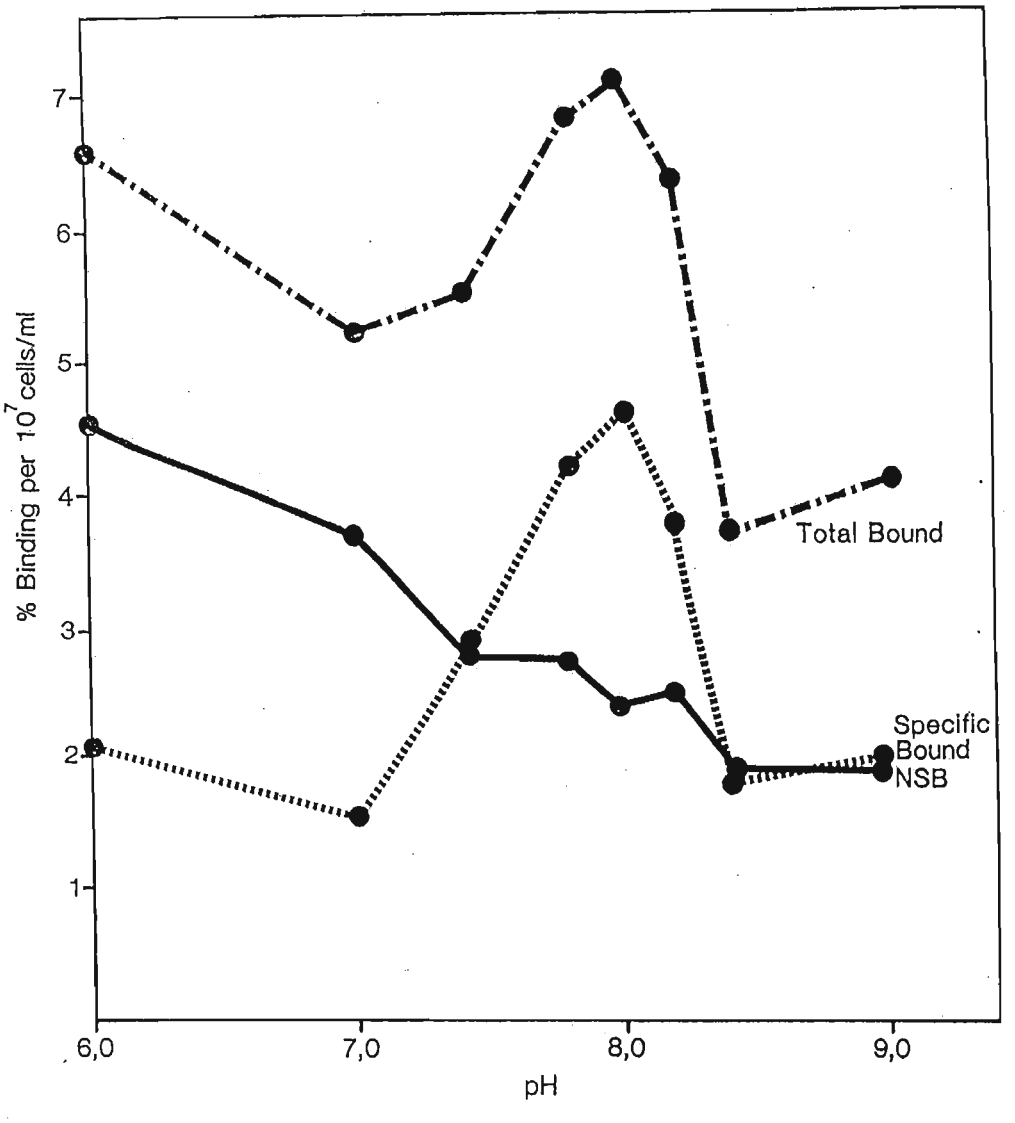


Fig. 2.6 **pH Optimum for insulin binding to Raji cells.** Each point is the mean of duplicate measurements. Specific binding is obtained by subtracting non-specific binding from total binding.

2.4.5 The Effect of Ca^{++} and Mg^{++} on Insulin Binding

In the concentration range 0-10 mmol/ℓ, neither calcium nor magnesium ions had any significant influence on insulin binding to the receptor (Fig. 2.7a). However, in HBB, which normally contains 1,2 mmol/ℓ $MgSO_4$, increasing concentrations of calcium ions apparently increased specific binding of insulin to its receptor (Fig. 2.7b).

2.4.6 Time and Temperature Dependence of Steady State Binding

Steady state binding was found to be both time and temperature dependent (Fig. 2.8). At 15°C maximum specific binding reached 5,5% after 120 min and remained at steady state until 180 min. By comparison, steady state binding was reached after 30-60 min at 37°C. Maximum binding, however, was only 3% after 60 min and then decreased to 2% after 120 min. At 4°C a maximum of 3% specific binding only was reached after 24 hours.

2.4.7 Specificity of Insulin Binding

The specificity of insulin binding to Raji cells is demonstrated by the competition curve in figure 2.9. For these data, non-specific binding was not subtracted from total binding. Approximately 50% of total bound ^{125}I-insulin was displaced by 10 ng/mℓ of unlabelled insulin, whereas only 7,5% was displaced by the same concentration of proinsulin. No significant displacement of bound ^{125}I-insulin occurred in the presence of either C-peptide or glucagon.

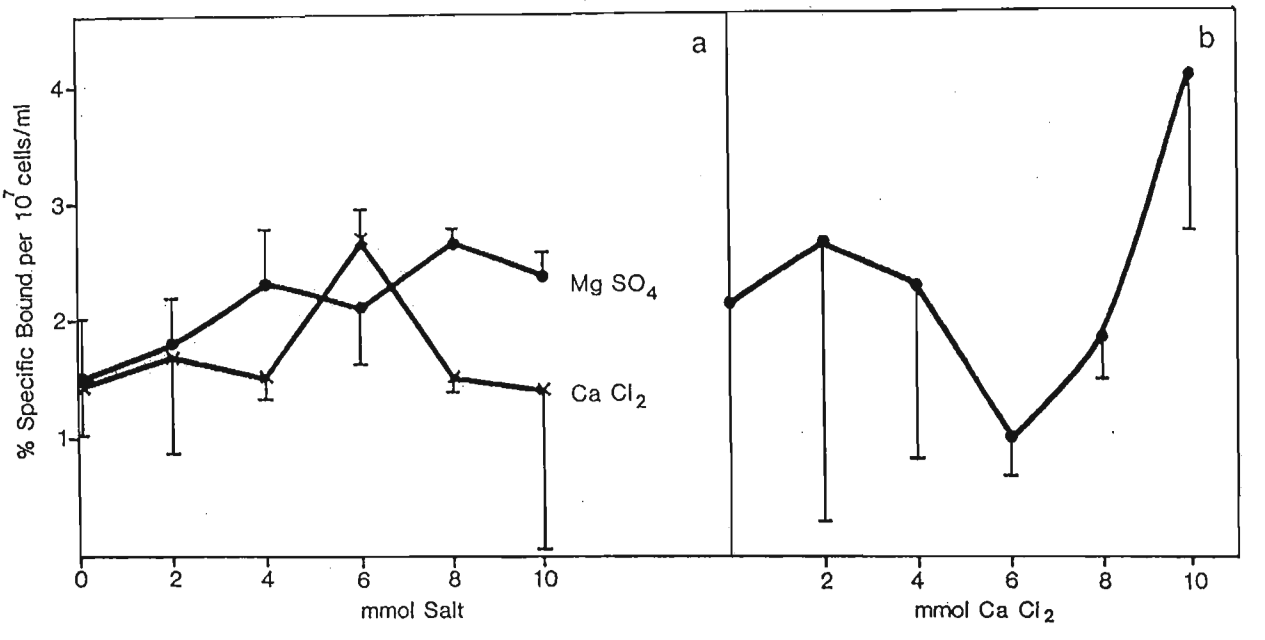


Fig. 2.7 (a) **The effect of Ca and Mg ions on insulin binding to Raji cells.** All points represent duplicate measurements of specific binding from two separate experiments.

Fig. 2.7 (b) **The effect of Ca ions on insulin binding in the presence of 1,2 m mol/l Mg SO_4.** Each point represents duplicate determinations from two different experiments.

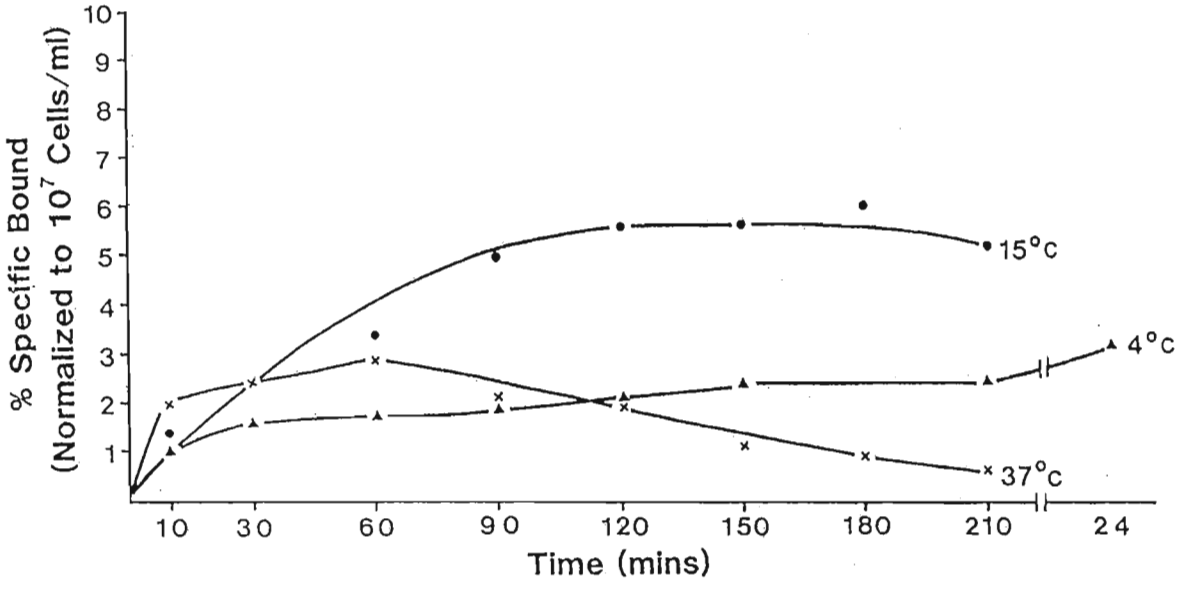


Fig. 2.8 **Temperature dependence of steady state binding to Raji cells.** Non specific binding has been subtracted from each point, which represents the mean of duplicate measurements.

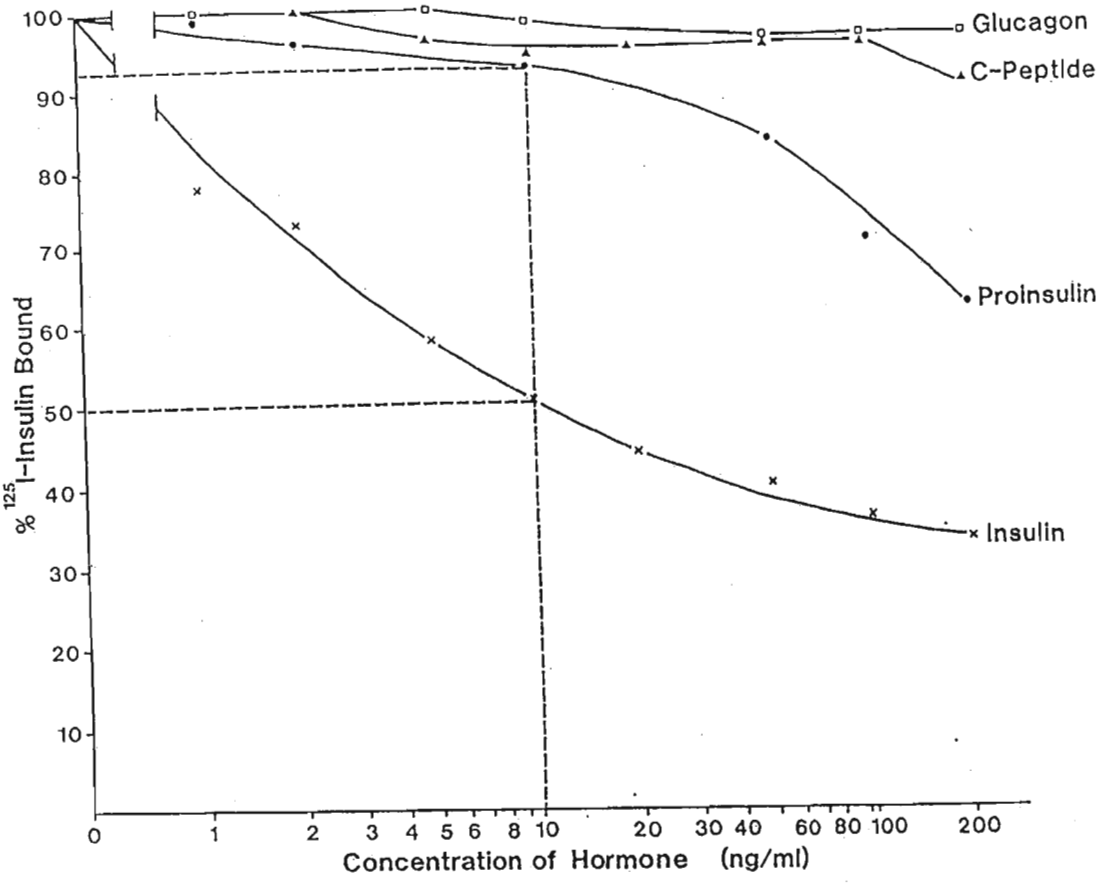


Fig. 2.9 **Specificity of insulin binding to Raji cells** is determined by competitive binding assays between radiolabelled insulin and increasing concentrations of unlabelled insulin, proinsulin, c-peptide and glucagon. All points represent the mean of duplicate determinations.

2.5 *DISCUSSION*

Based on these preliminary experiments the ideal conditions for insulin binding to Raji cells have been defined. For subsequent studies of insulin-receptor binding, Raji cells were used during stationary phase, unless otherwise stated. This ensured that insulin receptors were not downregulated and experimental observations were consistent and therefore, comparable.

The competition curve for ^{125}I-insulin and unlabelled insulin shows that about 7-8% of the radioactive insulin in the incubation mixture was specifically bound by Raji cells. At equilibrium, 50% of this bound radiolabelled hormone was displaced by $5,2 \times 10^{-10}$ mol/ℓ of unlabelled hormone. This concentration is the same order of magnitude as the K_d which was calculated by Scatchard analysis; this will be discussed in section 3.5.1. Thus, as mentioned previously in section 2.2.5, the ID_{50} gives a reasonably accurate estimate of the affinity constant (K_d).

Incubation of ^{125}I-insulin and Raji cells in TBB and HBB showed an increase in the affinity of the receptor-hormone interaction in the latter buffer. In addition, specific binding was markedly increased at pH8. At lower and higher pHs insulin dissociates from the receptor. Thus, incubation of cells in HBB at pH8 results in high affinity insulin-receptor binding. These conditions were used in all insulin binding studies and they are in agreement with findings on both IM-9 lymphocytes and adipocytes (Sonne & Gliemann, 1980).

It has also been demonstrated that the insulin binding requirement

for calcium and magnesium ions is related to ionic environment rather than to a dependence on specific ionic co-factors. Similar results have been reported for adipocytes (Cuatrecasas,1971).

From experiments on steady state binding it was found that the initial rate of binding increased as the temperature was raised, therefore, steady state conditions were reached more quickly at 37°C. However, the amount of insulin bound at steady state was lower at 37°C compared to 15°C. As the dissociation rate is accelerated to a greater degree than the association rate at higher temperatures, there is an overall decrease in insulin binding at 37°C (Olefsky, 1980). Furthermore, internalization and degradation of insulin is increased at 37°C compared to 15°C which results in an overall decrease in insulin binding (these aspects of insulin-receptor function will be discussed in more detail in Chapter 4). On the basis of these observations, optimal conditions for steady state binding were defined as 120 min at 15°C. Identical conditions have been used to study insulin binding on human monocytes (Beck-Nielsen & Pederson, 1979).

Competitive studies between bound ^{125}I-insulin and unlabelled proinsulin showed some displacement of receptor bound radiolabelled insulin. This suggests that there is some crossreactivity between the insulin receptor and proinsulin. Proinsulin is the precursor for insulin and there is significant structural homology between these peptides: thus some interaction between proinsulin and the insulin receptor is indeed possible. By comparison, peptides such as C-peptide and glucagon which are not structurally similar to insulin did

not interact with the insulin receptor. These studies have shown that the insulin receptor on Raji cells specifically recognises insulin and insulin-like peptides.

Previous reports of insulin receptors on cell lines derived from Burkitt's lymphoma have been conflicting. Initially, Gavin *et al.* (1972) noted the absence of insulin binding to lymphocytes derived from Burkitt's lymphoma. A later report by Schlickenrieder *et al.* (1985) demonstrated the presence of insulin receptors on a lymphocyte cell line (ALL) from Burkitt's lymphoma.

The experimental results presented in this study have clearly demonstrated the presence of specific, high affinity insulin receptors on EB-virus transformed lymphocytes which were derived from a Burkitt's lymphoma (Raji cell line).

CHAPTER 3

KINETIC ANALYSIS OF INSULIN BINDING TO RAJI CELLS

Nature and Nature's laws lay hid in night.
God said: "Let Newton be" and all was light.
-POPE

It did not last. The Devil, shouting "Ho!
Let Einstein be," restored the status quo.
-SQUIRE

3.1 INTRODUCTION

In section 1.4, the value of studying the kinetics of the binding of a ligand to its receptor was briefly reviewed, giving insight into the nature of the binding reaction.

The purpose of the studies outlined in this chapter is to report on the kinetics of insulin binding to receptors on the Raji cells. As indicated in section 1.2.2.1, normal lymphocytes have very low insulin receptor numbers, but following EB virus transformation, large numbers are induced on the surface of the transformed cells. If it can be shown that the kinetics of insulin binding to receptors on the Raji cells is similar to the kinetics of insulin binding in other tissues, it would be reasonable to assume that the transformations induced by the EB virus result in a true expression of the insulin receptor gene in human lymphocytes.

To facilitate understanding, the mathematical derivation of the graphical methods for describing insulin binding to its receptors will be dealt with in detail before presenting the experimental evidence.

3.2 *MATHEMATICAL DERIVATION OF THE SCATCHARD PLOT*

In order to obtain receptor-binding constants a model for the interaction between receptor and ligand must be assumed or established as plausible. A number of mathematical models has been developed for the treatment of data obtained from observations on the binding of small molecules to macromolecules (Hill, 1909; Scatchard, 1949; Berson & Yalow, 1959). Although the data from hormone-receptor binding studies do not often fit the idealised conditions proposed by the mathematical models, they do provide useful constants for describing the observed displacement curves. However, it is most important to bear in mind that the application of these mathematical analyses to binding assays requires a number of assumptions (Kahn *et al.*, 1974).

These are that:

(i) the hormone is present as a monomer;
(ii) the labelled and unlabelled hormones bind in an identical manner;
(iii) equilibrium conditions between hormone and receptor are obtained during the assay;
(iv) bound and free hormone can be separated without perturbing the equilibrium;
(v) no co-operative interactions exist between the receptors;
(vi) the hormone-receptor binding reaction is univalent.

If these assumptions are made in the analysis of insulin binding to a specific receptor, it is possible to describe the reaction by a series of equations derived from the Law of Mass action.

$$[R] + [H] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RH] \qquad \text{.....(1)}$$

where H is the hormone, R is the receptor, HR is the hormone receptor complex, k_1 and k_2 are the association and dissociation rate constants, respectively.

According to the Law of Mass action, the rate of formation of hormone-receptor complex is dependent on the concentration of both hormone and receptor and is, therefore, given by a second order rate equation,

$$\frac{d\,[HR]}{dt} = k_1\,[H]\,[R] \qquad \text{.....(2).}$$

The breakdown of the hormone-receptor complex is a first order reaction and is given by the rate equation,

$$\frac{-d\,[HR]}{dt} = k_2\,[HR] \qquad \text{.....(3).}$$

When the rate of formation of hormone-receptor complex is equal to its rate of breakdown, that is when the reaction system has entered steady state, which is defined as that state in which the concentration of HR remains constant, then

$$k_1[H][R] = k_2\,[HR] \qquad \text{.....(4).}$$

Rearranging equation 4,

$$K_a = \frac{k_1}{k_2} = \frac{[HR]}{[H][R]} \qquad \text{.....(5)}$$

where K_a is the equilibrium constant or association constant. From the rate equations (2) and (3) the units for k_2 are reciprocal time in seconds (S^{-1})and the units for k_1 are reciprocal concentration and time ($\ell.mol^{-1}.S^{-1}$). Therefore, from equation 5 K_a would be expressed as $\frac{(\ell.mol^{-1}.S^{-1})}{(S^{-1})}$ or ℓ/mol that is, reciprocal molarity.

Alternatively, the equilibrium of a hormone-receptor binding reaction can also be expressed in terms of the dissociation constant K_d, which is the reciprocal of the K_a and has the units mol/ℓ.

A second equation which describes the binding reaction at equilibrium is the conservation equation of the total receptor concentration $[R_T]$

$$[R] + [HR] = [R_T] \qquad \text{.....(6).}$$

To obtain the Scatchard plot of Bound/Free (B/F) vs Bound (B) from equations (5) and (6), it is necessary to define B/F and B in the same terms.

$$\frac{B}{F} \equiv \frac{[HR]}{[H]}$$

and bound

$$B \equiv [HR]$$

Thus, from equation (5) B/F can be expressed as

$$\frac{B}{F} \equiv \frac{[HR]}{[H]} = K_a [R]$$

Substituting for R from equation (6)

$$\frac{B}{F} = K_a \left([R_T] - [B]\right)$$

or .....(7).

$$\frac{B}{F} = \frac{1}{K_d} \left([R_T] - [B]\right)$$

Equation (7) is the Scatchard plot which is commonly used to linearise experimental data for graphical estimation of binding parameters. A Plot of B/F vs B produces a straight line with slope $-K_a$ and an intercept on the bound-axis equal to R_T(Fig. 3.1a & b). A single class of binding sites will result in a linear Scatchard graph (Fig. 3.1a), while non-linear plots represent two classes of independent binding sites which have different affinities for the ligand (Fig. 3.1b).

Determination of binding constants for curvilinear graphs is not straightforward. Many workers have used linear regression and extrapolated from each half of the curve to obtain these constants. However, this approach has been criticised by Light (1984) because when two binding sites compete for the same pool of ligand, the high affinity sites bind most of the ligand at low concentrations and reach saturation before the second site. Therefore, to analyse the data correctly, the competition between the two sites must be accounted for mathematically. For this reason, a simple linear regression is not suitable for analysing curvilinear graphs.

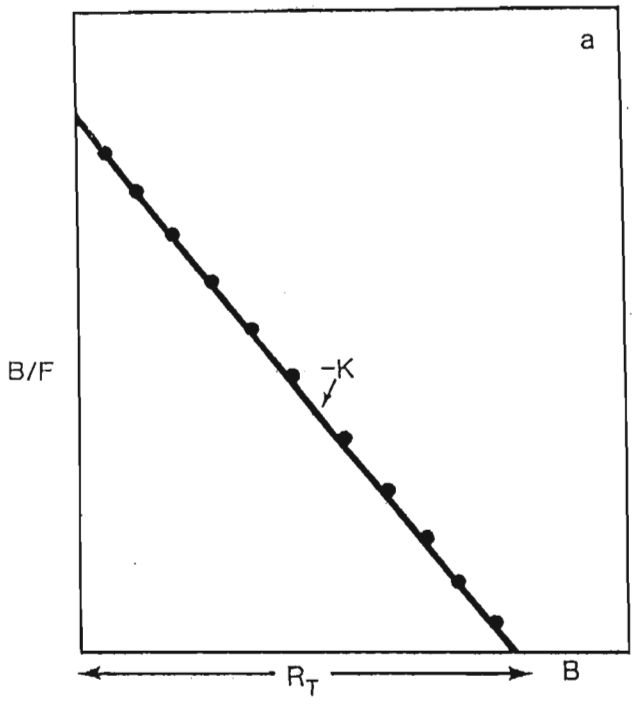


Fig. 3.1 (a) **Scatchard plot for ligand binding to a single class of independent sites** (see legend overleaf).

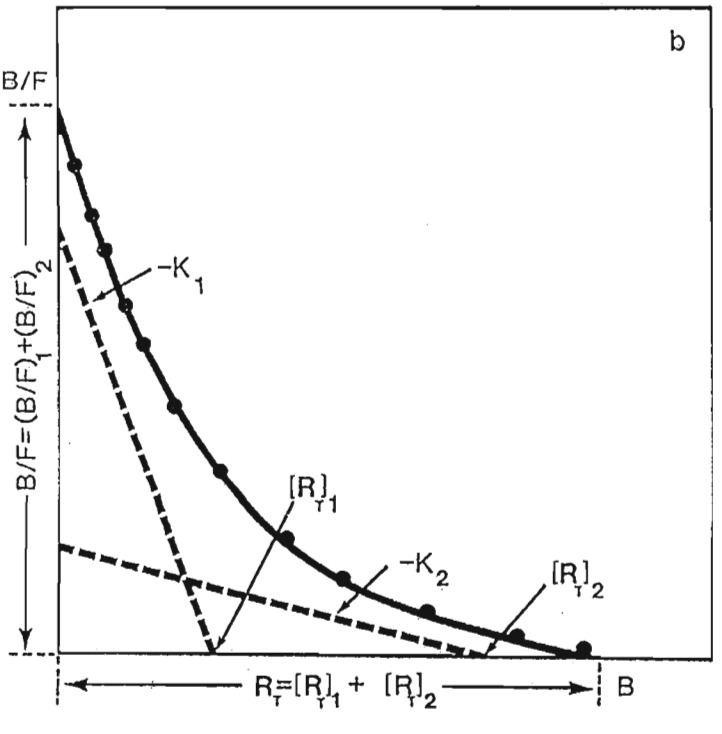


Fig. 3.1 (b) **Scatchard plot for ligand binding to two classes of independent sites** (see legend overleaf).

Fig. 3.1 (a) **Scatchard plot of ligand binding to a single class of independent sites.**
$B/_F = {}^{bound}/_{free}$ ligand,
B = molar concentration of bound ligand, K = association or equilibrium constant ($l.mol^{-1}$).
R_T = molar concentration of binding sites or binding capacity (reproduced from De Meyts and Roth, 1975).

Fig. 3.1 (b) **Scatchard plot of ligand binding to two classes of independent sites.** The curve is the sum of two subclasses of sites, one of high affinity (K_1), low capacity $(R_T)_1$, and one of low affinity (K_2), high capacity $(R_T)_2$ (reproduced from De Meyts and Roth, 1975).

Munson and Rodbard (1983) have developed a computer programme (LIGAND) which is based on non-linear or curvilinear regression. The data are analysed using a mathematical model relating bound concentration as a function of total ligand, affinity and receptor concentration. In this way the kinetic parameters which fit the experimental data can be determined. Statistical analysis of the data is done by weighted sum of squares and the "goodness-of-fit" is estimated using the analysis of variance criterion.

3.2.1 Scatchard Analysis of Insulin Binding Data on Raji Cells

In this section a Scatchard plot is applied to binding data from the insulin competition curve previously described in section 2.4.2. The same data is then analysed using the LIGAND programme to determine the binding parameters.

3.2.1.1 Scatchard Plot

Data obtained from the competition curve (Section 2.4.2) were used to determine the ratio of bound to free (B/F) and Bound (B). These parameters were calculated as follows:

$$B/_F = \frac{(\text{mean cpm bound} - \text{non-specific binding})}{(\text{Total cpm} - \text{mean cpm bound}).} \text{ per } 10^7 \text{ cells}$$

Bound concentration (B*) was calculated as:

B* (ng/mℓ) = (mean cpm bound - NSB) per 10^7 cells x Total insulin (nmol/ℓ).

A graph of B/F versus Bound (B*) nmol/ℓ was drawn for these data.

The number of receptors per cell (R_c) can be calculated by the following equation

$$R_c = \frac{\text{INSULIN BOUND (mol/}\ell\text{)} \times N}{\text{CELL CONCENTRATION PER LITRE}} \quad \ldots\ldots\ldots\ldots (8)$$

where Insulin Bound = the intercept of the curve with the abscissa and N (Avogadro's number) = $6{,}023 \times 10^{23}$ mole^{-1}.

3.2.1.2 Analysis using the Ligand Programme

Data from the competition curve were analysed using the LIGAND programme. The data were compared to binding models of increasing complexity and the best "fit" was determined by a particular subprogramme.

After each fit a table of final parameter estimates and their standard errors was printed as well as results from statistical analyses.

3.2.2 Results

A Scatchard plot for insulin binding to Raji cells is shown in figure 3.2. It is a typically curvilinear plot with upward concavity and no apparent intercept on the bound axis.

The same insulin binding data were analysed by the LIGAND programme to determine the equilibrium constants and the number of binding sites. A printout of the computer analysis is shown in Table 3.1. The first two sets of parameter estimates pertain to a single binding site and the second two sets to a two-site model. From statisti-

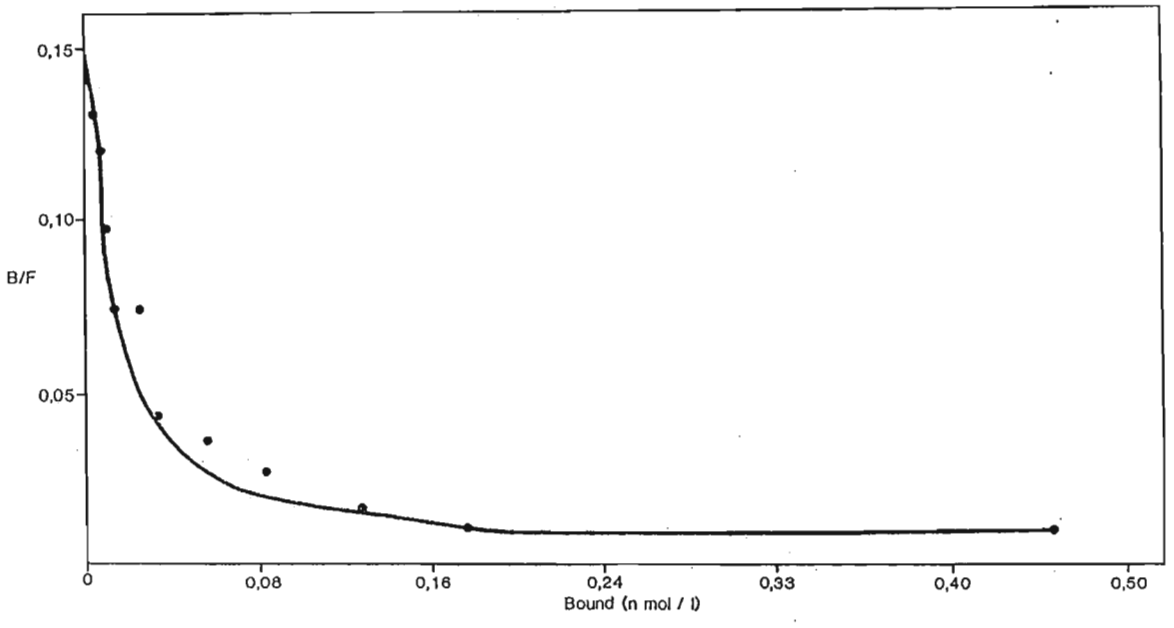


Fig. 3.2 **Scatchard plot for insulin binding to Raji cells.** Insulin binding data from the competion curve (section 2.4.2.) were used for Scatchard analysis. Each point is the mean of duplicate determinations from two experiments.

Table 3.1 Ligand analysis of insulin binding to Raji cells

Parameter estimates +/- standard error (approximate)

K11 = 0.484274E+01 +/- 0.238296E+01
K12 = 0.438509E-02 +/- 0.303955E-02
R1 = 0.137872E-01 +/- 0.469021E-02
R2 = 0.206094E+01 +/- 0.138208E+01
N1 = 0.147510E-02 +/- 0.982176E-03
C1 = 1

CURVE	SUM OF SQUARES	D.F.	MEAN SQ	F	RESIDUALS (+)	RESIDUALS (-)	RUNS
PRAC2	399.35	11	36.30432		8	8	12 (P>.05)
TOTAL	399.35	11	36.30432		8	8	

FIT	SUM SQ.	D.F.	MEAN SQ.	F	
1	4910.563	14	350.7545	41.42	(P=0)
2	1750.85	13	134.6808	18.61	(P=0)
3	463.4102	12	38.61751	1.76	(P= .164)
4	399.3475	11	36.30432	---	

Although non-specific binding (NSB) has been subtracted from the data there is still a small amount of NSB when using the two site model. Comparing the F value for the four "fits" it was apparent that the 5 parameter model (fit 4) was the appropriate one. In addition, the Runs Test was not significant ($P > 0.05$) which again supports these parameter estimates.

cal data calculated by the LIGAND programme, it was concluded that there are two binding sites for insulin with p = 0,000 indicating that there is no possiblity of a single site.

The equilibrium constants for the two sites were estimated by the LIGAND programme to be $K_{11} \sim 4{,}8 \times 10^{9}$ ℓ/mol ($K_d \sim 2{,}0 \times 10^{-10}$ mol/ℓ) and $K_{12} \sim 4{,}39 \times 10^{6}$ ℓ/mol ($K_d \sim 2{,}3 \times 10^{-7}$ mol/ℓ).

Intercepts R_1 (0,0138) and R_2 (2,061) represented the intercept on the bound axis for equilibrium constants K_{11} and K_{12}, respectively. Substituting the values for R_1 and R_2 in equation (8) it was shown that R_1 corresponds to approximately 2375 sites per cell and R_2 to about 355 000 sites per cell (Table 3.2).

3.3 *PRINCIPLE OF NEGATIVE CO-OPERATIVITY*

In some polypeptide hormones such as growth hormone and prolactin, the Scatchard plot is linear, indicating that a single homogeneous class of independent binding sites is present (Lesniak *et al.*, 1974; Shiu *et al.*, 1974). However, as was seen in the previous section, in the case of insulin a curvilinear plot with upward concavity is obtained. This phenomenon could be due to a number of factors. The best known examples of these are the presence of heterogeneous binding sites with different but fixed affinities, or interactions between homogeneous binding sites as in negative co-operativity. In the latter case the affinity of the receptors is not fixed but decreases with increasing receptor occupancy. Steady state data alone

Table 3.2. Equilibrium Constants for Insulin Binding to Raji Cells

	BINDING SITE 1	BINDING STIE 2
	Mean (Range)	Mean (Range)
Equilibrium constant (K_a) (ℓ/mol)	4,84 x 19^{9} (2,6-7,2)	4,4 x 10^{6} (1,4-7,4)
Dissociation constant (K_d) (mol/ℓ)	2,0 x 10^{-10} (1,4-1,4)	2,3 x 10^{-7} (1,35-7,2)
Number of binding sites/cell	831 (548-1114)	355000 (117200-592147)

do not discriminate between these two models, but an experimental technique to demonstrate negative co-operativity has been developed (De Meyts *et al.* (1973).

3.3.1 Experimental Demonstration of Negative Co-operativity

De Meyts *et al.* (1973) developed an original method to demonstrate the presence of site-site interactions which is based on the kinetics of dissociation of insulin from its receptor. The experimental method assumes that, in the case of negative co-operativity, the decrease in receptor affinity is partially due to an increase in the dissociation rate. This being the case, a tracer amount of ^{125}I-insulin is incubated with the receptor preparation to allow low receptor occupancy. After steady-state interaction is achieved, the cells are centrifuged and resuspended in the same volume of buffer. Portions are then removed and "infinitely diluted" (x100) in buffer (dilution only), or in buffer containing excess unlabelled insulin (dilution + insulin). At various time intervals the dissociation rate is measured in both assay systems. The rationale for the presence of excess insulin is that unoccupied sites will be filled by unlabelled insulin and the effect of receptor occupancy on the dissociation rate can be determined.

Since the interpretation of results is based on changes in dissociation rate constants, it is important that no measurable rebinding of labelled insulin occurs during the dissociation (De Meyts & Roth, 1975). The hormone-receptor complex which is diluted in the presence of insulin will have no measurable rebinding of labelled insulin

due to isotopic dilution of the dissociating labelled hormone. In the case of dilution only, no rebinding would occur if the dilution was truly infinite, as the free ^{125}I-insulin concentration would be infinitely small and hence negligible. Although 100-fold dilution is not infinite, labelled hormone concentration is initially very low and hence is reduced by a further two orders of magnitude. Indeed, De Meyts and Roth (1975) has shown that in dilutions of 50-fold and greater, no significant rebinding of free labelled hormone will occur in this experiment.

In the case of the dilution with unlabelled insulin, the unlabelled hormone will bind to the unoccupied receptor sites. This should not influence the rate at which the tracer dissociates from the occupied sites unless, of course, filling of empty sites induces interactions between receptor sites. Hence, it should be possible by an analysis of insulin binding under the stated conditions, to assess whether or not negative co-operativity between insulin receptors plays a significant role.

3.3.2 Affinity Profile : A Graphical Analysis

De Meyts and Roth (1975) developed a graphical presentation of insulin binding to its receptor which describes a single class of binding sites undergoing progressive site-site interactions with increasing occupancy.

The affinity of the binding sites is modified by site-site interactions and therefore, the affinity varies as a function of occupancy

Because the stoichiometry of this binding process is not known, the concept of the "average affinity" at each level of occupancy on the Scatchard plot was introduced (Fig. 3.3a). The changing affinity is represented by the decreasing slope of the successive lines through the intercept R on the Scatchard plot. Thus at a particular level of receptor occupancy the slope of the line joining B/F to R represents the average affinity of the receptors. However, the actual affinity of the receptor is not fixed, but is distributed between two conformations: a high affinity (K_e) or "empty sites" conformation and a low affinity (K_f) or "filled sites" conformation.

The data from the Scatchard plot in Fig. 3.3(a) can be used to plot the "average affinity" profile which gives the average affinity $\overline{K}$ as a function of the fractional saturation $\overline{Y}$ (Fig. 3.3c). The average affinity at each point is equal to (B/F)/(R -B) which can be calculated from Fig. 3.3(b). The occupancy is measured as B/R and is classically designated by Y.

From the average affinity profile four parameters $\overline{K}_e$, $\overline{K}_f$, $\overline{Y}_e$, $\overline{Y}_f$, are obtained which describe the binding system and the extent of site-site interactions.

3.3.3 Measurement of Insulin Dissociation Rates to Assess Site-Site Interactions in the Raji Cells

3.3.3.1 The Effect of Temperature

^{125}I-Insulin (3,8 x 10^{-11} mol/ℓ) was incubated with Raji cells (7,5 x 10^7 cells/mℓ) in Hepes binding buffer pH 8,0 in a total volume of 2,5

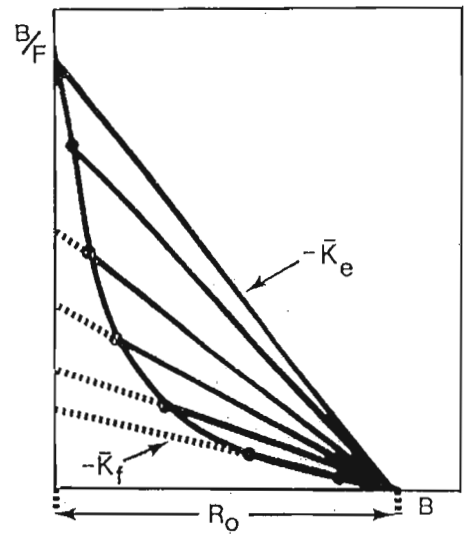


Fig. 3.3 (a) **Scatchard Plot** for ligand binding to a single class of sites with negative cooperativity. When occupancy increases the "average affinity" decreases from a limiting high affinity ($\bar{K}_e$), to a limiting low affinity ($\bar{K}_f$).

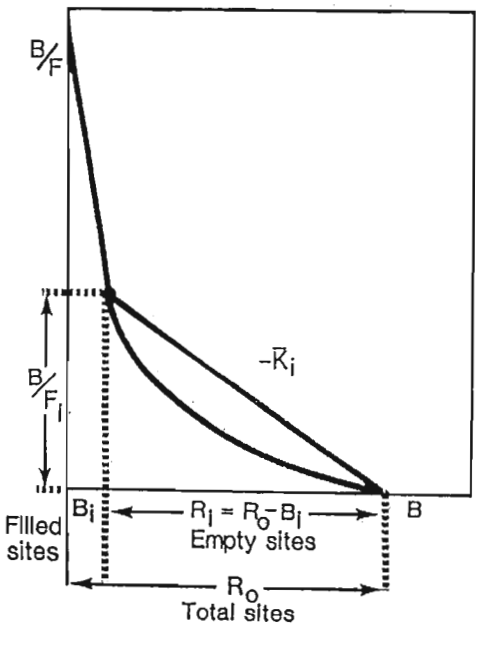


Fig. 3.3 (b) **Calculation of the average affinity** $\bar{K}$. At a given point on the curve, i, the level of bound hormone, B_i, corresponds to a given $^{bound}/_{free} = {}^{B}/F_i$.

The value of the average affinity $\bar{K}$ for that occupancy is given by the negative of the slope $-\bar{K}_i$.

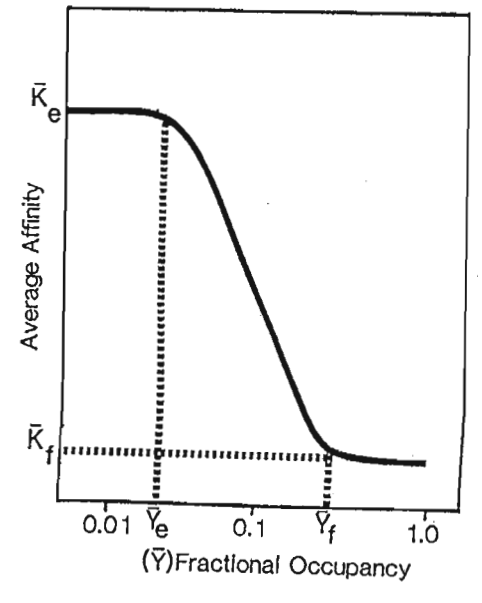


Fig. 3.3 (c) **The average affinity profile.** $\bar{K}$, the average affinity, calculated as $(^{B}/_{F})/(R_o - B)$, is plotted as a function of log Y = log $(^{B}/_{Ro})$

(See key overleaf)

Fig. 3.3 (c)

$\overline{Y}$ = 0 when all sites are empty and $\overline{Y}$ = 1 when all sites are occupied.

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

$\overline{K}_f$ = affinity of the "filled sites" conformation.

$\overline{Y}_e$ = threshold for measurable site-site interactions.

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

(Reproduced from De Meyts and Roth, 1975).

mℓ for 30 min at 15°C. After incubation the cells were centrifuged at 200 *g* for 5 min at 4°C. The supernatant was discarded and cells were resuspended in an equal volume of cold buffer (4°C). A portion (100 µℓ) of the incubation mixture was removed for measurement of bound ^{125}I-insulin ("time zero", t = o). Further portions (100 µℓ) were transferred to a series of test tubes which contained 10 mℓ of buffer at 15°C (dilution only). In a parallel assay 100 µℓ samples were added to 10 mℓ of buffer containing 1,7 x 10^{-7} mol/ℓ unlabelled insulin. At various time intervals duplicate tubes from each set were removed and centrifuged at 200 *g* at 4°C. The cell pellet was counted in a gamma counter and radioactivity was expressed as a percentage of the radioactivity present at zero time. This was then plotted against dissociation time.

To assess the effect of temperature on the rate of insulin dissociation in the presence and absence of unlabelled insulin, the same procedure was carried out at 37°C and 4°C.

3.3.3.2 Receptor Occupancy During Dissociation

A more direct measurement of the effect of initial receptor occupancy on the dissociation of bound ^{125}I-insulin was carried out according to the method of Pollet *et al.* (1977).

The same procedure was followed using 5 x 10^{-9} mol/ℓ ^{125}I-insulin to give high receptor occupancy and 5 x 10^{-11} mol/ℓ ^{125}I-insulin for low receptor occupancy. Dissociation of bound labelled hormone was measured at 15°C in the presence or absence of 5 x 10^{-9} mol/ℓ unlabelled insulin. This concentration was chosen so that during dissocia-

tion, the receptor occupancy remained the same (5×10^{-9} mol/ℓ insulin) or increased from 5×10^{-11} mol/ℓ to 5×10^{-9} mol/ℓ insulin.

3.3.4 Results

3.3.4.1 Dissociation Experiments

Dissociation of unlabelled insulin from the receptor at different temperatures is shown in Fig. 3.4. At 4°C, the dissociation rate appeared to follow first order kinetics although only 20% of bound ^{125}I-insulin dissociated in 120 min. The dissociation rate at 15°C was slightly increased as 40% dissociated in 120 min. However, at this temperature the dissociation rate was no longer a first order reaction. When the temperature was increased to 37°C there was a marked acceleration in the dissociation rate (nearly 70% dissociated in 120 min) and the dissociation curve became multiexponential.

A comparison of the dissociation rate in the presence or absence of unlabelled insulin at the same temperatures is given in Figs. 3.5a, b and c. In all three experiments the rate of dissociation was increased in the presence of excess unlabelled insulin compared to dilution only. At 4°C (Fig. 3.5a) the amount of dissociated ^{125}I-insulin increased from 20% to nearly 50%. A similar increase from 40% to 70% was found at 15°C (Fig. 3.5b). However, at 37°C there was only a 5 to 10% increase in the dissociation rate in the presence of excess insulin (Fig. 3.5c).

The effect of increased receptor occupancy on the dissociation rate is shown in Fig. 3.6. The dissociation curves in the absence of

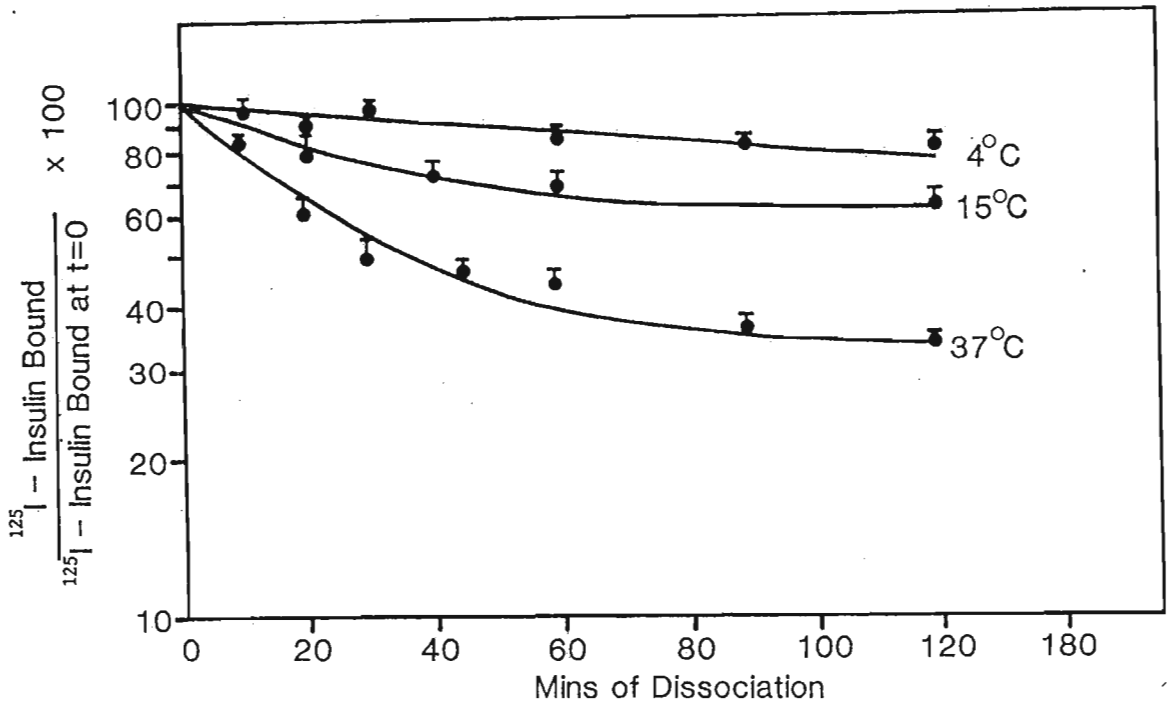


Fig. 3.4 **Dissociation of membrane bound ^{125}I-insulin at different temperatures.** After ^{125}I-insulin was bound to the receptor, the dissociation rate of labelled ligand was measured at 4°C, 15°C and 37°C. All points are mean values of duplicate measurements with one S.D.

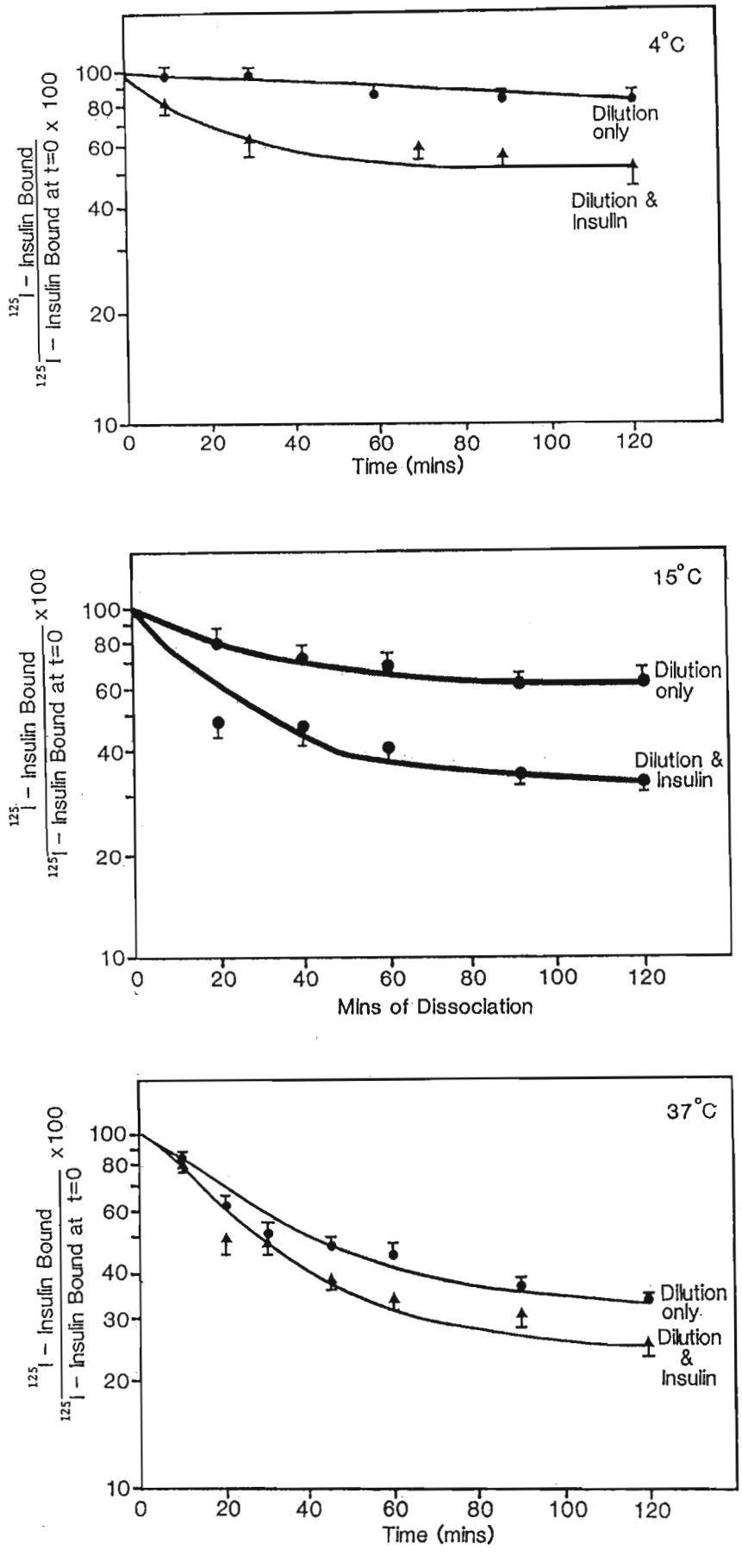


Fig. 3.5 The effect of unlabelled insulin on the dissociation of ^{125}I-insulin at 4° C, 15°C and 37° C.(see legend overleaf).

Fig. 3.5 **The effect of unlabelled insulin on the dissociation of ^{125}I-insulin at 4° C, 15°C and 37°C.** In these experiments the dissociation of radiolabelled insulin was measured in the absence (dilution only) or presence (dilution + insulin) of $1,7 \times 10^{-7}$ mol/l unlabelled insulin. Each point is the mean of duplicate determinations and one S.D.

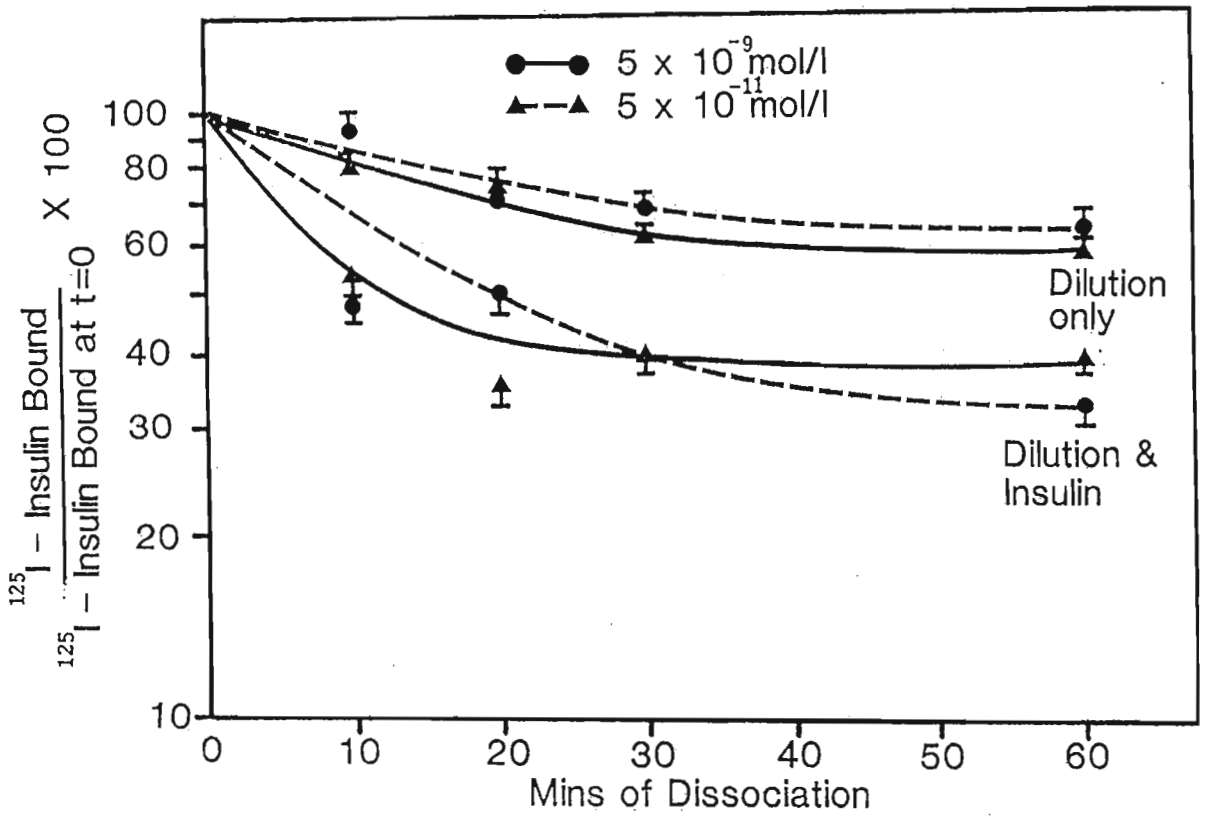


Fig. 3.6 **Receptor occupancy does not affect dissociation of bound ^{125}I-insulin.** The dissociation of ^{125}I-insulin (5×10^{-11} and 5×10^{-9} mol/l) was measured in the absence of unlabelled insulin (dilution only) and in the presence of 5×10^{-9} mol/l unlabelled insulin. This concentration of insulin resulted in an increase in receptor occupancy (5×10^{-11} to 5×10^{-9} mol/l insulin) or constant receptor occupancy (5×10^{-9} mol/l insulin). All values represent the mean and one S.D. of duplicate measurements.

unlabelled insulin (dilution only) are the same for both high and low binding site occupancy (5×10^{-9} mol/ℓ and 5×10^{-11} mol/ℓ ^{125}I-insulin, respectively).

In the presence of 5×10^{-9} mol/ℓ unlabelled insulin the dissociation rate increased equally for both concentrations of ^{125}I-insulin. At high receptor occupancy (5×10^{-9} mol/ℓ ^{125}I-insulin) the dissociation of labelled hormone was increased in the presence of unlabelled insulin although the receptor occupancy remained the same.

3.3.4.2 Graphical Analysis

A plot of the average affinity versus fractional occupation for the Raji cells is given in Fig. 3.7. The high affinity sites (K_e) represented 1 to 1,3% of the total number of receptors and this percentage of receptors represented the threshold for measurable site-site interactions (Υ_e). After 30 to 35% receptor occupancy, the site-site interactions were maximal and a limiting low affinity (K_f) was obtained.

3.4 DISCUSSION

Curvilinear regression analysis using the LIGAND programme, showed that insulin binding data on Raji cells fits a two-site model. A relatively small number of sites bind insulin with a high affinity, whereas the majority of the binding sites appear to have a lower affinity for insulin.

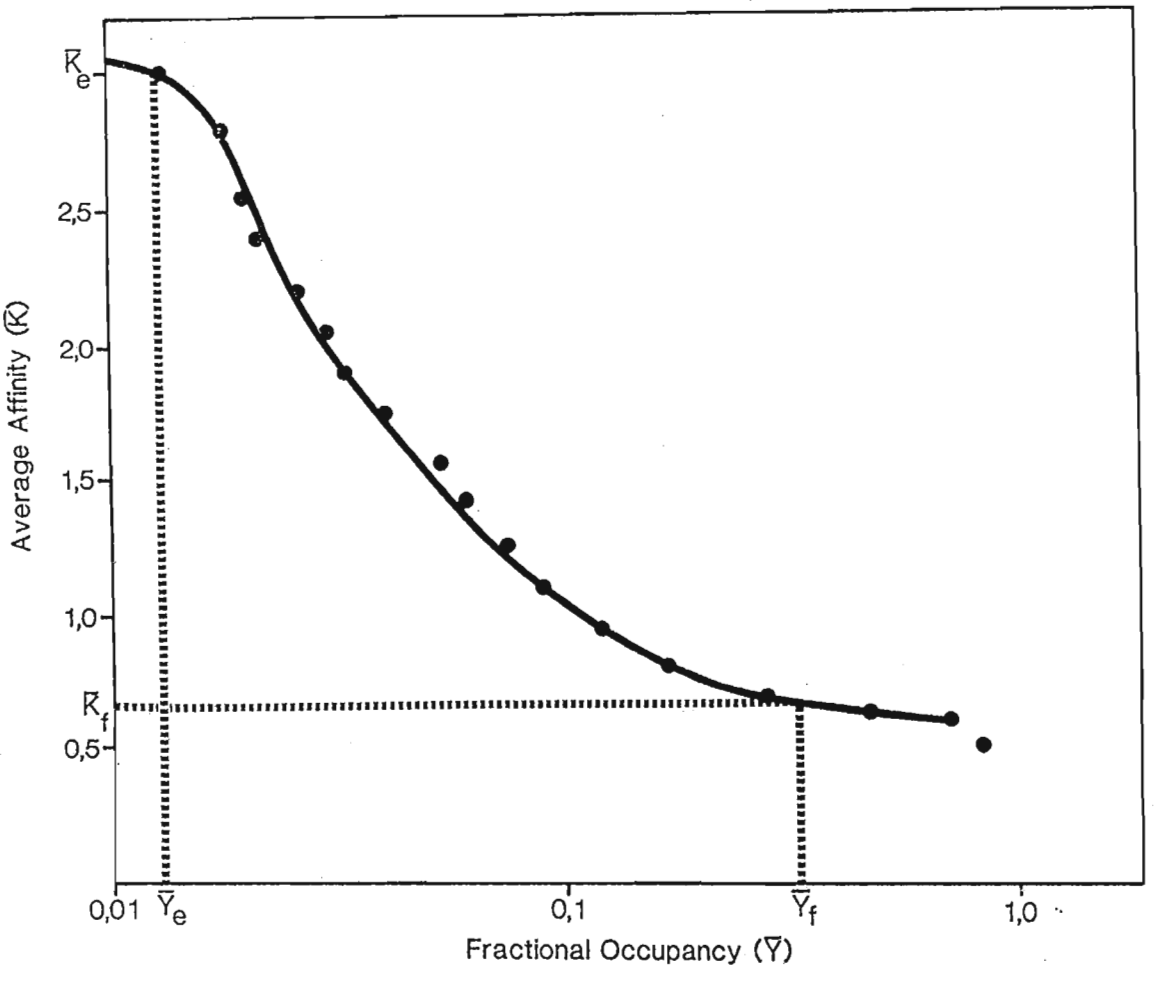


Fig. 3.7 **The average affinity profile of insulin binding to Raji cells.** K_e = affinity of the "empty sites" conformation, K_f = affinity of the "filled sites" conformation, Y_e = threshold for measurable site-site and Y_f = fractional occupancy at which site-site interactions are maximal. This plot is computed from the Scatched plot and therefore the parameters used to describe insulin binding are further removed from the primary data.

The K_d for the high affinity insulin binding site on Raji cells was similar to published data on liver membranes, isolated adipocytes and IM-9 lymphocytes (Hammond *et al.*, 1972; Kahn *et al.*, 1974 and Pollet *et al.*, 1977). However, the lower affinity K_d was about two orders of magnitude greater than published data on the same tissues, while the total number of sites per cell was ten times higher. The reason for this discrepancy was, probably, because binding data for the Raji cells was determined over a wider range of insulin concentrations which enabled more accurate determination of these constants (Klotz, 1982; Light, 1984). Using a novel method for determining binding constants, Pang and Schafer (1984) have demonstrated high affinity (K_d ~6 x 10^{-10} mol/ℓ) and low affinity dissociation constants (K_d ~0,8 x 10^{-7} mol/ℓ) for purified insulin receptors from human placenta which are similar to those reported here for Raji cells.

Dissociation of insulin from its receptor at 4°C followed first order kinetics suggesting that at low temperatures the conformation of binding sites is homogeneous. At higher temperatures however, dissociation was multi-exponential and implied heterogeneity of binding sites or interaction between binding sites. De Meyts *et al.* (1976) have argued that first order dissociation at 4°C precludes pre-existing heterogeneity of binding sites and favours site-site interactions.

Experimental evidence from the Raji cells showed that the rate of ^{125}I-insulin dissociation from occupied sites was increased by filling empty receptor sites with unlabelled insulin. Acceleration of

the dissociation rate by increasing receptor occupancy has been interpreted as evidence for site-site interactions of a negatively co-operative nature (De Meyts *et al.*, 1973).

Based on this phenomenon, De Meyts *et al*, 1976 proposed a model in which the receptor sites can exist reversibly in either a slow dissociating or a fast dissociating conformational state. As the number of occupied sites increases they are able to undergo a transition from the slow dissociating state to a fast dissociating state. This transition is also facilitated by temperature.

In Raji cells the difference between the dissociation rate of bound ^{125}I-insulin at low receptor occupancy (dilution only) and when the sites are filled (dilution + insulin) is decreased at 37°C compared to 15°C and 4°C. This implies that the interaction between receptors is decreased at physiological temperatures. However, it has been suggested that at low receptor occupancy the increase in temperature represents the energy necessary to shift receptors from the slow dissociating state to the fast dissociating state. The nature of this transition from one state to another is considered to be a conformation change in individual receptor subunits or clustering of receptors in the fluid membrane, or a combination of both (De Meyts *et al*. 1976). Support for the existence of different conformational states has recently been demonstrated with monoclonal antibodies to the insulin receptor. Two monoclonal antibodies which compete with insulin for the same binding site increase the rate of dissociation of bound ligand and appear to stabilize the low affinity fast dissociating conformation state (Forsayeth *et al*. 1987). On the other

hand, two different monoclonal antibodies which precipitate the receptor but do not compete with insulin seem to favour the high affinity slowly dissociating state of the insulin receptor (Gu *et al.* 1988).

The interpretation of the kinetics of insulin binding to its receptor on the basis of negative co-operativity was challenged by Pollet *et al.* (1977). These authors demonstrated that the dissociation rate of ^{125}I-insulin from IM-9 lymphocytes was not dependent on receptor occupancy. Results of the current studies on the Raji cells have shown that the increase in the dissociation rate is unchanged at high receptor occupancy and when receptor occupancy is kept constant. Thus, the increased dissociation rate in these cells is not directly related to receptor occupancy.

To date, the experimental methods of differentiating between the negative co-operativity model and the two-site binding model have been inconclusive.

Recently a new receptor model was proposed based on experimental evidence from double probe labelling (Pang & Schafer, 1984). It was found that a single receptor unit bound two insulin molecules but, the two binding sites had markedly different affinities for insulin.

Negatively co-operative interactions between the two binding sites could account for the observed increase in dissociation of bound insulin in the presence of unlabelled insulin. Thus, the curvilinear nature of the Scatchard plot might result from both receptor hetero-

geneity and negative co-operativity which are intrinsic to the insulin receptor structure.

As discussed earlier (section 1.5.2), structural studies on the insulin receptor have shown that the membrane receptor is bivalent. This would enable the receptor to bind two insulin molecules. These observations appear to support the insulin receptor model proposed by Pang & Schafer (1984).

If the insulin receptor is bivalent and these two binding sites interact with one another, then two of the assumptions made in section 3.2 are no longer valid. Consequently, the binding constants derived from Scatchard analysis would have no precise physico-chemical meaning. Despite the apparent ambiguities in this receptor model, these binding constants have been useful in comparative studies on different tissues and in disease states where insulin binding to its receptor is impaired.

Clearly, the LIGAND programme provides a sophisticated tool for analysing insulin binding data. Although the kinetic and structural data suggest that the traditional mathematical models are inadequate for analysing insulin binding, the LIGAND programme has the advantage of giving consistent results on kinetic studies.

Analysis of the kinetics of insulin binding to whole cells is further complicated by the presence of insulin-like growth factor receptors which appear to bind insulin with a lower affinity. The presence of type I IGF receptors are investigated in detail in Chapter 7.

3.5 CONCLUSION

Quantitative analysis of insulin binding data on the Raji cells suggested the presence of heterogeneous sites with similar kinetic constants to those reported by Pang & Schafer (1984).

However, the average affinity profile for insulin binding to Raji cells was similar to data reported by De Meyts & Roth (1975) in support of the negative co-operativity model. The rate of dissociation was increased in the presence of insulin, although this did not appear to be dependent on receptor occupancy. In addition, there was some evidence that increased temperature induced a change in the conformation of the receptor.

From the information presented here the insulin receptor on the Raji cells is best described by the model proposed by Pang & Schafer (1984). This model incorporates both receptor heterogeneity and site -site interactions which could induce different conformational states.

CHAPTER 4

ENDOCYTOSIS OF INSULIN AND THE MEMBRANE RECEPTOR IN RAJI CELLS

All organic beings originate from and consist of vesicles or cells. These, when detached, and regarded in their original process of production, are ***the infusorial mass or primeval slime*** *whence all larger organisms fashion themselves or are evolved.*

-OKEN, 1805.

4.1 INTRODUCTION

Having established that the kinetics of insulin binding to Raji cells is similar to published data on both target tissue and non-target tissue, the functional aspects of insulin binding to its receptor were investigated.

As mentioned in section 1.6.2, morphological studies have shown that once insulin has bound to its receptor, the insulin-receptor complex is internalised by the process of endocytosis. The internalised complex is then associated with a number of intracellular vesicles; dissociation of insulin from the receptor probably occurs in the endosome. Carpentier *et al.* (1986) have suggested that this dissociation might be the first step in receptor recycling and, in some cell types, the dissociated insulin is subsequently degraded intracellularly in the lysosomes. At present, it appears that endocytosis of the insulin-receptor complex is a mechanism to remove the hormone from the cell surface and terminate its signal. Impor-

tant functional consequences of this process would appear to be hormone internalisation followed by a loss of membrane receptors, a process known as downregulation.

In this chapter, internalisation of radiolabelled insulin and insulin-induced receptor downregulation have been studied in the Raji cell, using well established biochemical techniques.

4.2 *MEASUREMENT OF ^{125}I-INSULIN INTERNALISATION BY RAJI CELLS*

4.2.1 Principle of the Experimental Method

Previous studies have demonstrated that mild acid treatment of cells can be used to rapidly strip off about 90% of cell surface receptor-bound hormone while removing less than 10% of the internalised hormone (Haigler *et al.*, 1980; Shimizu *et al.*, 1981). In this experiment, internalisation of insulin into the Raji cells is demonstrated by exploiting this simple finding. Cells incubated with ^{125}I-insulin are harvested after various time intervals and the relative amounts of ^{125}I-insulin remaining in the cell after acid treatment or similar treatment in buffer can be measured. Hence, the relative amount of ^{125}I-insulin internalised by the cell can be calculated. By carrying out the experiments at various temperatures it is possible to reach conclusions concerning the energy dependence of the internalisation process.

4.2.2 Materials and Methods

In this experiment Raji cells were grown as previously described in section 2.2.2.2 and were used during the stationary phase of growth at a density of 1-2 x 10^6 cells/mℓ. The chemical composition of Hepes binding buffer (HBB) was previously described in section 2.2.3.1. A mild acetic acid solution (MAAS) consisting of 0,2 mol/ℓ acetic acid (pH 2,5) and 0,5 mol/ℓ NaCl was used to remove cell-surface bound ^{125}I-insulin.

4.2.3 Procedure

After washing and resuspending the cells in HBB, 400 µℓ portions were incubated in a series of test tubes with 0,6 ng/mℓ (1 x 10^{-10} mol/ℓ)^{125}I-insulin in 50 µℓ, in the presence or absence of 10^5 ng/mℓ (1 x 10^{-5} mol/ℓ) of unlabelled insulin. Incubations were carried out at 37°C, 15°C and 4°C.

At various time intervals, duplicate 200 µℓ portions of the incubation mixture were removed and layered onto an equal volume of HBB at 4°C, followed by centrifugation at 200 *g* for 5 min. The cells were washed three times with 500 µℓ of HBB and were then treated with 700 µℓ of MAAS at 4°C. After 6 min the cells were centrifuged at 200 *g* for 5 min. The supernatant was discarded and the cell pellet was washed with another 500 µℓ MAAS. Once again the supernatant was discarded and the cell pellet was counted in a Berthold gamma counter as described in section 2.2.3.2.

A control study was carried out in a parallel series of test tubes, with HBB being substituted for MAAS. The amount of radiolabelled insulin which was internalised (MAAS treated) was expressed as a percentage of insulin associated with HBB treated cells and presented graphically by plotting against time.

4.2.4 Results

A time course of ^{125}I-insulin internalisation by Raji cells at 37°C 15°C and 4°C is presented in fig. 4.1. At all three temperatures ^{125}I-insulin was internalised most rapidly in the first 30 min and thereafter reached a plateau. However, at 37°C approximately 90% of the ^{125}I-insulin was taken up by the cells, whereas at 15°C and 4°C only 40-50% of the radiolabelled hormone was internalised.

4.3 *MEASUREMENT OF INSULIN-INDUCED LOSS OF RECEPTORS (DOWNREGULATION)*

4.3.1 Principle of the Experimental Method

Raji cells are pre-incubated with unlabelled insulin in the first instance in order to downregulate the number of insulin binding sites. Following on this, insulin which is bound to the receptor is removed by incubating cells in a PBS buffer pH 6 at 30°C. This procedure promotes dissociation of endogenous insulin bound to the receptor without damaging the membrane receptor (Kosmakos & Roth, 1980). This mild treatment which promotes dissociation of receptor bound unlabelled hormone, will enable subsequent measurement of

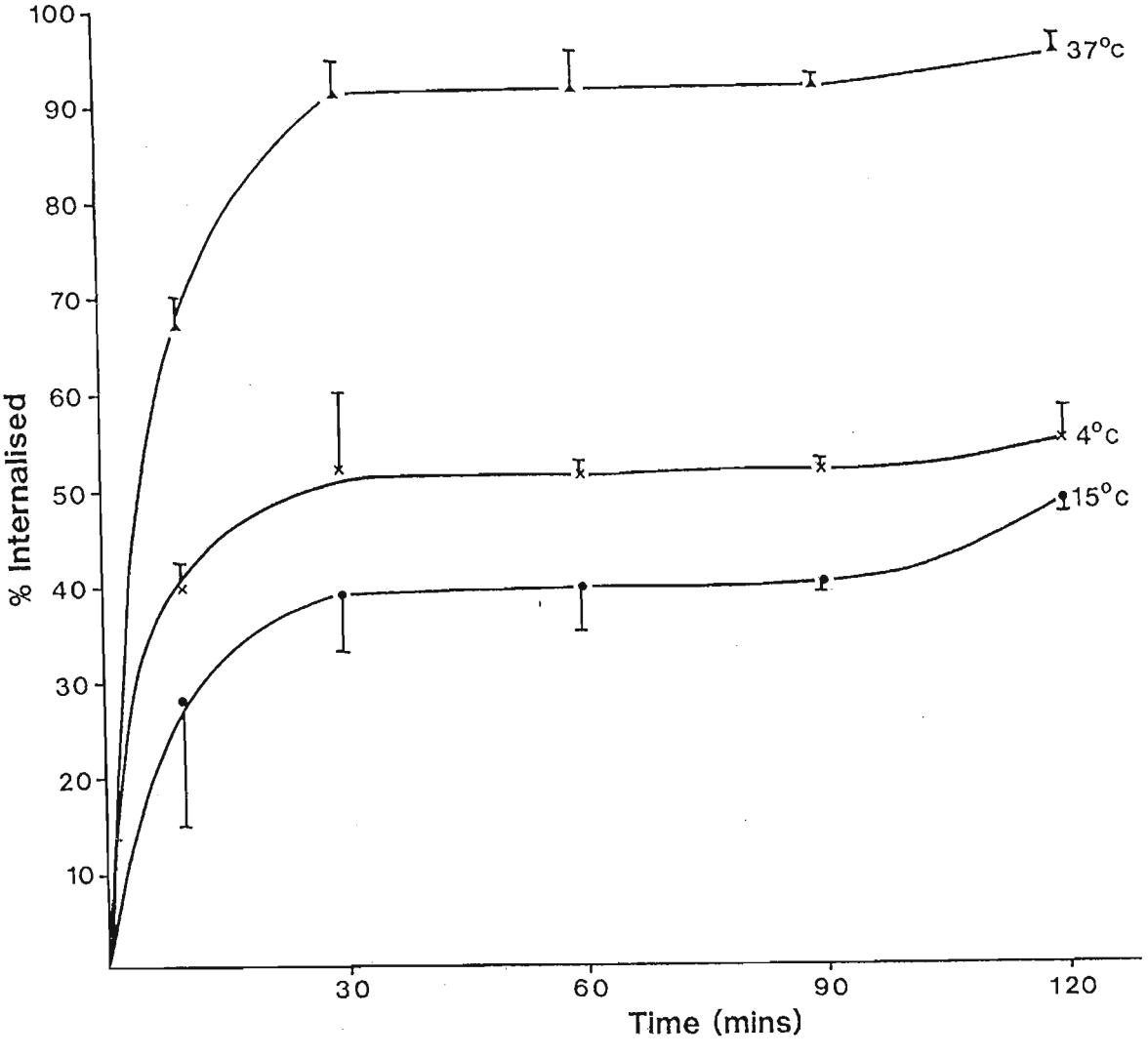


Fig. 4.1 **The effect of temperature on internalisation of membrane bound insulin.** At specific time intervals the amount of acid resistant cell-associated ^{125}I-insulin was determined as a percentage of bound radiolabelled hormone. Each point represents the mean and one S.D. of duplicate measurements from two similar experiments.

unoccupied receptor sites remaining on the cell surface. Insulin binding studies are then carried out in the normal way as described in section 2.2.3 and the results compared with Raji cells which were carried through the same procedure, but not exposed to insulin. A diagramatic presentation of the principle on which the procedures are based is set out in Fig. 4.2.

4.3.2 Materials and Methods

As previously mentioned in section 2.2.2.2, Raji cells were used during stationary growth phase. All procedures, except the insulin binding assay, were carried out under sterile conditions. The composition of HBB was previously described in section 2.2.3.1. Phosphate buffered saline (PBS) pH 6,0 contained 0,07 mol/ℓ NaH_2PO_4, 0,07 mol/ℓ Na_2HPO_4, 0,325 mol/ℓ NaCl and 0,1% BSA.

4.3.3 Procedure

Raji cells were removed from RPMI-1640 medium by centrifugation at 200 *g* for 5 min and then resuspended in an equal volume of fresh growth medium. Equal portions (60 mℓ) were transferred to three culture flasks and insulin was added to two of the flasks at concentrations of 1×10^{-8} mol/ℓ and 1×10^{-6} mol/ℓ. The third flask, without added insulin, served as a control. Series of flasks were incubated for varying time intervals ranging from 0-24 hours at 37°C in an atmosphere of 5% CO_2 and 95% air. On completion of the incubation period, 5 mℓ portions of cells were pelleted by centrifugation at 200 *g* for 5 min. To dissociate endogenous insulin bound

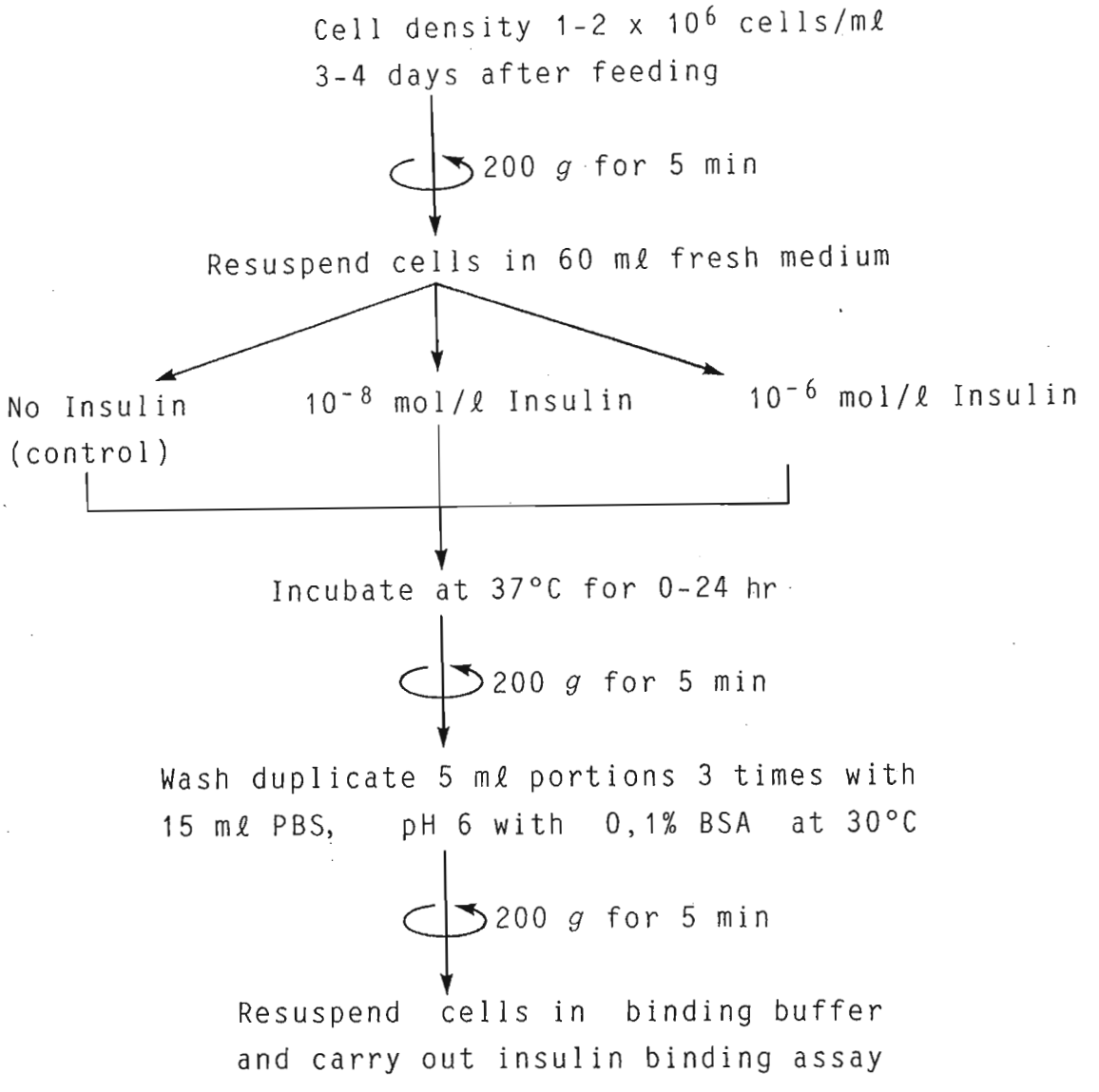


Fig. 4.2 The experimental procedure for measurement of down-regulation of insulin receptors by addition of exogenous insulin to the Raji cell medium

to the receptors, cells were washed in 15 mℓ PBS pH 6 at 30°C and then centrifuged at 200 *g* for 5 min. Cells were then incubated for 25 min at 30°C in the same volume of PBS and re-centrifuged. This procedure was carried out twice. After the washing procedure cells were resuspended in 5 mℓ HBB and the insulin binding assay was carried out as described in section 2.2.3.

4.3.4 Calculations

The amount of insulin specifically bound to the cells immediately before addition of exogenous insulin (zero time, t = 0) was taken as maximum specific insulin binding. The results of insulin binding after pre-incubation of cells with insulin at various time intervals (t = n) were expressed as a percentage of this value.

$$\frac{\text{cpm-NSB at } t = n \times 100}{\text{cpm-NSB at } t = 0.}$$

These results are presented graphically as a plot of % ^{125}I-insulin bound versus time.

4.3.5 Results

Fig. 4.3a shows the dose-response of pre-incubating Raji cells with unlabelled insulin and subsequent measurement of specific insulin binding. At t = 0, 7,85% ± 0.24 of ^{125}I-insulin was specifically bound to the cells. After only two hours pre-incubation there was about a 15% decrease in receptors in the control. By comparison, pre-incubation of cells with 1×10^{-8} mol/ℓ and 1×10^{-6} mol/ℓ insulin for 2 hours resulted in a receptor loss of 40-50% and 75%, respectively.

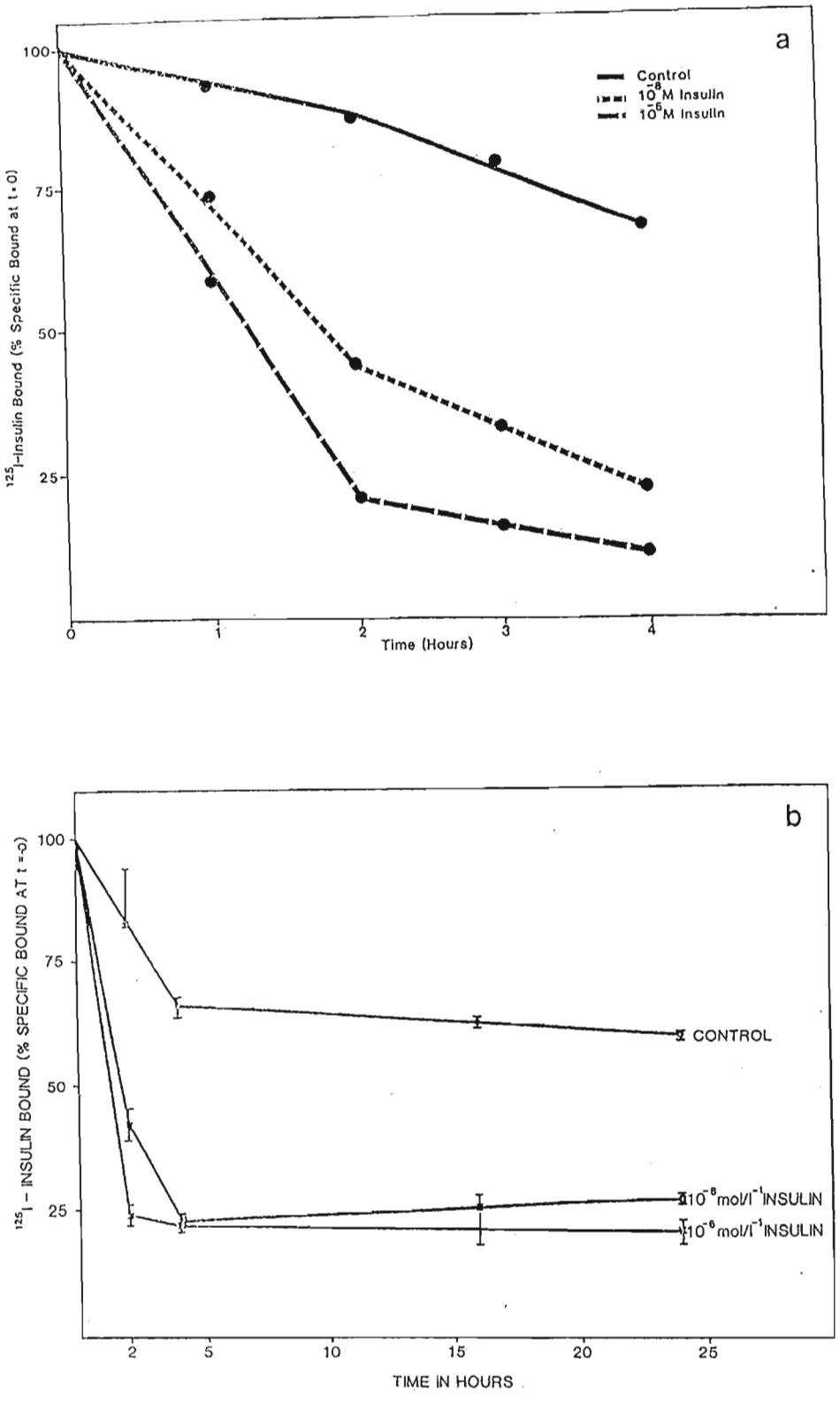


Fig. 4.3 (a) Loss of membrane receptors after 1-4 hours incubation with exogenous insulin.

(b) Downregulation after 1-24 hours pre-incubation with exogenous insulin (see legend overleaf).

Fig. 4.3 **Loss of membrance receptors after 1-4 hours (a) and 1-24 hours (b) pre-incubation with exogenous insulin.** The experimental details have been outlined in detail in fig. 4.2. All points in (a) are the mean of duplicate determinations. In (b) each point is the mean and one S.D. of two separate experiments.

In all cases receptor loss reached a nadir after about 4 hours and this level was maintained for 24 hours. During this period receptor loss in the control was 35-40%. For this same period, both 1×10^{-8} mol/ℓ and 1×10^{-6} mol/ℓ insulin induced an 80% loss in receptors. There was no significant difference in receptor decrease between these insulin concentrations (Fig. 4.3b).

4.4 *DISCUSSION*

Results of the internalisation studies showed that after binding to the receptor, insulin was rapidly taken up by the Raji cells. The initial rate of insulin internalisation was time-dependent. However, after 30 min a steady state was reached. Establishment of an equilibrium between insulin binding and internalisation after 30 min was common to all three temperatures studied. At the more physiological temperature the initial rate of internalisation was increased compared to 4°C and 15°C. In addition, the amount of insulin internalised at 37°C was greater than that at lower temperatures. By contrast, there was no apparent difference in the rate and amount of insulin internalised at 4°C and 15°C. These observations suggest that the movement of insulin into the cell is not via simple diffusion, but by an energy-dependent, metabolic process. This is in agreement with previous studies which have shown that endocytosis is inhibited by treatment of cells with metabolic poisons which decrease cellular ATP levels (Steinmann *et al.*, 1974).

The mechanism of insulin internalisation in the Raji cells is probably receptor-mediated. Indirect evidence of this mechanism is that pre-incubation of Raji cells with insulin, induced an 80% membrane receptor loss after 24hr while a loss of only 30-40% of membrane receptors was shown in the control after the same period. This decrease of receptors in the control study was probably due to the presence of insulin in FCS in the fresh medium which was added to the cells at the beginning of this experiment (about $3,5 \times 10^{-10}$ mol /ℓ insulin). Thus the control experiment may, in fact, be interpreted if not entirely, then certainly in part, as an indication of the receptor loss induced by $3,5 \times 10^{-10}$ mol/ℓ insulin.

In both the control incubations, and in cells exposed to exogenous insulin, there was an initial rapid loss in the number of receptors until a new steady state set in. The initial rate of receptor loss was directly related to the concentration of exogenous insulin. In addition, after 4 hrs the new state of equilibrium between insulin binding and receptor concentration was higher in the control incubation than in the incubations in which insulin was added, which further suggests that receptor loss is related to insulin levels. However, after 4 hrs pre-incubation there appeared to be a nadir steady state at 10^{-8} mol/ℓ insulin beyond which increasing concentrations of insulin did not decrease receptor number. Despite a 100-fold increase in insulin concentration (10^{-6} mol/ℓ), receptor number did not decrease further than 70-80%. These findings are consistent with the results of other workers who have shown that 18 hr exposure of human adipocytes and monocytes to physiological concentrations of insulin (10^{-10} - 10^{-9} mol/ℓ) induced an 80% de-

crease in receptor numbers (Schimke, 1975; Bar *et al.*, 1976). In cultured human lymphocytes (IM-9), a similar decrease in insulin receptors was induced by 10^{-6} mol/ℓ insulin (Kosmakos & Roth, 1980). It would seem Raji cells are slightly less sensitive to insulin than adipocytes and monocytes, but are more sensitive than cultured human lymphocytes (IM-9).

Although these experiments have shown that both the hormone and receptor were initially internalised in a time-dependent manner, both processes eventually reached saturation and a new steady state was established.

So far it has been difficult to establish a direct relationship between hormone internalisation and receptor downregulation as it has been shown that the receptor can rapidly be re-cycled back to the cell membrane (Carpentier *et al.*, 1984). It can be speculated that the remaining 20-30% insulin binding which was not lost following pre-incubation with high concentrations of insulin represents the amount of receptor which was recycled.

As both the hormone and receptor are internalised, it is clear that this process plays an important role in removing insulin from the cell surface and regulating the membrane receptor concentration. Since this mechanism of receptor regulation has been demonstrated in the Raji cells it would seem that the virus-induced insulin receptor is functionally competent in the physiological sense. In addition, downregulation of the receptor occurred at relatively low insulin levels which suggests that this cell line is sensitive to physiological insulin concentrations.

CHAPTER 5

INSULIN DEGRADATION BY RAJI CELLS

"The biochemist strives to obtain a mental picture of that invisible dance and transformation of molecules which in every organ or tissue underlies its activities."
-HOPKINS, 1929

5.1 *INTRODUCTION*

In Chapter 1 (section 1.6.2.2) the significance of intracellular insulin degradation was discussed, and in the previous chapter, the process of insulin internalisation was demonstrated in Raji cells. At present, receptor-mediated degradation of insulin is thought to be the primary physiological process for termination of insulin action. However, measurement of this pathway in an *in vitro* system such as a cultured cell line, is not straightforward. At least three sites of insulin degradation have been identified. Firstly, non-specific degradation of insulin by extracellular proteases in the cell medium (Sonne & Gliemann, 1980). Secondly, degradation of insulin by specific cell surface proteases (Yokono *et al.*, 1982) and thirdly, as mentioned previously in section 1.6.2.2, insulin can be internalised by the cell and eventually be degraded intracellularly in the lysosomes. In the latter case, the degradative products of lysosomal insulin degradation are rapidly extruded by the cell into the surrounding medium.

As the proteolytic products of all three degradative pathways are found in the extracellular medium it is difficult to distinguish the contribution of each process to overall degradation.

In this chapter, all three mechanisms of ^{125}I-insulin degradation by Raji cells have been investigated, using a series of independent biochemical techniques.

5.2 *DEGRADATION OF ^{125}I-INSULIN BY EXTRACELLULAR PROTEASES*

5.2.1 Principle of the Method

Studies on ^{125}I-insulin degradation by extracellular proteases were carried out according to the methods of Sonne & Gliemann (1980). The amount of labelled insulin degraded after incubation in the medium was determined by precipitation of incubation medium with cold trichloroacetic acid (TCA). In dilute solution, TCA denatures and precipitates both large proteins and short oligopeptides. As free amino acids are soluble in TCA, the measurement of the degradative product iodotyrosyl, can be used to quantitate insulin degradation. A schematic presentation of the experimental procedure is given in figure 5.1.

5.2.2 Materials and Methods

HBB was previously described in section 2.2.3.1. Radiolabelled insulin and unlabelled insulin were described earlier (section 2.2.1).

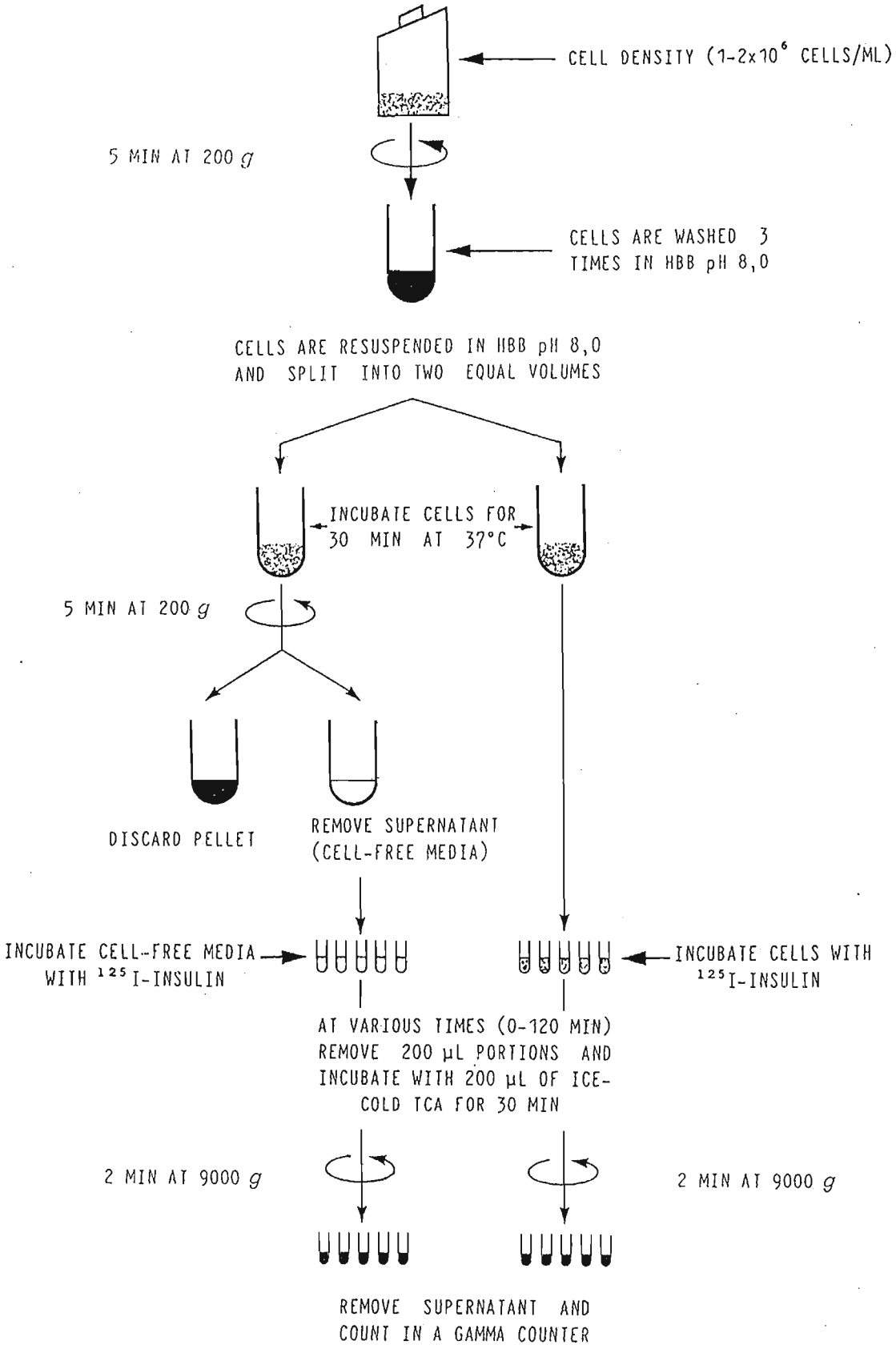


Fig. 5.1 **The experimental procedure for measuring ^{125}I-insulin degradation by extracellular proteases compared to cellular degradation.**

5.2.3 Procedure

Raji cells (1×10^7 cells/mℓ) were washed three times and then re-suspended in HBB pH 8,0 as detailed in section 2.2.3.2. Two equal volumes (60 mℓ) of cells were then pre-incubated at 37°C for 30 min. In order to determine whether proteases were released by the cells into the surrounding medium during this 30 min incubation, cells from one of the incubation mixtures were removed by centrifugation at 200 *g* for 5 min. Portions (400 µℓ) of the cell-free medium were then incubated with 0,6 ng/mℓ ^{125}I-insulin (1×10^{-10} mol/ℓ) in the presence or absence of 10 ng/mℓ unlabelled insulin. In a parallel series, 400 µℓ portions of the second incubation mixture containing cells were incubated with ^{125}I-insulin. This cell incubate represented total insulin degradation from all three sites previously described (section 5.1). By comparing the degradation of ^{125}I-insulin in these two incubates, it was possible to determine the relative contribution of extracellular proteases to total degradation.

After various time intervals, duplicate portions (200 µℓ) from each incubation mixture were removed and added to an equal volume of ice-cold 25% TCA. Samples were kept on ice and then centrifuged at 4°C in a Beckman microfuge. The supernatant was removed and radioactivity in both the supernatant and the pellet was counted in the Berthold gamma counter.

The amount of ^{125}I-insulin which was soluble in TCA was expressed as a percentage of total counts in the incubation mixture. Degradation

of ^{125}I-insulin in the presence of a large excess of unlabelled insulin was taken as non-specific degradation.

This experiment was repeated at 15°C to determine the amount of degradation which occurs during the steady state binding assay (section 2.4.2).

5.2.4 Results

Degradation of ^{125}I-insulin after 120 min at 37°C in the presence of cells and in the cell-free medium reached 9,5% and 14%, respectively (Fig. 5.2a). At 15°C (Fig 5.2b) cell degradation of insulin decreased to 4,5% and degradation of insulin by cell-free medium was 3%. Thus, compared to 37°C, the amount of insulin degraded by the cells at 15°C was greater than that degraded by the extracellular proteases.

5.3 *RECEPTOR-MEDIATED DEGRADATION OF ^{125}I-INSULIN*

5.3.1 Principle of the Method

The possible correlation between ^{125}I-insulin binding to the receptor and degradation of labelled hormone by the Raji cells was investigated using the method of Taylor SI,(personal communication).

This technique relies on the consideration that, if insulin binding and its subsequent degradation are related, then the degradation of

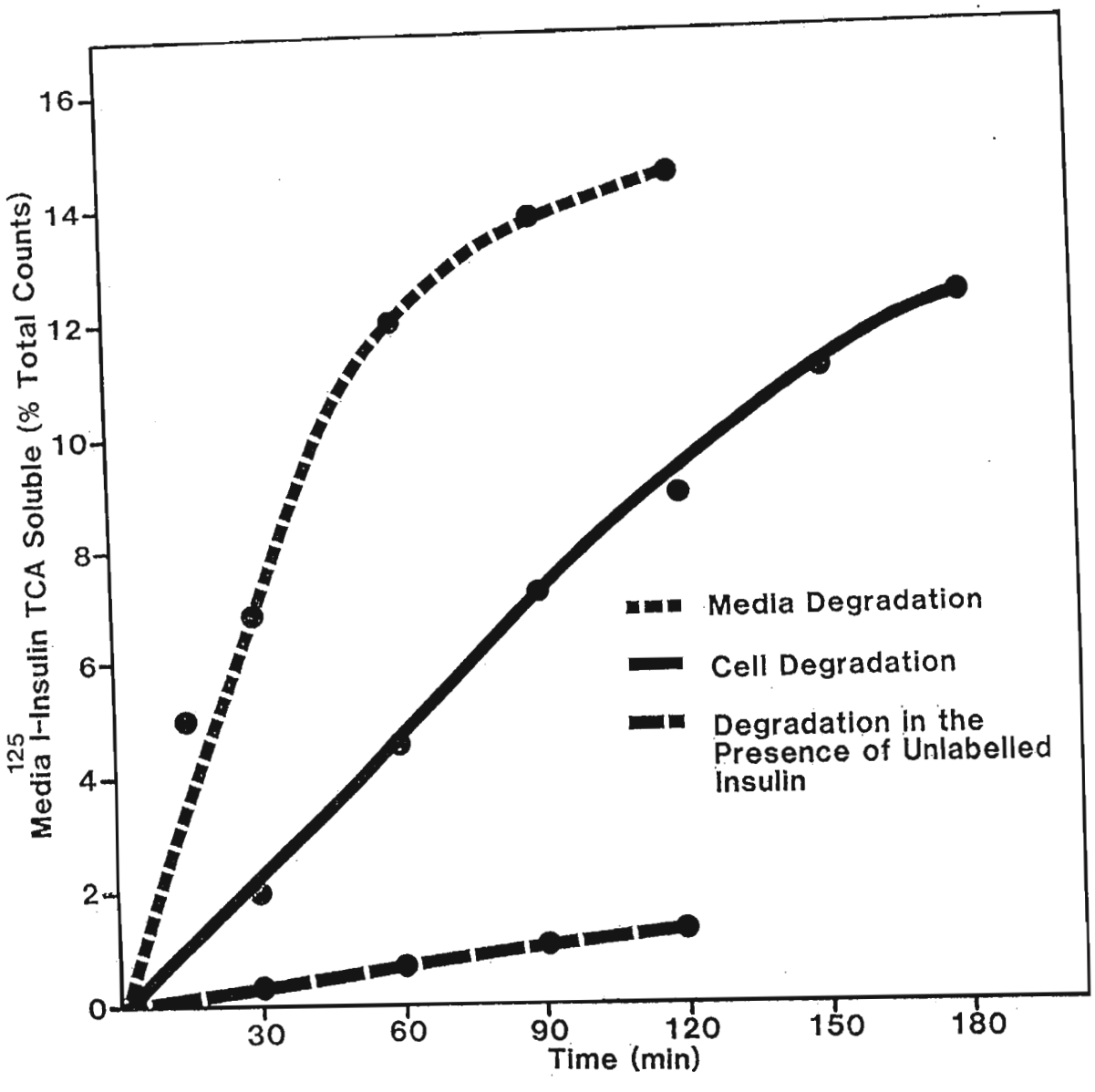


Fig. 5.2(a) **Degradation of ^{125}I-insulin by extracellular proteases at 37°C.** The relative amount of TCA soluble iodotyrosyl from extracellular proteases (media degradation) and cellular degradation was determined as outlined in figure 5.1. Degradation in the presence of unlabelled insulin was subtracted at each point to give specific degradation. All points represent the mean of duplicate determinations.

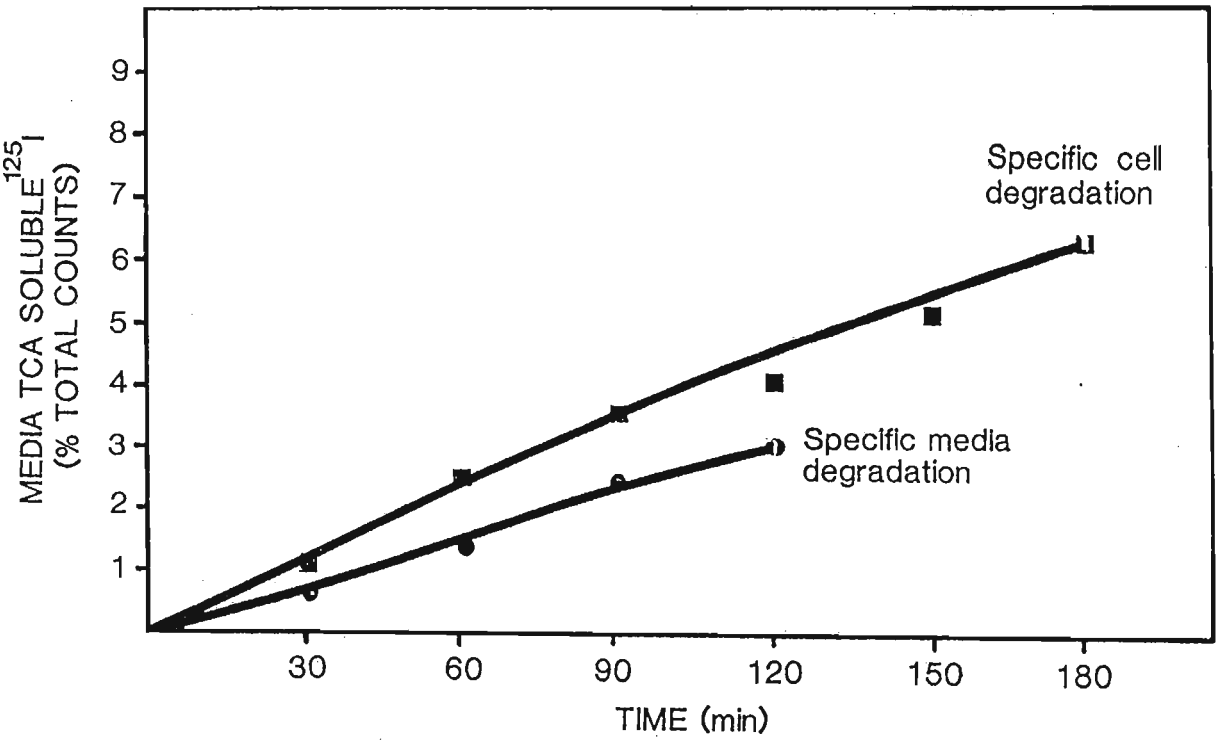


Fig. 5.2 (b) **Degradation of ^{125}I-insulin by extracellular proteases at 15° C.** Degradation of ^{125}I-insulin by extracellular proteases and cellular degradation was determined by measuring TCA soluble iodotyrosyl at specific time intervals. Degradation of radiolabelled insulin in the presence of unlabelled insulin was less than 0,1% and has been subtracted from the data to give specific degradation. Each point is the mean of duplicate measurements.

labelled insulin under steady state binding conditions must decrease progressively when increasing concentrations of unlabelled hormone are added to the incubation mixture. Specificity of the degradative process can be defined if labelled glucagon, which does not bind to the insulin receptor, is substituted for labelled insulin. If the degradation is specific for insulin bound to its receptor, then increasing concentrations of unlabelled insulin should not affect the glucagon degradation.

5.3.2 Materials and Methods

HBB was described in section 2.2.3.1. Details of radiolabelled insulin and unlabelled insulin were previously mentioned in section 2.2.1. (3-[^{125}I]-*iodotyrosyl*^{10}) glucagon was obtained from Amersham, UK.

5.3.3 Procedure

In this experiment 0,28 ng/mℓ ^{125}I-glucagon ($7,6 \times 10^{-11}$ moℓ/ℓ) and ^{125}I-insulin (5×10^{-11} moℓ/ℓ) were incubated with Raji cells (1×10^{7} cells/mℓ) in HBB pH 8,0 at 15°C. Increasing concentrations of unlabelled insulin (0-10^{5}ng/mℓ) were added to the incubation medium in both studies. After 120 min at 15°C the amount of hormone degraded was determined by precipitating the incubation medium (200 µℓ) with an equal volume of 25% TCA as described in section 5.2.1. The TCA soluble radioactivity in the absence of unlabelled hormone was

considered maximal degradation and results were expressed as a percentage of maximal degradation.

5.3.4 Results

Degradation of ^{125}I-insulin and ^{125}I-glucagon in the presence of increasing concentrations of unlabelled insulin is shown in Fig. 5.3. Maximal degradation of insulin and glucagon was 24,1% ± 0,28 and 65,05% ± 0,49, respectively. In the range 10 - 10^5 ng/mℓ of unlabelled insulin the degradation of ^{125}I-insulin was inhibited by about 80%. By comparison the degradation of ^{125}I-glucagon was not affected by this concentration of unlabelled insulin.

5.4 *DETERMINATION OF CELL SURFACE AND INTRACELLULAR ^{125}I-INSULIN DEGRADATION BY GEL FILTRATION*

5.4.1 Principle of the Method

To determine whether cell surface bound and internalised insulin was intact or degraded, the cells were incubated with ^{125}I-insulin and then subjected to a mild acid wash. As described in the previous chapter (section 4.2.1), MAAS can be used to extract cell-surface bound ^{125}I-insulin leaving behind intracellular radiolabelled hormone. Exactly the same procedure was carried out for this experiment, except the relative amounts of extractable and non-

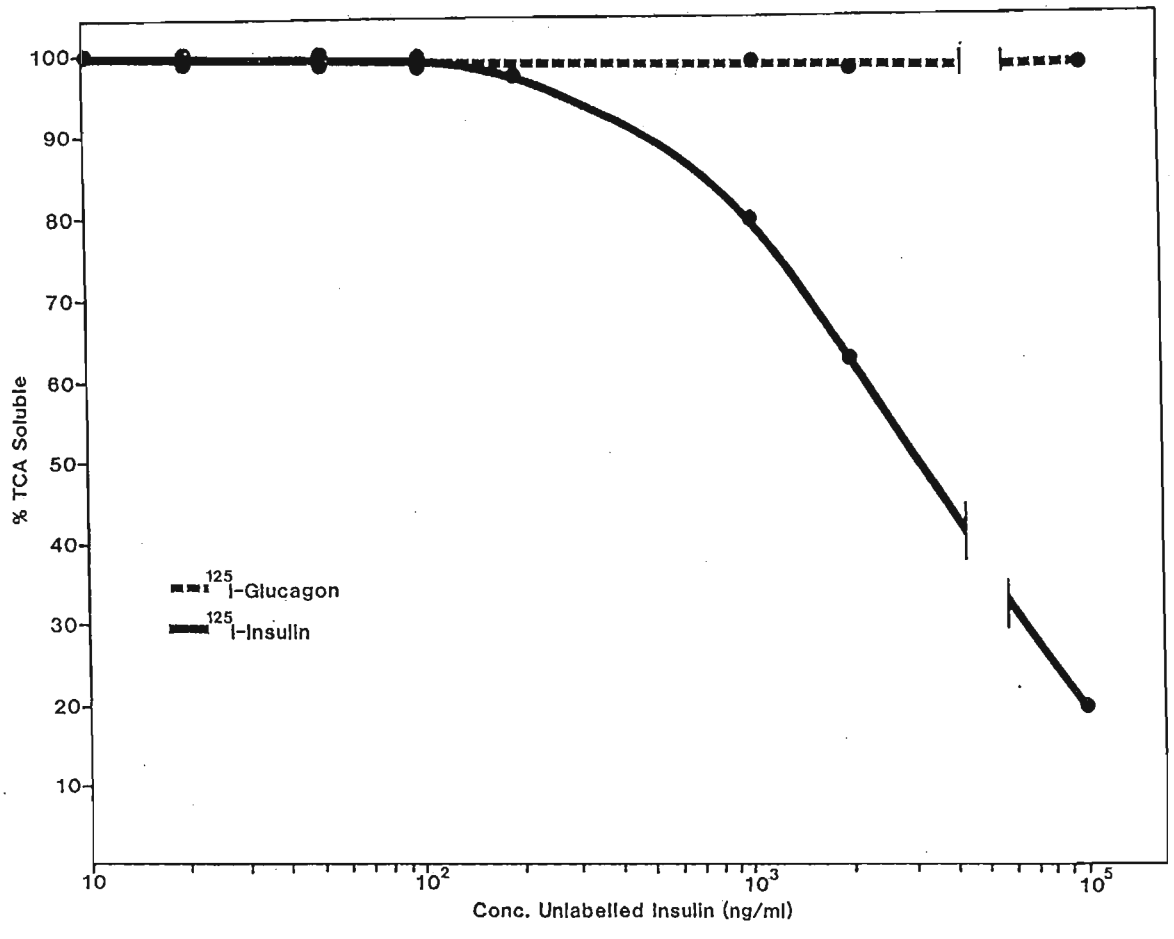


Fig. 5.3 **The relationship between insulin binding and insulin degradation.** The effect of increasing concentrations of unlabelled insulin on ^{125}I-insulin and ^{125}I-glucagon degradation, was determined after steady state binding at 15^{o}C. Degradation of both radiolabelled hormones in the absence of unlabelled hormone was taken as maximal. Each point represents the mean of duplicate determinations.

extractable radioactivities were determined by gel filtration.

5.4.2 Materials and Methods

Gel filtration was carried out on a Sephadex G-50 column (1,5 x 90 cm) which was pre-equilibrated with the elution buffer, 0,1% (w/v) BSA in 0,5 mol/ℓ acetic acid (Sonne & Gliemann, 1980). The flow rate of the column was 64 mℓ/hr and fractions were collected at 3 min intervals. ^{125}I-insulin (20 000 cpm) was applied to the column to determine the elution volume (Ve) of intact insulin, and the void volume (Vo) was determined using the elution volume of dextran blue.

5.4.3 Procedure

Raji cells were washed 3 times as described in section 2.2.3.2. The cells (10^7 cells/mℓ) were then incubated with 1 ng/mℓ ^{125}I-insulin in HBB for 60 min at 37°C. After incubation cells were centrifuged at 200 *g* for 5 min and washed twice with HBB to remove free ^{125}I-insulin. Radiolabelled insulin bound to the cell surface was extracted with MAAS as described in section 4.2.1 and the acid extract was layered on to the Sephadex G-50 column. The cell pellet was dissolved in 6 mol/ℓ urea, 3 mol/ℓ acetic acid and 0,1% triton X-100 and then centrifuged at 400*g* for 5 min to remove cell debris. The supernatant from the solubilised cells was removed and placed on the Sephadex G-50 column. Fractions were collected and radioactivity was counted in a Berthold gamma counter. In addition, 500 µℓ portions from each fraction in the radioactive peaks were removed

and precipitated with 25% TCA as described in section 5.2.1. The relative amounts of radioactivity soluble or insoluble in TCA were used as an indication of insulin degradation.

5.4.4 Results

The void volume (Vo) of the column as determined by dextran blue was 75 mℓ and the elution volume (Ve) of ^{125}I-insulin was about 147 mℓ (Fig. 5.4a and b). From the column dimensions the total volume was estimated as about 159 mℓ.

Radiolabelled insulin, which was removed from the cell surface with MAAS and then subjected to gel-filtration, demonstrated two peaks (I and II, Fig. 5.4a). Peak I eluted at the same volume as intact ^{125}I-insulin. By contrast, peak II eluted after the total volume of the column and most of the radioactivity was found in this peak.

As mentioned previously in section 5.2.1, iodotyrosyl is the insulin degradation product which is soluble in TCA. From Fig. 5.4a, it appears that portions of peak I contained no TCA soluble material, whereas the portions (500 µℓ) taken from fractions in peak II consisted of about 95% TCA soluble material.

The intracellular radioactivity comprised three peaks on gel filtration (Fig. 5.4b). The first peak (I) eluted at the void column, peak II eluted with intact ^{125}I-insulin and peak III eluted after the total column volume. There was a slight shoulder to the right of peak II which eluted at the total column volume.

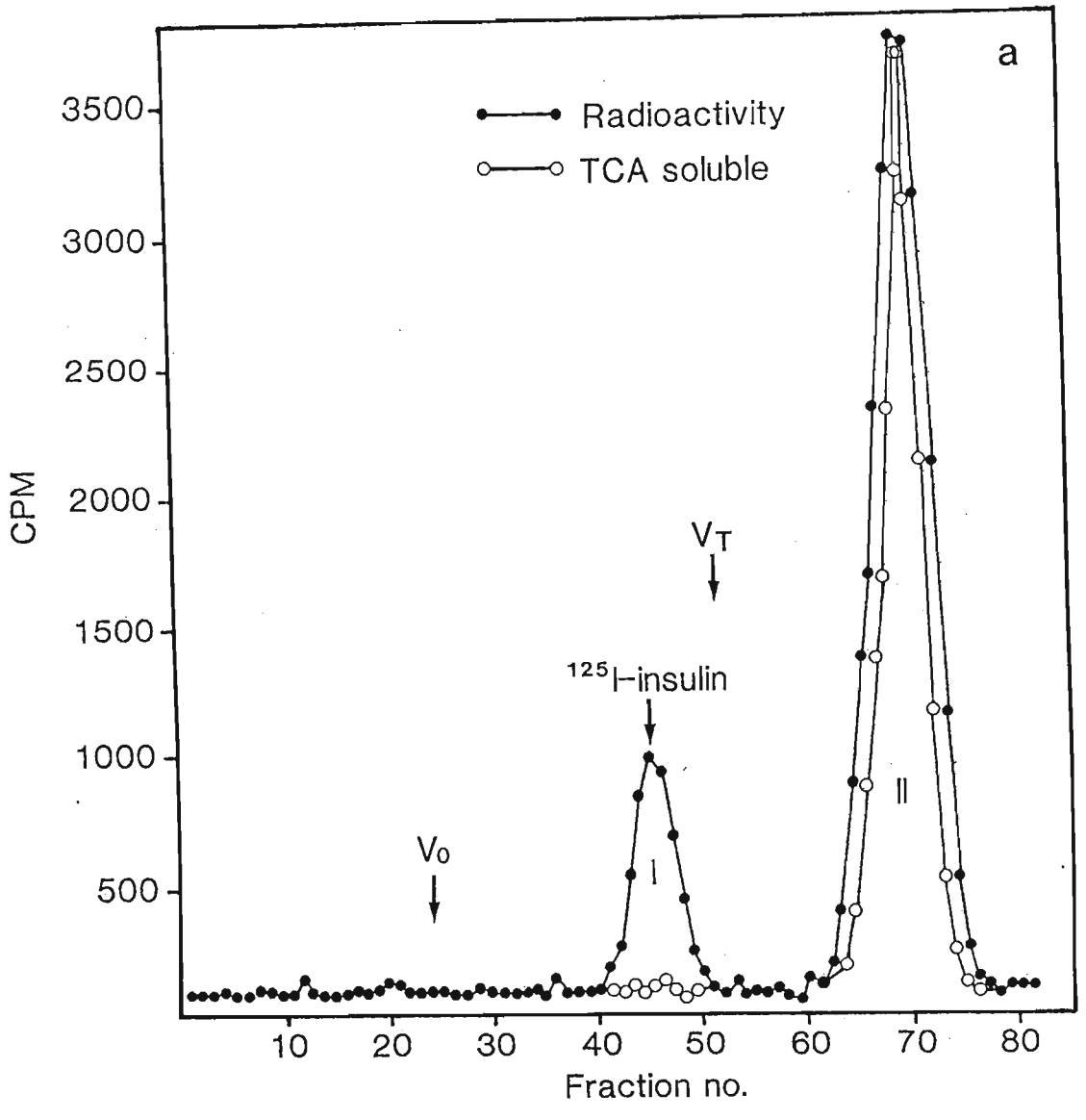


Fig. 5.4 (a) **Gel filtration of cell surface bound ^{125}I-insulin.** Radiolabelled insulin bound to cell surface receptors after 1 hour incubation was dissociated with a mild acid wash and then subjected to gel filtration on a sephadex G-50 column. The closed circles represent total radioactivity in the fractions and open circles correspond to the amount of TCA soluble radioactivity.

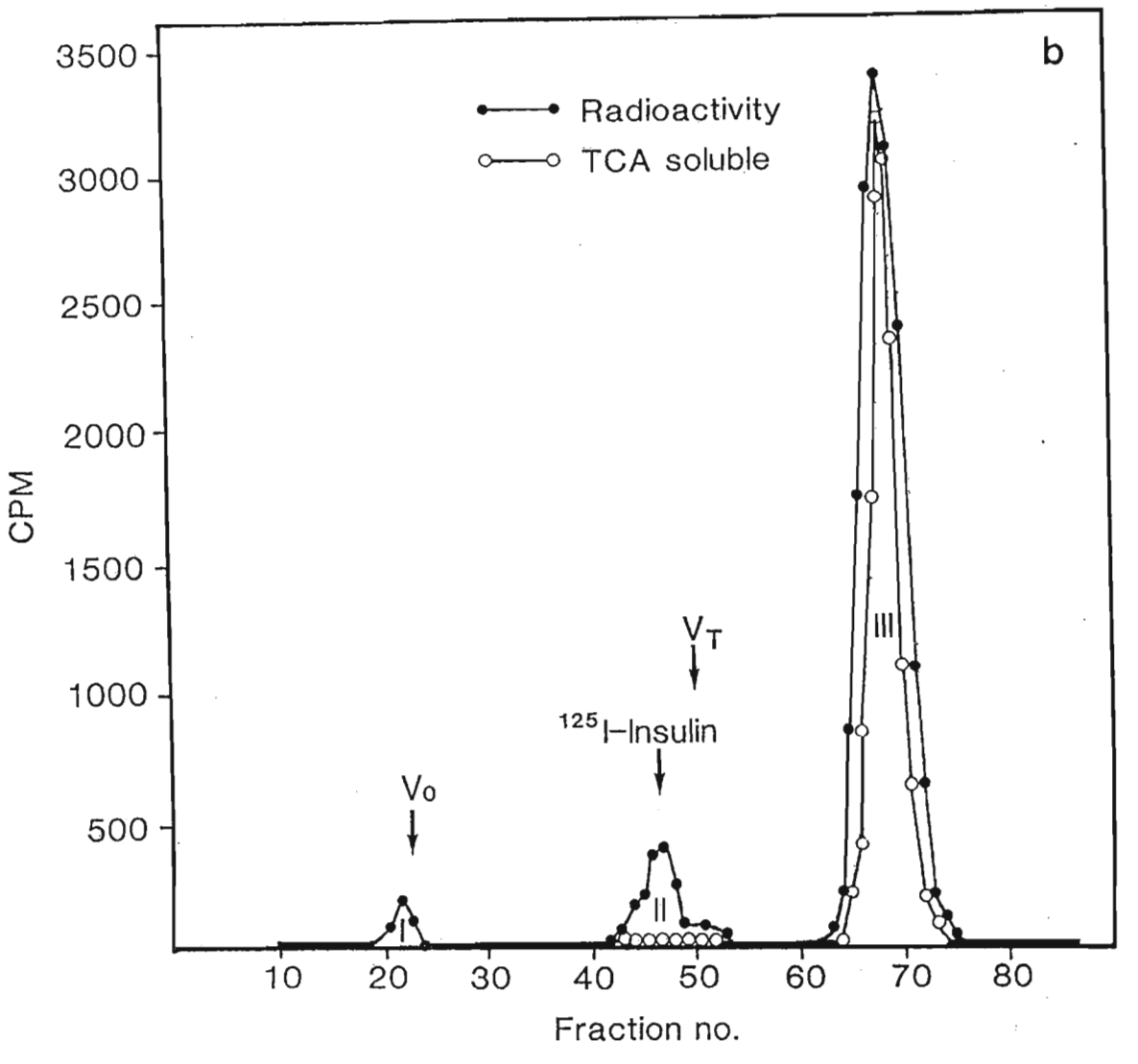


Fig. 5.4 (b) **Intracellular ^{125}I-insulin was determined by gel filtration of** solubilised cell extracts. After dissociation of surface bound radiolabelled insulin the cells were solubilised and then applied to a sephadex G-50 column as described in the text. Radioactivity in the fractions is shown by closed circles and open circles represent TCA soluble radioactivity.

Peak II, which represents intact ^{125}I-insulin, had no TCA soluble material. Portions of the third peak, however, contained only TCA soluble material.

5.5 *LYSOSOMAL DEGRADATION OF ^{125}I-INSULIN*

5.5.1 Principle of the Method

As previously mentioned in section 1.6.2.2, pre-treatment of adipocytes with chloroquine, inhibits lysosomal degradation of insulin, resulting in an increase of cell-associated (intact) ^{125}I-insulin (Olefsky *et al.*, 1982).

5.5.2 Materials and Methods

Chloroquine disulphate was purchased from Sigma Chemicals, USA.

HBB was described in section 2.2.1.

Details of ^{125}I-insulin and unlabelled insulin were given in section 2.2.1.

5.5.3 Procedure

To demonstrate the intracellular degradative pathway, Raji cells (1×10^7 cells/mℓ) were pre-incubated at 37°C in the presence or absence of 0,1 mmol/ℓ chloroquine diphosphate for 30 min. ^{125}I-Insulin (0,6 ng/mℓ) was then added to the incubation medium and after

various time intervals, the cells were centrifuged at 200 *g* for 5 min and washed with HBB. After recentrifugation the cell-associated radioactivity was determined. Non-specific binding defined as insulin binding in the presence of 10^5 ng/mℓ unlabelled insulin was determined in both the presence and absence of chloroquine treatment and subtracted from total binding.

This experimental procedure was repeated at 15°C in the presence or absence of 1 mmol/ℓ chloroquine to assess the effect of temperature and an increased chloroquine concentration on lysosomal degradation of insulin.

5.5.4 Results

Pre-incubation of Raji cells at 37°C with 0,1 mmol/ℓ chloroquine (Fig. 5.5.a) had no significant effect on cell-associated radioactivity. In both the control cells and cells pre-incubated with chloroquine, the maximum specific binding was about 2% at 60 min and remained at this level for a further 60 min.

Decreasing the temperature to 15°C resulted in an overall increase in insulin binding (Fig. 5.5b), although a ten-fold increase in chloroquine did not affect the amount of cell-associated radioactivity.

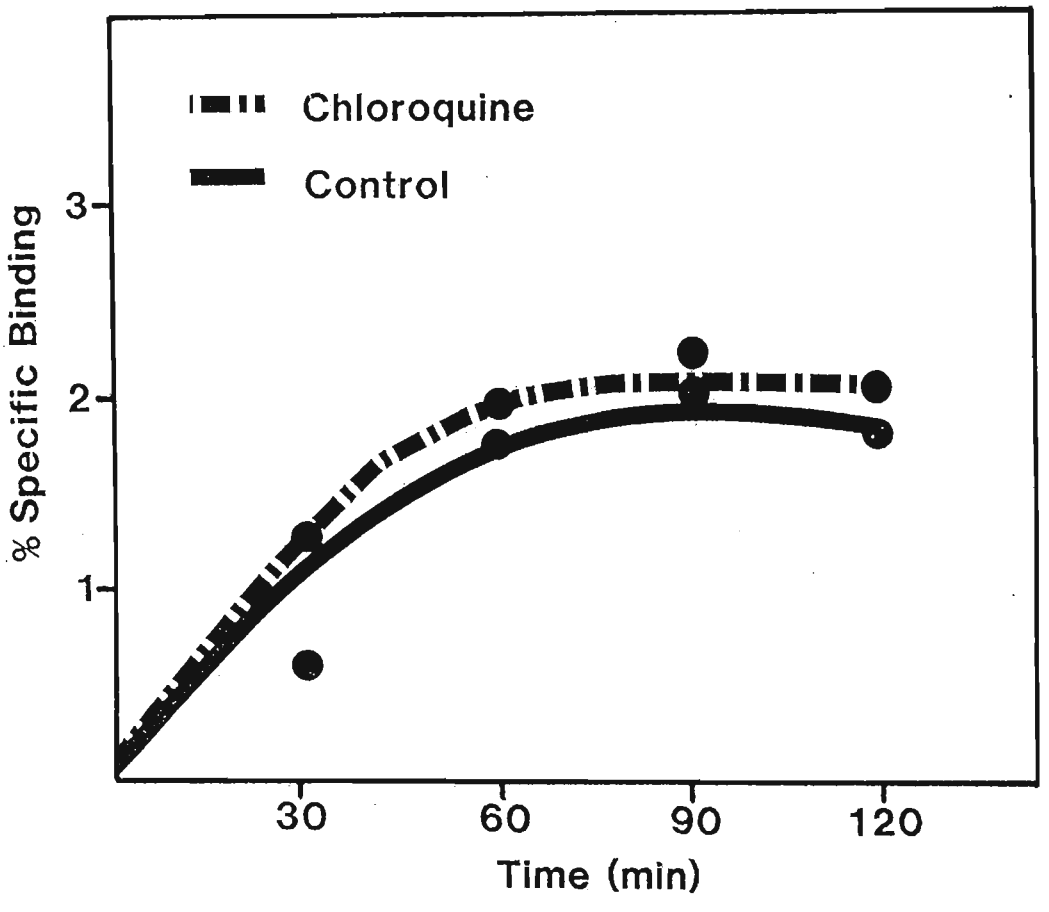


Fig. 5.5 (a) **Determination of lysosomal degradation of insulin at 37° C.** Raji cells were pre-incubated with 0,1 mmol/l chloroquine and the amount of cell-associated radioactivity was determined after various time intervals. Each point is the mean of duplicate measurements.

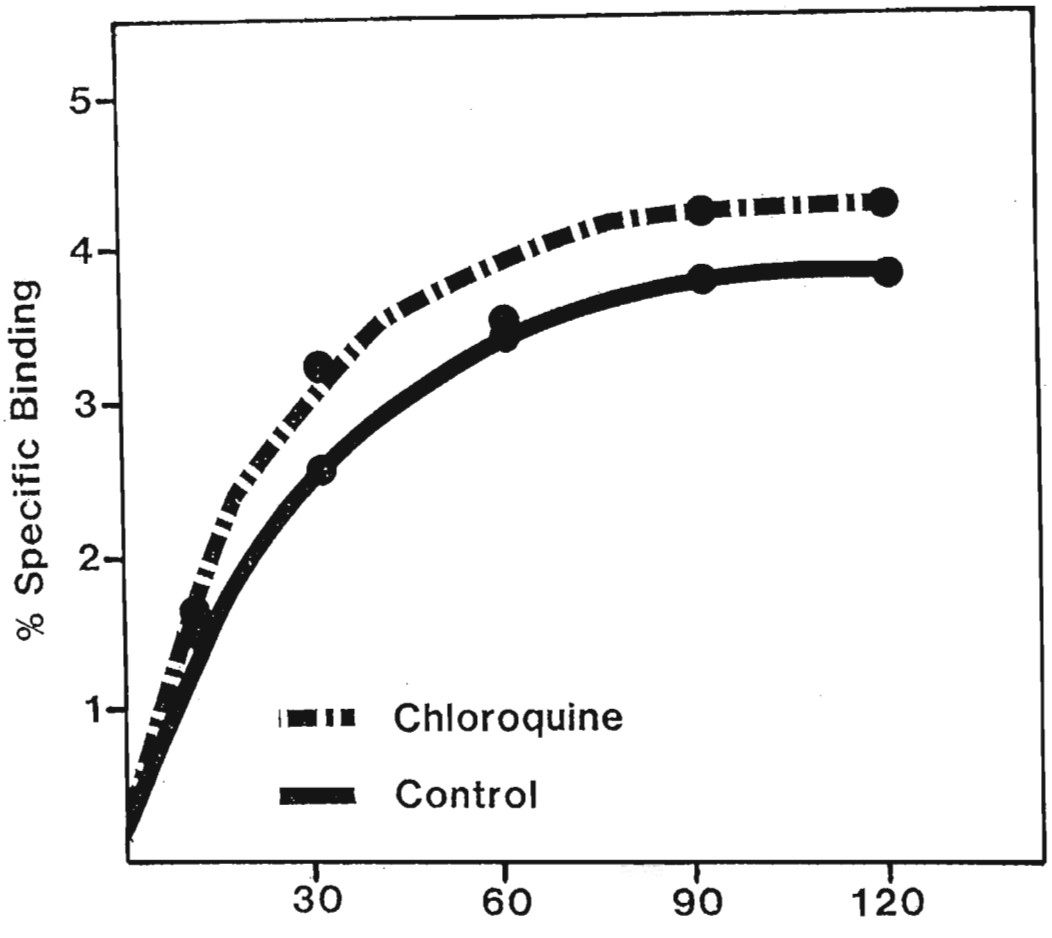


Fig. 5.5 (b) **Determination of lysosomal degradation of insulin at 15° C.** The cells were incubated with an increased concentration of chloroquine (1mmol/l). All values represent duplicate determinations.

5.6 *DISCUSSION*

These studies have shown that in Raji cells incubated at 37°C, most of the insulin was degraded by extracellular proteases in the cell-free medium. The presence of these proteases in the medium could be the result of either cell death or cell surface enzymes which were released by the cells into the medium (Roth *et al.*, 1985). By contrast, at 15°C cellular degradation of insulin was increased compared to extracellular degradation. It was previously shown (section 2.6.6), that the amount of insulin bound to the receptor almost doubled at 15°C as compared to 37°C. It therefore seems likely that the increase in cellular insulin degradation seen at 15°C was a result of increased insulin binding to the receptor which, in turn, suggests a receptor-mediated degradative pathway for insulin.

Further evidence for this mechanism was demonstrated by the decreased degradation of radiolabelled insulin in the presence of increasing concentrations of unlabelled hormone. The finding that ^{125}I-glucagon degradation was not affected by the addition of unlabelled insulin to the medium is clear evidence that insulin degradation is closely related to insulin receptor binding.

Gel filtration of acid extractable cell surface bound ^{125}I-insulin revealed that only about a third of the radioactivity measured was in the form of intact insulin; the remainder of the radioactivity was eluted after the total column volume indicating that most of

the radioactivity on the cell surface was in the form of free amino acids. This evidence suggests that insulin bound to the cell membrane was degraded by a specific cell surface enzyme or enzymes. Such a phenomenon has previously been demonstrated by Yokono *et al.* (1982) on the cell membrane of IM-9 lymphocytes.

The chromatography pattern of the intracellular extract showed a small proportion of internalised radioactivity which was associated with the Vo. This fraction could represent ^{125}I-insulin-receptor complex which had been internalised or insulin associated with subcellular organelles. The second peak constituted about 1/6th of the cell-associated radioactivity: the material eluted with the same volume as intact insulin and it was precipitated by 25% TCA.

Most of the radioactivity was eluted after the total volume had passed through the column. Its solubility in TCA and retention by the column suggest that the radioactivity was associated with free amino acids. A reasonable conclusion from these results would be that the intracellular radioactivity was associated with intact insulin and also degradation products of insulin which were smaller than oligopeptides.

In Chapter 4, it was shown that insulin was internalised by the cell via a receptor-mediated mechanism. It would seem reasonable to assume that internalised insulin is the only source of intracellular intact insulin and degradation products. However, in the Raji cells, internalised insulin did not appear to be degraded in the lysosomes as evidenced by the inability of chloroquine to inhibit

insulin degradation.

At present, therefore, it is not clear whether only intact insulin is internalised or whether cell surface degraded insulin is also internalised by endocytosis. If the latter occurs, then the degraded insulin within the cell might be the result of cell surface proteases. This possibility has been suggested by Yaso *et al.* (1987) from studies on insulin degradation in Bri-7 cultured lymphocytes. On the other hand, an alternative degradative pathway might exist within the cell. Indeed Goldstein & Livingston (1981) have suggested that in adipocytes insulin is degraded mainly by neutral cytosolic proteases and not by lysosomal proteases.

In summary, insulin appears to be degraded at the cell surface by a specific protease. However, unlike the IM-9 lymphocytes (Sonne & Gliemann, 1980) and RPMI-1788 cultured lymphocytes (Maegawa *et al.*, 1983), the Raji cells have a receptor-mediated pathway for intracellular degradation. As the internalised insulin is not degraded in the lysosomes, the insulin degradation products within the cell could either be a result of endocytosis of cell surface degraded insulin or degradation by neutral cytosolic proteases.

CHAPTER 6

IDENTIFICATION OF THE INSULIN BINDING SITE ON THE RAJI INSULIN RECEPTOR

"With accurate experiment and observation to work upon, imagination becomes the architect of physical theory."
-TYNDALL

6.1 *INTRODUCTION*

Two major aspects of insulin-receptor interactions have been described in previous chapters. Firstly, in Chapters 2 and 3, it was shown that the kinetics of insulin binding to the receptor in Raji cells were similar to published binding data for insulin target cells. Secondly, it was found that the receptor on the Raji cells functions normally, although the site of insulin degradation was different in these cells compared to adipocytes (Chapters 4 and 5).

On this evidence it seems reasonable to assume that the virus-induced receptors on B-lymphocytes are indeed kinetically and functionally identical to target tissue insulin receptors. However, the assumption will be measurably strengthened if it can be shown that the receptor is structurally similar to target tissue receptors. In this chapter, some evidence of the structural components of Raji cell insulin receptors is presented.

6.2 *STRUCTURAL COMPONENTS OF THE RAJI CELL INSULIN RECEPTORS*

In section 1.5 the structure of the insulin receptor was discussed. The evidence for the structure is based on covalently linking labelled insulin to the receptor components using a bifunctional crosslinking agent, followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Visualisation of the electrophoretic components is achieved by exposure to a sensitive photographic plate.

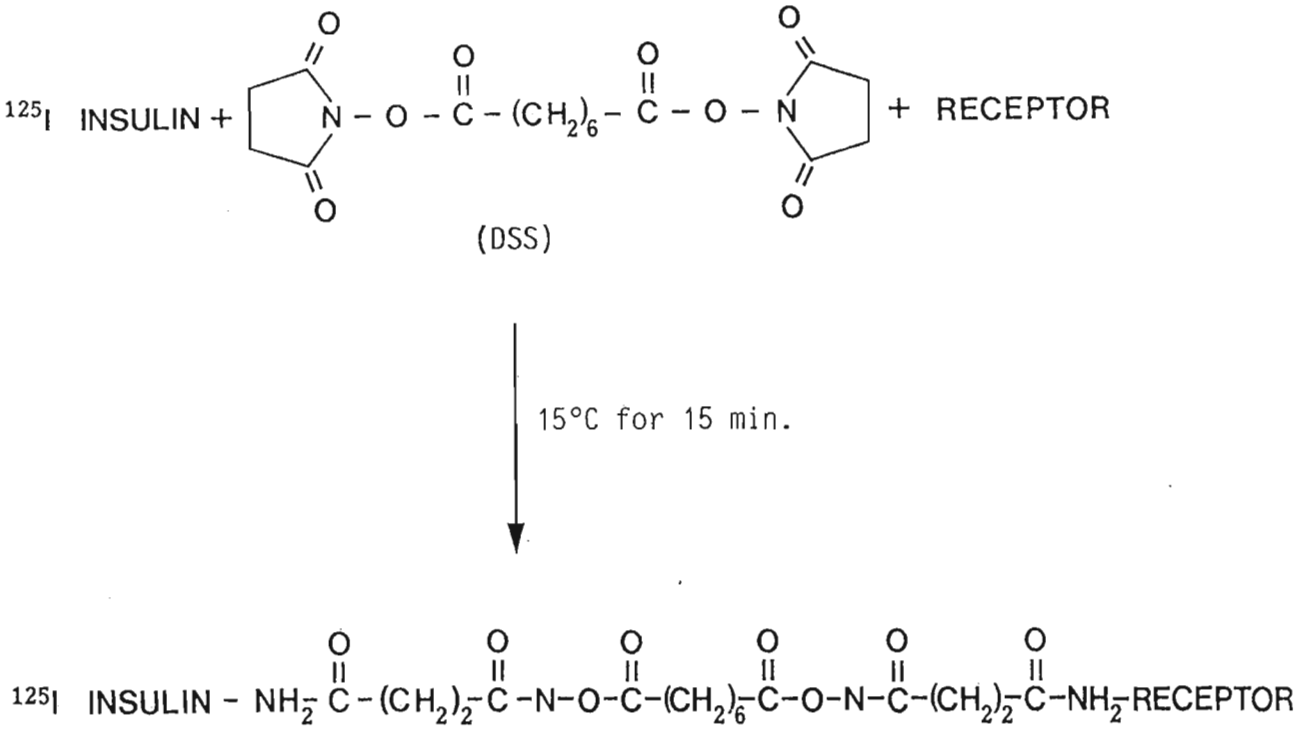
6.2.1 Principle of Crosslinking of ^{125}I -Insulin to Insulin Receptors

The bifunctional crosslinking agent, disuccinimidyl suberate (DSS) can be used to covalently link two protein molecules which are closely bound. This reaction results in an amide bond between DSS and the ε-amino groups of the basic amino acids in the two protein molecules (Fig. 6.1). Addition of Tris-EDTA prevents further crosslinking, as excess DSS reacts with the primary amines on the Tris molecule. Optimum conditions for crosslinking ^{125}I-insulin to the receptor on intact cells have previously been described by Pilch & Czech (1980).

6.2.2 Materials

Disuccinimidyl suberate was obtained from Pierce Chemical Company and dimethyl sulphoxide (DMSO) from Merck Chemicals.

The incubation buffer was Hepes binding buffer (HBB), described in



(^{125}I-insulin crosslinked to the membrane receptor).

Fig. 6.1 **Crosslinking reaction** between disuccinimidyl suberate and the ε-amino groups of basic amino acids on the receptor molecule and the insulin molecule.

section 2.2.3.1. Hepes binding buffer without BSA was used for the incubation with DSS.

Buffer containing 10 mmol/ℓ Tris-HCl and 1 mmol/ℓ EDTA pH 7,4 was used to stop the reaction.

Raji cells were used during stationary phase and washed as previously described in section 2.2.3.

The SDS-PAGE reducing application buffer contained sodium phosphate buffer (10 mmol/ℓ; pH 7,0), SDS (2%), glycerol (10%), dithiothreitol (DTT) (100 mmol/ℓ) and bromophenol blue (0,001%). The non-reducing application buffer was the same, but DTT was omitted. Solubilising buffer (pH 7,4) contained 50 mmol/ℓ HEPES, 10 mmol/ℓ $MgSO_4$, 1% Triton x-100, 2 mmol/ℓ phenylmethylsulphonyl fluoride (PMSF) and 1000 U/mℓ aprotinin.

6.2.3 Procedure

6.2.3.1 ^{125}I-Insulin Crosslinked to Raji Cells in the Presence or Absence of Unlabelled Insulin

Cells were incubated (10^8 cells/2,5 mℓ) in HBB with ^{125}I-insulin (3×10^{-10} mol/ℓ) in the presence or absence of unlabelled insulin (1×10^{-5} mol/ℓ). Incubation was carried out at 15°C for 120 min after which ice-cold HBB (10 mℓ) without albumin was added to the incubation mixture. The cells were then pelleted by centrifugation at 200 *g* for 10 min at 4°C. The supernatant was discarded and cells were resuspended in HBB without albumin (1 mℓ) at 15°C.

Disuccinimidyl suberate dissolved in DMSO was added to the cell suspension to give a final concentration of 0,5 mmol/ℓ. After incubation at 15°C for 15 min, the crosslinking reaction was stopped by addition of 4 mℓ of Tris-HCl buffer. Cells were incubated for a further 5 min and then centrifuged at 200 *g* for 10 min at 4°C. The cell pellet was dissolved in 250 µℓ of solubilising buffer for 1 hr at room temperature. The supernatant was partially clarified by centrifugation in a Beckman microfuge (8740*g*) for 10 min at 4°C. After centrifugation, an equal volume of reducing SDS-PAGE application buffer was added and the solution was boiled for 3 min. Electrophoresis of these samples was carried out using a discontinuous buffer system (Laemmli , 1970) with a 4% stacking gel and a 7,5% resolving gel.

6.2.3.2 Determination of ^{125}I-Insulin Crosslinked to Raji Cells using Non-Reducing Conditions

In this experiment the procedure was the same as in section 6.2.3.1. However, in the final step solubilised cells were added to 250 µℓ of non-reducing SDS-PAGE application buffer. The samples were then subjected to SDS-PAGE using a 4% stacking gel and a 5% resolving gel.

6.2.3.3 Crosslinking ^{125}I-Insulin to Raji Cells in the Presence of Unlabelled IGF-I or Insulin

In section 1.3.1.2 it was mentioned that the structure of the type I IGF receptor and the insulin receptor is identical. By definition the insulin receptor is the molecule which has a greater affinity for insulin. Therefore crosslinking ^{125}I-insulin to the receptor in

the presence of unlabelled IGF-I should not decrease the intensity of the insulin receptor band.

Raji cells were incubated (10^8/2,5 mℓ) in HBB containing ^{125}I-insulin (8×10^{-10} mol/ℓ) in the presence of unlabelled IGF-I (1×10^{-7} mol/ℓ), or unlabelled insulin (1×10^{-7} mol/ℓ), or in the absence of unlabelled peptide. The crosslinking procedure was followed as described in section 6.2.3.1.

6.3 *SDS-PAGE OF THE CROSSLINKED ^{125}I-INSULIN-RECEPTOR COMPLEX*

To determine the molecular weight of the crosslinked ^{125}I-insulin-receptor complex, the sample was subjected to SDS-PAGE using a discontinuous buffer system (Laemmli , 1970).

6.3.1 Reagents

BIS-Acrylamide: 30% Acrylamide
0,8% BIS (N N-Methylene-bis-acrylamide)

Stacking Gel Buffer: 0,1 mmol/ℓ Tris-PO_4
0,2% SDS
4 mmol/ℓ EDTA
pH 6,7

Resolving Gel Buffer: 0,75 mmol/ℓ Tris-HCl
0,2% SDS
4 mmol/ℓ EDTA
pH 8,9

TEMED: N, N, N, N Tetramethylethylenediamine

Catalyst: 4% Ammonium Persulphate

Electrophoresis Buffer: 50 mmol/ℓ Tris
380 mmol/ℓ Glycine
0,1% SDS
2 mmol/ℓ EDTA
pH 8,3

Glycerol

Gel Apparatus: The Protean Dual Slab Cell was supplied by Bio-Rad.
GelBond was obtained from FMC Corporation.

^{14}C-Molecular Weight Standards:
^{14}C-labelled high molecular weight protein standards (Bio-Rad, USA):

Myosin	200 000
Galactosidase	116 250
Phosphorylase B	92 500
Bovine serum albumin	66 200
Ovalbumin	45 000

Unlabelled Molecular Weight Standards:
Unlabelled protein standards with the same molecular weight range were also used (Bio-Rad, USA)

Autoradiography: Hyperfilm-MP was obtained from Amersham, UK

Casette: A cassette with intensifying screens was used to store the film during exposure to the dried gel (Kodak, USA).

6.3.2 Procedure

6.3.2.1 Casting Discontinuous Gels

Discontinuous gels consist of a resolving gel (7,5% or 5%) and a stacking gel (4%). Monomer solutions for the two gels were made up

as shown in Table 6.1. After addition of the catalyst, the resolving gel was poured between the glass plate sandwich until the level of the gel was 3 cm from the top of the plate. Using a syringe, water was immediately overlayed on the resolving gel to give a smooth interface after polymerisation. After 30 min, the water was poured off and the comb was placed between the plates. The stacking gel monomer solution was then poured to about 1 cm from the top of the plates and the comb pushed down. Polymerisation of the stacking gel occurred after about 30 min.

6.3.2.2 Sample Application

After removal of the comb from the gel, each well was filled with electrophoresis buffer. A Hamilton syringe was used to underlay the sample and the molecular weight standards into individual wells.

6.3.2.3 Running the Gel

The glass plate assembly was placed in the Protean buffer chamber containing electrophoresis buffer. The water cooling system was turned on and the apparatus attached to the power supply. Samples were run at constant current (7,5 mA) until the dye front (incorporated in the application buffer) reached the end of the gel.

6.3.2.4 Autoradiography

After removing the gel which was attached to gelbond, it was clipped to a glass plate to prevent the edges from curling over and allowed to dry overnight at room temperature. The dried gel was then placed in the cassette between the film and the intensifying screen. Cassettes were stored at -70°C for 6-8 weeks.

Table 6.1 Monomer Solutions for Discontinuous Gel Electrophoresis

REAGENT	4% STACKING GEL	5% Resolving gel	7,5% RESOLVING GEL
Acrylamide/BIS	2 mℓ	7 mℓ	7,5 mℓ
Buffer	7,5 mℓ	15 mℓ	15 mℓ
Glycerol	-	6 mℓ	3 mℓ
Water	5,3 mℓ	13 mℓ	4,3 mℓ
TEMED	10 µℓ	20 µℓ	20 µℓ
Ammonium persulphate (added after solutions had been degassed for 2 min)	150 µℓ	300 µℓ	300 µℓ

6.3.2.5 Developing and Fixing

Films were developed manually according to the recommendations of the manufacturer.

6.4 *RESULTS*

6.4.1 Affinity Crosslinked ^{125}I-Insulin in the Presence and Absence of Unlabelled Insulin using Reducing Conditions

An autoradiograph of ^{125}I-insulin covalently linked to the membrane receptor is shown in Fig. 6.2. Tracks A and C contain the same crosslinked material in the absence of excess unlabelled insulin whereas, tracks B and D consist of similar material in the presence of excess unlabelled hormone (10^{-5} mol/ℓ). The ^{14}C-labelled molecular weight standards were run in track E. Unlabelled high molecular weight protein standards were run in track F.

A single band with an apparent molecular weight of 130 kD is seen in tracks A and C. In both tracks there are bands at the application points and at the interface of the two gels, suggesting large molecular weight material which cannot enter the gels.

In the presence of excess unlabelled insulin (tracks B and D) the 130 kD band is absent. In addition, the high molecular weight bands are no longer apparent.

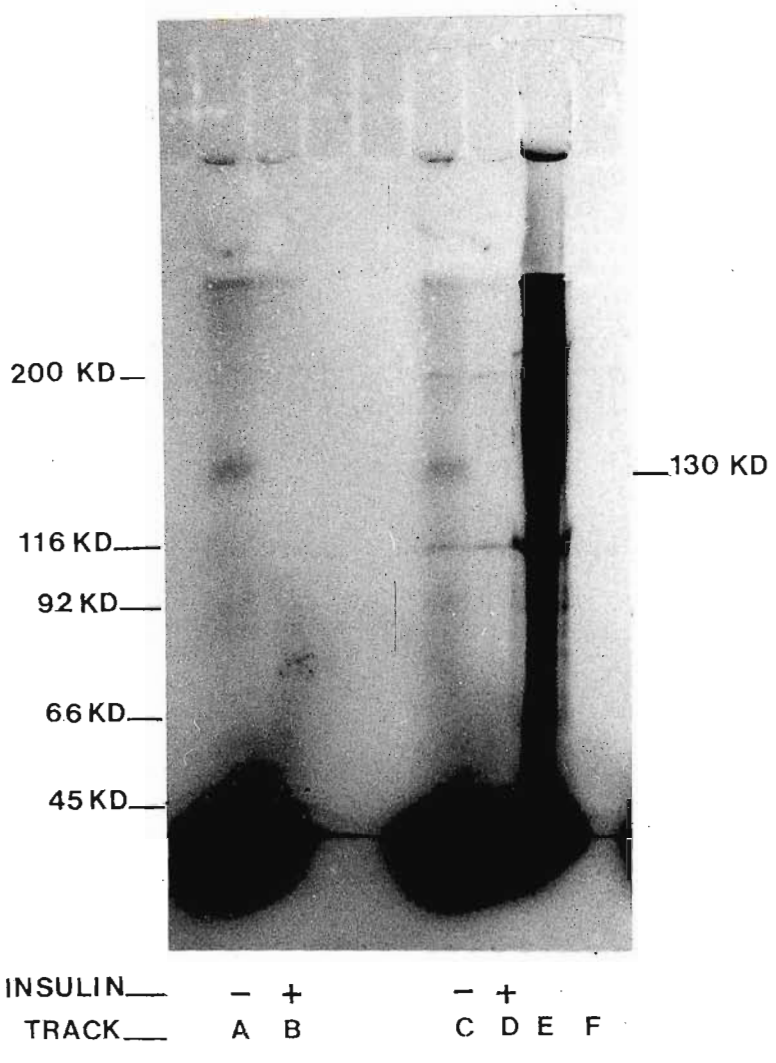


Fig. 6.2 **An autoradiograph of ^{125}I-insulin covalently cross-linked to the insulin receptor on Raji cells.** ^{125}I-insulin (3 x 10^{-10} mol/l) was bound to Raji cells in the absence (A, C) or presence (B, D) of unlabelled insulin. The hormone-receptor complex was then covalently cross-linked using D.S.S., solubilised and reduced in the electrophoresis application buffer. Samples were subjected to SDS-PAGE on 7,5% gel and finally autoradiographed. Molecular weight standards are indicated on the left of the autoradiograph.

6.4.2 ^{125}I-Insulin Crosslinked to the Receptor under Non-Reducing Conditions

Using non-reducing conditions a single, large molecular weight band of about 300 - 350 kD was observed (Fig. 6.3, track A). Once again this band was not observed in the presence of excess unlabelled insulin (track B).

There was also some evidence of large molecular weight products in track A, which have not entered the resolving gel.

6.4.3 Crosslinked ^{125}I-Insulin in the Presence of IGF I or Insulin

In the presence of unlabelled IGF-I three distinct bands were apparent (Fig. 6.4). These bands had approximate molecular weights of 130 kD, 105 kD and 66 kD (track C).

Similarly, all three bands were observed in the absence of excess unlabelled insulin (track A). By contrast, only traces of the 105 kD and 66 kD bands were observed in the presence of 10^{-7} mol/ℓ unlabelled insulin (track B). The concentration of ^{125}I-insulin was slightly increased in this experiment resulting in these low molecular weight bands.

In all three experiments there was a dark band at the interface of the two gels, indicating large molecular weight products labelled with ^{125}I-insulin.

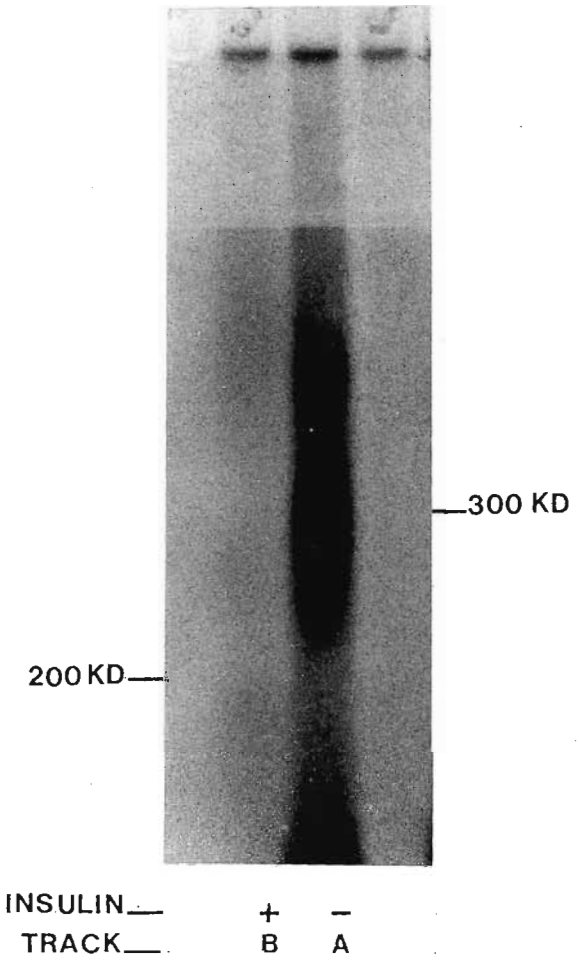


Fig. 6.3 **^{125}I-insulin covalently cross-linked to the membrane receptor and subjected to SDS-PAGE under non-reducing conditions.** Radiolabelled insulin was cross-linked as described in fig. 6.2. However, in this case, the sample was not reduced and SDS-PAGE was carried out on a 5% gel. Material in track (A) was cross-linked in the absence of unlabelled insulin and track (B) was similar material cross-linked in the presence of insulin.

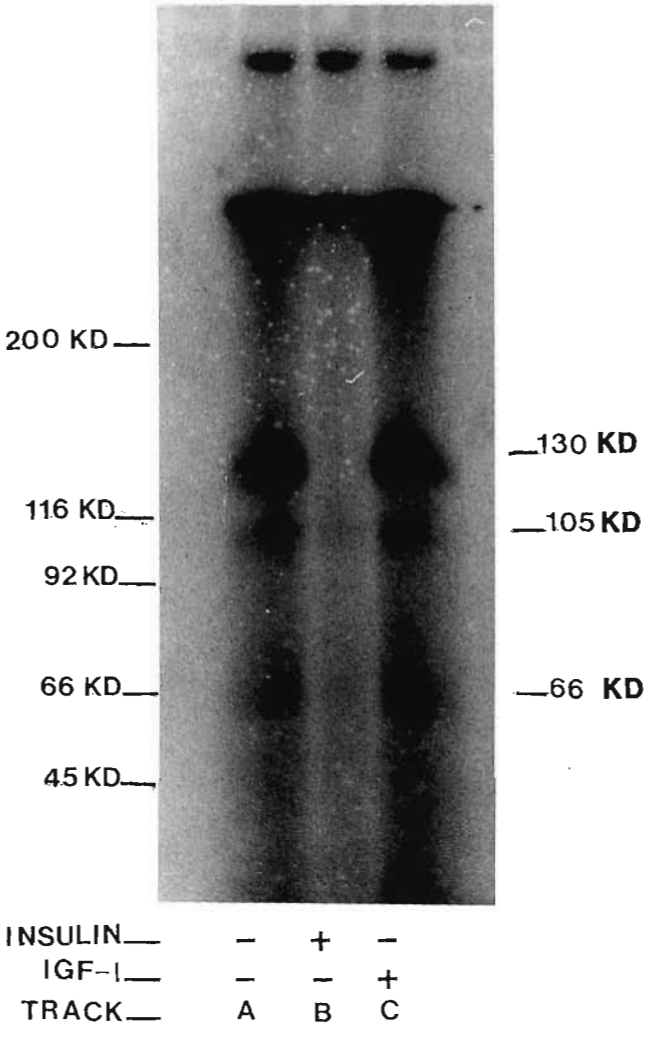


Fig. 6.4 **Cross-linked ^{125}I-insulin in the presence of unlabelled IGF-I and insulin.** The cross-linking procedure was carried out as described in section 6.2.3.3 with subsequent SDS-PAGE of reduced samples. The autoradiograph shows radiolabelled insulin crosslinked in the presence of 1 x 10^{-7} mol/l IGF-I (C) and 1 x 10^{-7} mol/l insulin (B), and in the absence of unlabelled peptide (A). Molecular weight standards are shown on the left hand side.

6.5 *DISCUSSION*

Covalently linking ^{125}I-insulin to the receptor with subsequent reduction, SDS-PAGE and autoradiography identified a radiolabelled molecule with a molecular weight of about 130 kD. As this was the major species crosslinked to ^{125}I-insulin, these results suggest that the insulin binding site is found on this subunit. The large molecular weight species which appeared to bind insulin might be intact receptors, which probably result from partial purification of the solubilised preparation. Addition of unlabelled insulin during the hormone binding and crosslinking procedure, resulted in the loss of both bands which further supports the suggestion that these bands represent specific insulin binding sites.

Using a non-reducing sample application buffer showed that radiolabelled insulin binds to a high molecular weight (M.Wt) species with an apparent M.Wt of 300 kD. In this case the 130 kD binding site was absent. From the findings it is clear that the low molecular weight binding site is a subunit of the insulin receptor, and in its native form the receptor exists as a disulphide-linked complex.

As discussed in section 1.5.2, previous studies on fat cell and liver membranes have shown that insulin binds to an α subunit which has a M.Wt of about 130 kD (Pilch & Czech, 1980). The native receptor complex has an apparent M.Wt of 300 kD and it is composed of two α and β-subunits which are linked by disulphide bonds (Czech, 1985). The present study showed that Raji cells have an insulin receptor which has the same molecular structure as receptors on target cell membranes.

In section 1.3.2.1 the cross-reactivity between insulin and IGF-I was discussed in detail. It is now apparent that membrane receptors for the two peptides have very similar structures (Massagué & Czech 1982). In the present study, 10^{-7} mol/ℓ unlabelled IGF-I did not affect the intensity of the 130 kD species which binds insulin. By contrast, the same concentration of unlabelled insulin (10^{-7} mol/ℓ) resulted in complete loss of the α subunit. This indicates that the Raji cells have a high affinity binding site which binds insulin but does not bind IGF-I.

The low molecular weight bands 105 kD and 66 kD appeared to be low affinity insulin binding sites which were not entirely displaced by 10^{-7} mol/ℓ of unlabelled insulin. Similar findings have been reported on fat cell and liver membranes (Pilch & Czech, 1980). However, the physiological relevance of these binding sites has not been described.

Affinity crosslinking of ^{125}I-insulin to the membrane receptor on Raji cells has shown that the α subunit has a M.Wt of 130 kD, is specific and has a high affinity for insulin. In addition, the molecular structure of the intact receptor is similar to insulin receptors on classical target tissue.

CHAPTER 7

CHARACTERISTICS OF TYPE I IGF RECEPTORS AND THE METABOLIC RESPONSE TO IGF-I AND INSULIN STIMULATION OF THE RAJI CELLS

Among natural systems and phenomena,
resemblances occur in great numbers.
-INGLE, 1968

7.1 INTRODUCTION

In the previous chapters, the kinetics of insulin binding to its receptor, as well as the function and structure of this receptor were studied in detail. It was concluded that the E-B virus did indeed induce insulin receptors which had all the properties described for receptors in target organs.

However, as discussed in section 1.3.1.2, there is some cross-reactivity between insulin and the type I IGF-receptor and between IGF-I and the insulin receptor. Because insulin, IGF-I and IGF-II can produce the same metabolic effects at appropriate concentrations and, because many cell types possess combinations of these three receptors (Czech 1982), it has been difficult to assess which receptor is mediating a specific response.

The purpose of this chapter is the determination of the relative IGF and insulin receptor numbers and the biological dose-response of Raji cells to respective stimulation by insulin and IGF-I.

7.2 STEADY-STATE BINDING OF ^{125}I-IGF I TO RAJI CELLS

In section 2.4.6, it was shown that steady-state binding of the insulin to its receptor was dependent on time. A similar study was carried out with ^{125}I-IGF I to determine the optimum time for steady -state binding to the type I IGF receptor at 15°C.

7.2.1 Materials

(3-[^{125}I] iodotyrosyl) Insulin-like growth factor I [Thr^{59}](specific activity ~ 2000 Ci/mmol) and unlabelled growth factor were supplied by Amersham International, UK.

Details of ^{125}I-insulin and unlabelled insulin were previously given in section 2.2.1.

Hepes binding buffer (HBB) pH 8 (section 2.2.3.1) was used throughout in binding assays.

Raji cells were grown until the early stationary phase of growth as described in section 2.4.1. Immediately before use, the cells were washed three times with HBB and resuspended at a concentration of about 1 x 10^7 cells/mℓ.

7.2.2 Procedure

Portions (400 µℓ) of the cell suspension were added to test tubes containing 2,5 x 10^{-11} mol/ℓ ^{125}I-IGF I (50 µℓ) in the presence or absence of 4 x 10^{-7} mol/ℓ of unlabelled IGF I (50 µℓ). The incuba-

tion mixture was maintained at 15°C. After 10 min and then at 30 min intervals, 200 µℓ portions were removed and layered on to 200-µℓ ice cold HBB in microfuge tubes. The tubes were centrifuged (8740 *g*), the supernatant was discarded and the cell pellet was counted in a Berthold multi-head gamma counter.

The remaining 100 µℓ from each tube were pooled and 200 µℓ portions were counted to give total counts per minute (cpm). Results were expressed as a percentage of total cpm. Non-specific binding (NSB) was taken as the percentage ^{125}I-IGF I bound in the presence of 4 x 10^{-7} mol/ℓ unlabelled IGF-I. Specific binding to the type I IGF receptor was determined by subtracting NSB from total bound.

7.2.3 Results

A plot of bound ^{125}I-IGF I versus time is shown in Fig. 7.1. Steady-state binding to type I IGF receptors was achieved after 120 min at 15°C and this level of binding was maintained for 180 min.

Maximum binding ranged between 3 and 3,5% of total radioactivity, whereas NSB was about 2%. As a result specific binding of ^{125}I-IGF I to the Raji cells was only 1-2% of total radioactivity.

7.3 *COMPETITIVE BINDING OF IGF-I AND INSULIN TO RAJI CELLS*

The presence of specific membrane receptors for IGF-I was investigated by allowing ^{125}I-IGF I to bind to the cells in the presence of

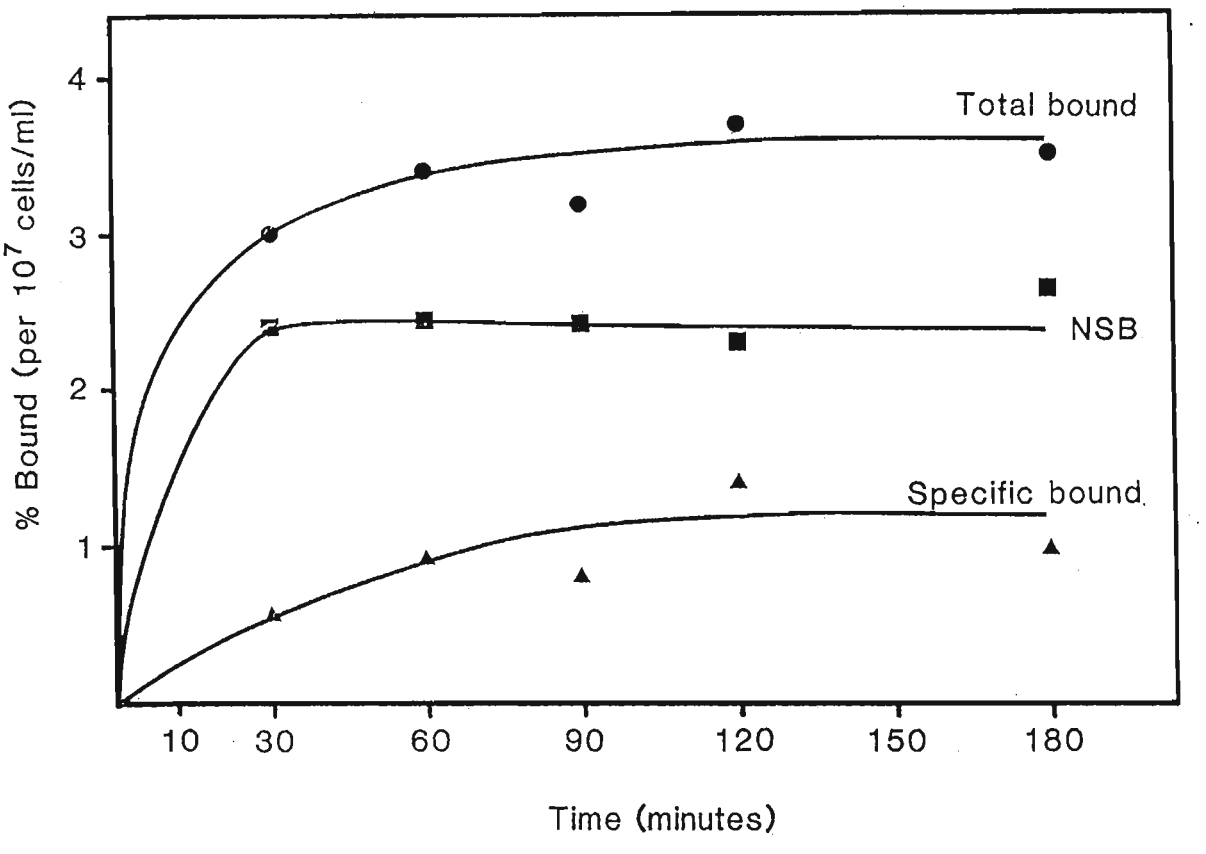


Fig. 7.1 **Steady state binding of ^{125}I-IGF-I to Raji cells was determined by incubating radiolabelled peptide with cells in the presence or absence of unlabelled IGF-I.** Specific binding was determined by subtracting NSB from total binding. All measurements were done in duplicate.

increasing concentrations of unlabelled IGF-I and insulin. The rationale for this competitive binding assay was considered in section 1.4.1.

7.3.1 Procedure

Cells were washed and resuspended in HBB as previously described in section 7.2.1. Portions (400 µℓ) of these cells were incubated at 15°C until steady state was reached (120 min) with 50 µℓ ^{125}I-IGF I ($2,5 \times 10^{-11}$ mol/ℓ) and 50 µℓ of buffer containing increasing concentrations of unlabelled IGF-I (10^{-11} - 10^{-6} mol/ℓ) or insulin (10^{-11} - 10^{-5} mol/ℓ). Maximum binding was determined by incubating cells with ^{125}I-IGF I in the absence of unlabelled peptide.

In a parallel study ^{125}I-insulin (3×10^{-11}mol/ℓ) was added to 400 µℓ of the same cell suspension in the presence of the same increasing concentrations of unlabelled IGF-I and insulin.

After incubation, duplicate portions (200 µℓ) were removed and layered on to 200 µℓ of ice cold HBB. These samples were then centrifuged in a Beckman microfuge. The supernatant was removed and the cell pellet was counted.

Results were calculated as previously detailed in section 2.2.4. However, in these experiments non-specific counts were not subtracted from total binding, because the amount of radiolabelled peptide bound in the presence of excess unlabelled peptide is an important indication of receptor specificity.

7.3.2 Results

7.3.2.1 Competitive Binding for ^{125}I-insulin

Competitive binding between ^{125}I-insulin and unlabelled insulin and IGF-I, is shown in figure 7.2(a). Increasing concentrations of unlabelled insulin in the assay mixture resulted in a progressive decrease in the amount of ^{125}I-insulin bound to the cells. About 33% of radiolabelled insulin remained bound in the presence of excess insulin (10^{-5} mol/ℓ).

By comparison, low concentrations of IGF-I (10^{-11} - 10^{-8} mol/ℓ) did not compete with bound radiolabelled insulin. However, nearly 50% of bound ^{125}I-insulin was displaced by unlabelled IGF-I in the concentration range 10^{-8} to 10^{-6} mol/ℓ. Approximately 50% of ^{125}I-insulin remained bound to the cells in the presence of 10^{-6} mol/ℓ IGF-I.

In both assays, maximum ^{125}I-insulin bound in the absence of unlabelled peptide gave a mean value of 13,53 ± 0,18% of total radioactivity added to the incubation mixture. Non-specific binding in the presence of excess unlabelled insulin and IGF-I was 4,5% and 7%, respectively.

The concentration of insulin which caused half-maximal displacement of bound ^{125}I-insulin (ID_{50}) was in the region of 3×10^{-10} mol/ℓ, whereas the same effect was induced by 1×10^{-7} mol/ℓ unlabelled IGF-I (Table 7.1). Thus, the ratio between the competitive potencies of insulin and IGF-I for radiolabelled insulin binding was about 1:300.

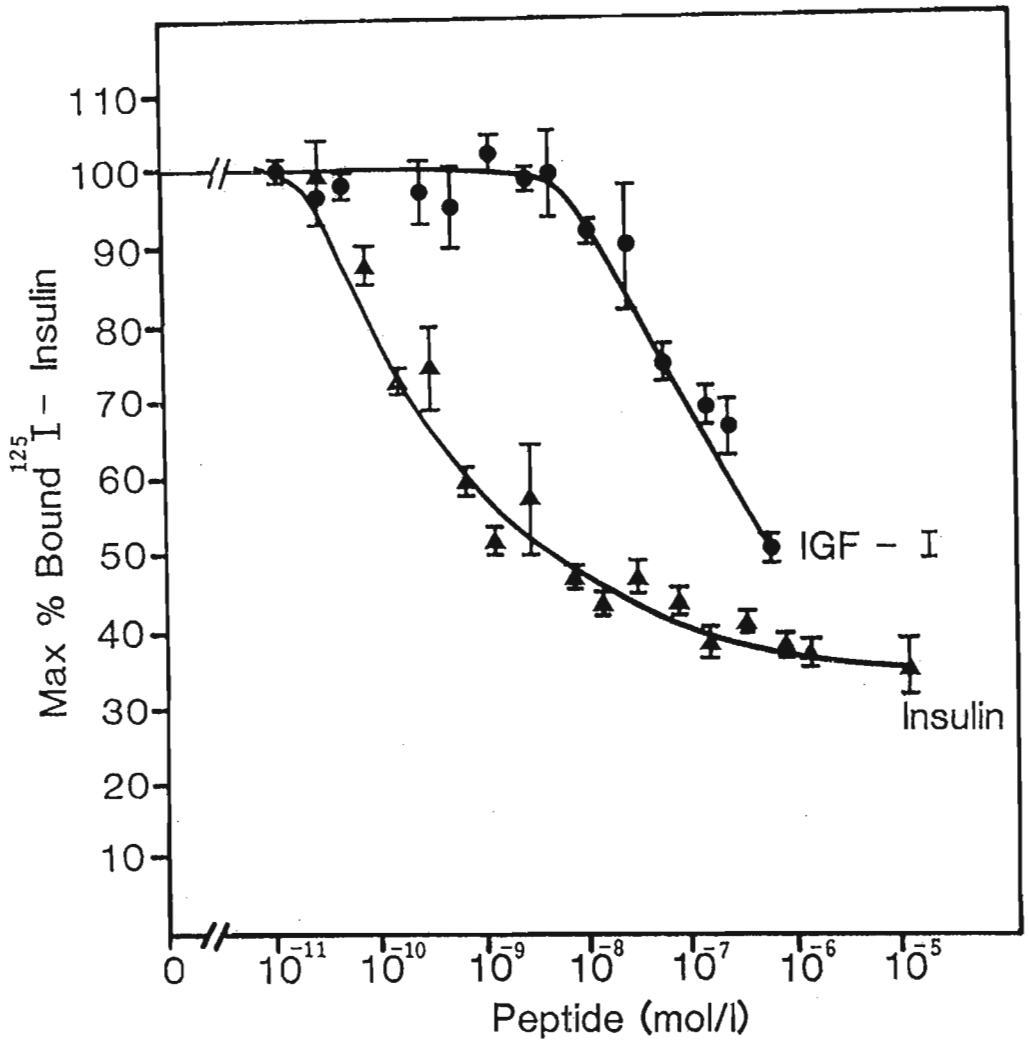


Fig. 7.2 (a) **Competition curve of ^{125}I-insulin and increasing concentrations of unlabelled insulin and IGF-I.** Radiolabelled insulin ($3x10^{-11}$ mol/l) was incubated with increasing concentrations (10^{-11} -10^{-5} mol/l) of unlabelled insulin and IGF-I for 120min at 15°c. Each point is the mean and S.D. of two experiments. Prior to use cells were grown in RPMI abd 10% FCS.

Table 7.1 Insulin and IFG-I Concentrations causing Half-maximal Displacement of ^{125}I-insulin or ^{125}I-IFG-1 (ID_{50})

	Concentration of Unlabelled Peptide	
Raji cells grown in RPMI 1640 and 10% FCS	Insulin (mol/ℓ)	IGF-1 (mol/ℓ)
Displacement of ^{125}I-insulin	3×10^{-10}	1×10^{-7}
Displacement of ^{125}I-IGF-1	augments binding	augments binding
Raji cells grown in SFM		
Displacement of ^{125}I-insulin	4×10^{-10}	-

7.3.2.2 Competitive Binding for ^{125}I-IGF I

Binding of ^{125}I-IGF I to Raji cells grown in 10% FCS, is shown in Figure 7.2 (b). Unlabelled IGF-I in the range 10^{-11} - 10^{-9} mol/ℓ increased binding of labelled IGF-I by about 20% with a peak at 2 x 10^{-10} mol/ℓ. Increasing the concentration of unlabelled IGF-I by one order of magnitude produced an overall decrease of 40% in bound labelled peptide. A further increase in the concentration of IGF-I (10^{-8} - 10^{-6} mol/ℓ) resulted in a second peak at 5 x 10^{-8} mol/ℓ, which represented a 15% increase in ^{125}I-IGF I binding. In the presence of 10^{-6} mol/ℓ unlabelled IGF-I nearly 70% of the radiolabelled peptide was still bound to the cells.

Unlabelled insulin had a similar effect on radiolabelled IGF-I binding to the Raji cells. However, the initial increase in ^{125}I-IGF I binding in the range 10^{-11}- 10^{-9} mol/ℓ unlabelled insulin was slightly lower than that induced by a similar concentration of unlabelled IGF-I. In addition, both peaks occurred at insulin concentrations ten-fold higher than unlabelled IGF-I.

In the presence of a large excess of unlabelled insulin (10^{-5} mol/ℓ approximately 75% of ^{125}I-IGF I remained bound to the cells.

The total amount of ^{125}I-IGF I bound in the absence of unlabelled peptide in both assays gave a mean value of 5,25 ± 0,15% and this was considered maximal. Non-specific binding of labelled peptide was 3,5% in the presence of excess IGF-I (10^{-6} mol/ℓ) and 4% for excess insulin (10^{-5} mol/ℓ).

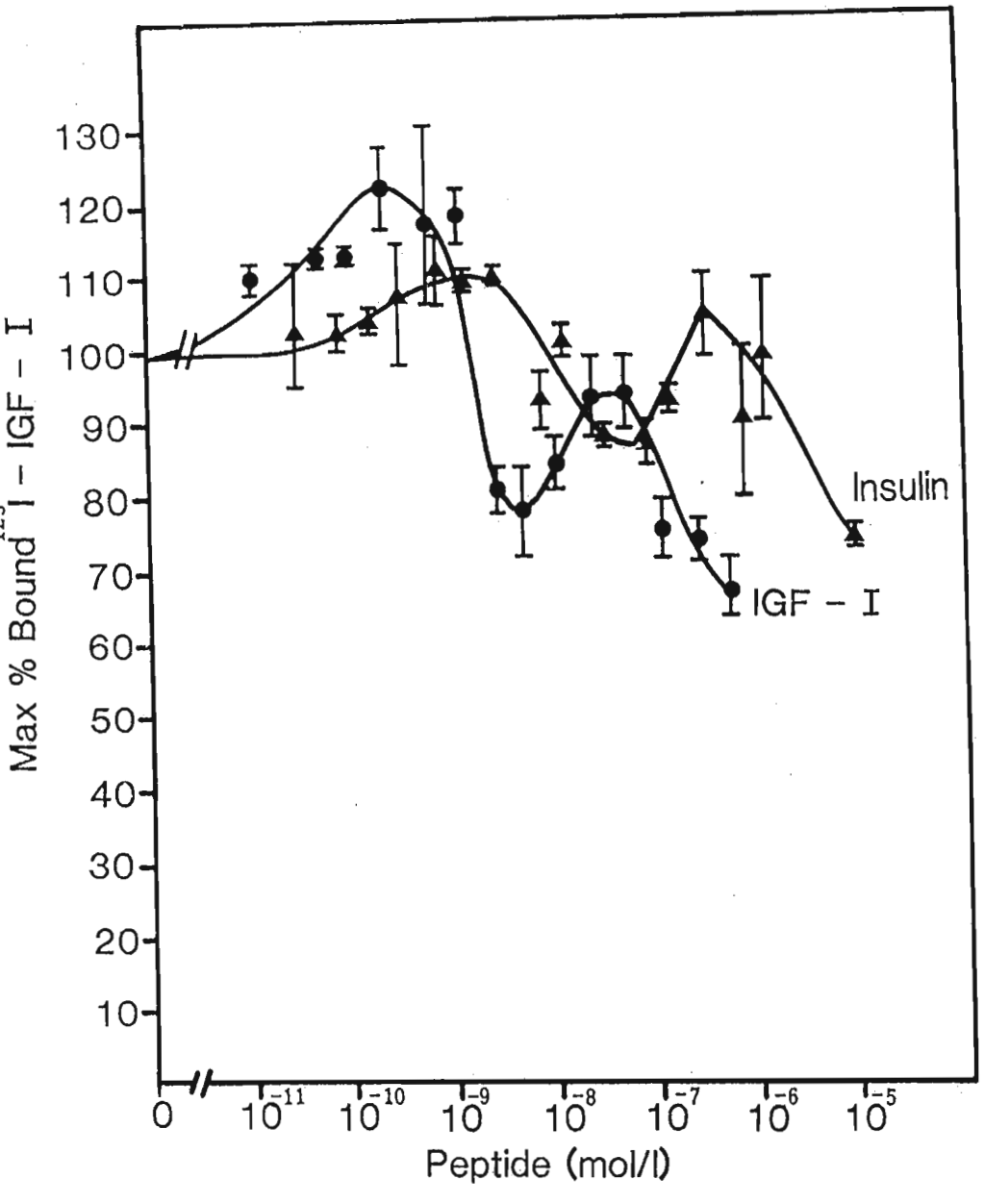


Fig. 7.2 (b) **Competition curve of ^{125}I-IGF-I and increasing concentrations of unlabelled IGF-I and insulin.** ^{125}I-IGF-I (2,5 x 10^{-11} mol/l) was incubated with unlabelled IGF-I and insulin in the range (10^{-11}-10^{-5} mol/l) until steady state at 15°c. Individual points represent the mean and one SD of duplicate assays. Cells were grown in RPMI and 10% FCS.

7.3.2.3 Analysis of Binding Data Using the LIGAND Programme

Analysis of insulin binding data using the LIGAND programme was discussed in detail in section 3.2.1.2. It was found that the Raji cells have two binding sites; a high affinity receptor (K_d 2 x 10^{-10} mol/ℓ) and a low affinity receptor (K_d 2,27 x 10^{-7} mol/ℓ). ^{125}I-IGF I binding data does not fit either the one site binding model or the two site binding model in the LIGAND Programme.

7.3.3 **Discussion**

From the ^{125}I-insulin competition curve it is apparent that physiological concentrations of insulin displaced the labelled ligand, whereas IGF-I only achieved displacement at concentrations three orders of magnitude greater than insulin. Half-maximal displacement of ^{125}I-insulin (Table 7.1), showed that insulin was 300 times more potent than IGF-I in competing for this particular binding site. Thus, it would appear that this cell-line has an insulin receptor which binds insulin with a high affinity, but it also has the ability to bind IGF-I at much higher concentrations. As the ID_{50} for unlabelled insulin and the high affinity K_d, determined by the LIGAND programme have very similar concentrations, the binding site with the highest affinity for insulin is clearly the insulin receptor.

The ^{125}I-IGF I competition curve for Raji cells grown in 10% FCS showed that low concentrations of unlabelled IGF-I and insulin did not compete for ^{125}I-IGF I binding, but rather augmented it. Similar findings have been demonstrated in adipocytes, where physiological

concentrations of insulin were shown to increase binding of labelled IGF-I and IGF-II (Zapf *et al.*, 1978). More recent studies have indicated that when insulin bound to the insulin receptor, the number of IGF-II receptors were increased by inducing a rapid redistribution of type II IGF receptors cycling between an intracellular pool of receptors and the plasma membrane (Wardzala *et al.*, 1984).

As mentioned in section 1.3.3.1, IGF-I cross-reacts with the type II receptor. The increase in ^{125}I-IGF I binding to Raji cells which was induced by insulin may well have been the result of IGF I binding to the type II IGF receptor. However, this finding does not explain the increase in radiolabelled IGF-I binding in the presence of similar low concentrations of unlabelled IGF-I.

In the Raji cells, it was shown that IGF-I only competes for insulin binding sites at concentrations greater than 10^{-8} mol/ℓ and, therefore, it is unlikely that this increase in ^{125}I-IGF I binding is mediated by IGF-I action on the insulin receptor. In fact, lower concentrations of IGF-I were more effective than insulin in augmenting ^{125}I-IGF I binding to these cells. Because IGF-I does not normally upregulate type II IGF receptors (Rechler & Nissley, 1985) the implication is that the receptor which bound labelled IGF-I is not a true type II receptor specifically controlled by insulin action on the insulin receptor.

The marked decrease in ^{125}I-IGF I binding in the presence of 10^{-9} - 10^{-8} mol/ℓ unlabelled IGF-I therefore suggests that this growth factor binds to the same receptor it has induced. Insulin was slightly

less effective than IGF-I in competing for the same binding site. A reasonable conclusion could be that IGF-I, and to a lesser extent insulin, induced a growth factor receptor which bound both peptides in the same concentration range.

Normally, insulin does not bind to the type II IGF receptor; IGF-II is bound to the insulin receptor with a very low affinity (section 1.3.1.2).

Recently, however, Misra *et al.* (1986) reported an unusual growth factor receptor in IM-9 lymphocytes which had the same affinity for insulin and IGF-II, but bound IGF-I with a slightly lower affinity. Jonas and Harrison (1985) identified two distinct type I IGF receptors in material from human placentae. There is therefore, evidence that receptor subtypes for IGF-I and insulin might exist.

Raji cells would appear to have a growth factor receptor which is induced by IGF-I and insulin. The affinity of this receptor for both peptides was closely related to their stimulating effect. Although the insulin receptor system is known to modulate type II IGF receptors in adipocytes (Wardzala *et al.*, 1984), it is not clear from these studies whether the growth factor receptor on Raji cells is an atypical type I or type II IGF receptor. Another possibility is that this growth factor receptor is related to a specific B lymphocyte growth factor.

The large amount of radiolabelled ligand bound in the presence of excess unlabelled insulin and IGF-I, is manifestly unusual. A plau-

sible explanation could be that ^{125}I-IGF I binds to two receptors, one of which is displaced by unlabelled IGF-I and insulin. An analogous situation has been reported for ^{125}I-IGF II binding in the presence of excess IGF-I in human fibroblasts (Rechler *et al*., 1980). It was found that this binding pattern was not consistent and was probably dependent on cell culture conditions.

In the Raji cells it was shown that high concentrations of unlabelled IGF-I and insulin induced a second peak of ^{125}I-IGF I binding (Fig. 7.2 b), and in the following section it will be demonstrated that this effect was influenced by the presence of foetal calf serum in the culture medium.

7.4 *COMPETITIVE BINDING ASSAYS FOR ^{125}I-IGF I AND ^{125}I-INSULIN ON RAJI CELLS GROWN IN SERUM-FREE MEDIUM*

Serum is an important component of most growth media used in tissue culture. Many of the growth effects of serum have been attributed to the presence of appropriate growth factors, hormones and growth inhibitors (Zapf *et al*., 1981). To minimise the effects of exogenous growth factors on the Raji cells, the cells were grown in RPMI 1640 and the percentage FCS was gradually decreased from 10% to 0% over a period of two weeks. Competitive binding assays were then carried out to establish the relative numbers of insulin and type I IGF receptors on cells grown in serum free medium (SFM).

7.4.1 Procedure

Competitive binding assays between ^{125}I-IGF I and unlabelled IGF-I, and ^{125}I-insulin and unlabelled insulin, were carried out as outlined in section 7.3.1.

7.4.2 Results

Insulin binding to Raji cells grown in SFM is shown in Figure 7.3 (a). In the presence of increasing concentrations of unlabelled insulin, the amount of bound ^{125}I-insulin gradually decreased.

Half-maximal displacement of bound radiolabelled insulin occurred in the presence of 4×10^{-10} mol/ℓ unlabelled insulin (Table 7.1).

The amount of ^{125}I-insulin bound in the absence of unlabelled hormone was 13,6 ± 0,08% of total radioactivity and this was considered maximal NSB was 5,5%.

Competitive inhibition of ^{125}I-IGF I binding to Raji cells grown in SFM is shown in Figure 7.3 (b). Radiolabelled IGF-I binding to these cells was increased by approximately 15% in the presence of 3×10^{-11} - 10^{-10}mol/ℓ unlabelled IGF-I. After this initial increase at low concentrations of IGF-I, there was about a 20% decrease in binding at concentrations of 10^{-10} - 10^{-9} mol/ℓ; thereafter increased IGF-I concentrations did not materially affect labelled IGF-I binding.

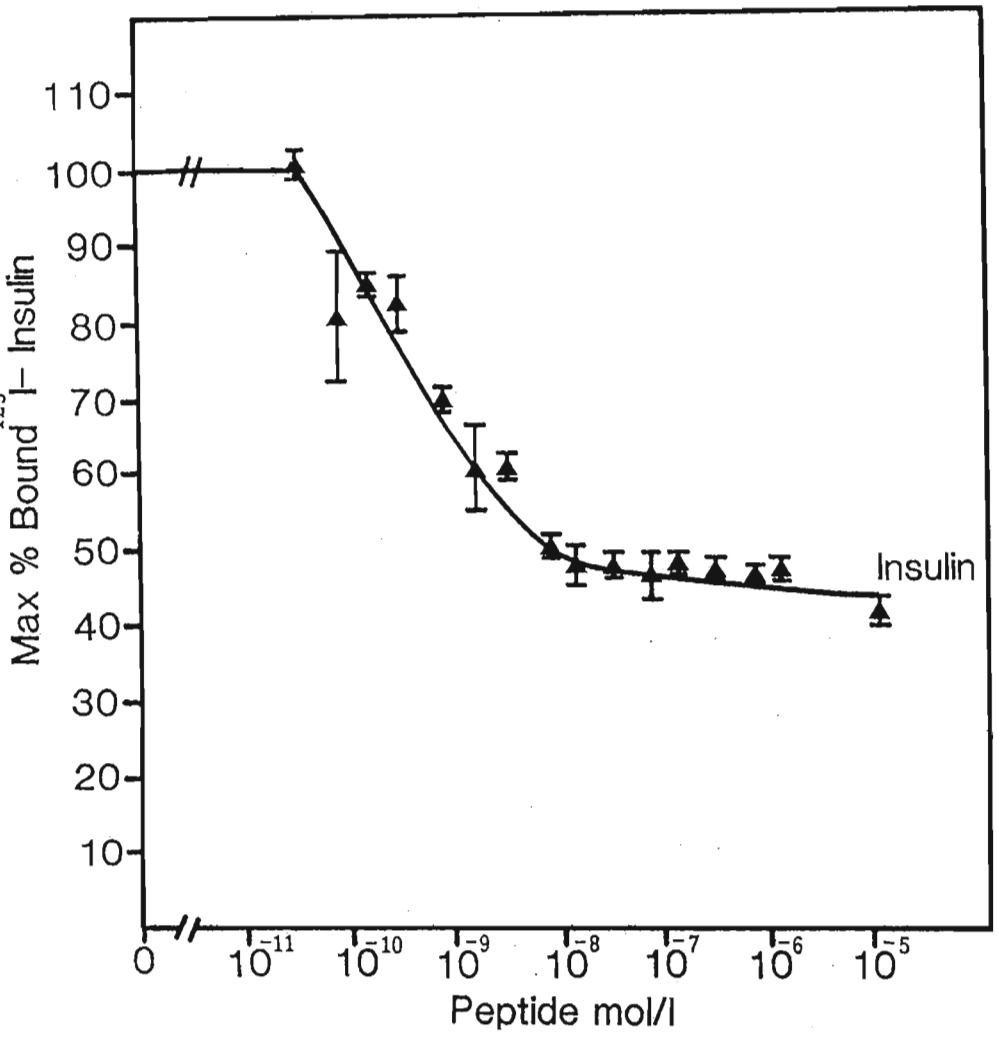


Fig. 7.3 (a) **The insulin competition curve on cells grown in SFM.** Raji cells were grown in SFM prior to measurement of insulin binding under steady state conditions. Binding of radiolabelled insulin ($3x10^{-11}$ mol/l) in the presence of increasing concentrations of unlabelled insulin was determined in duplicate samples.

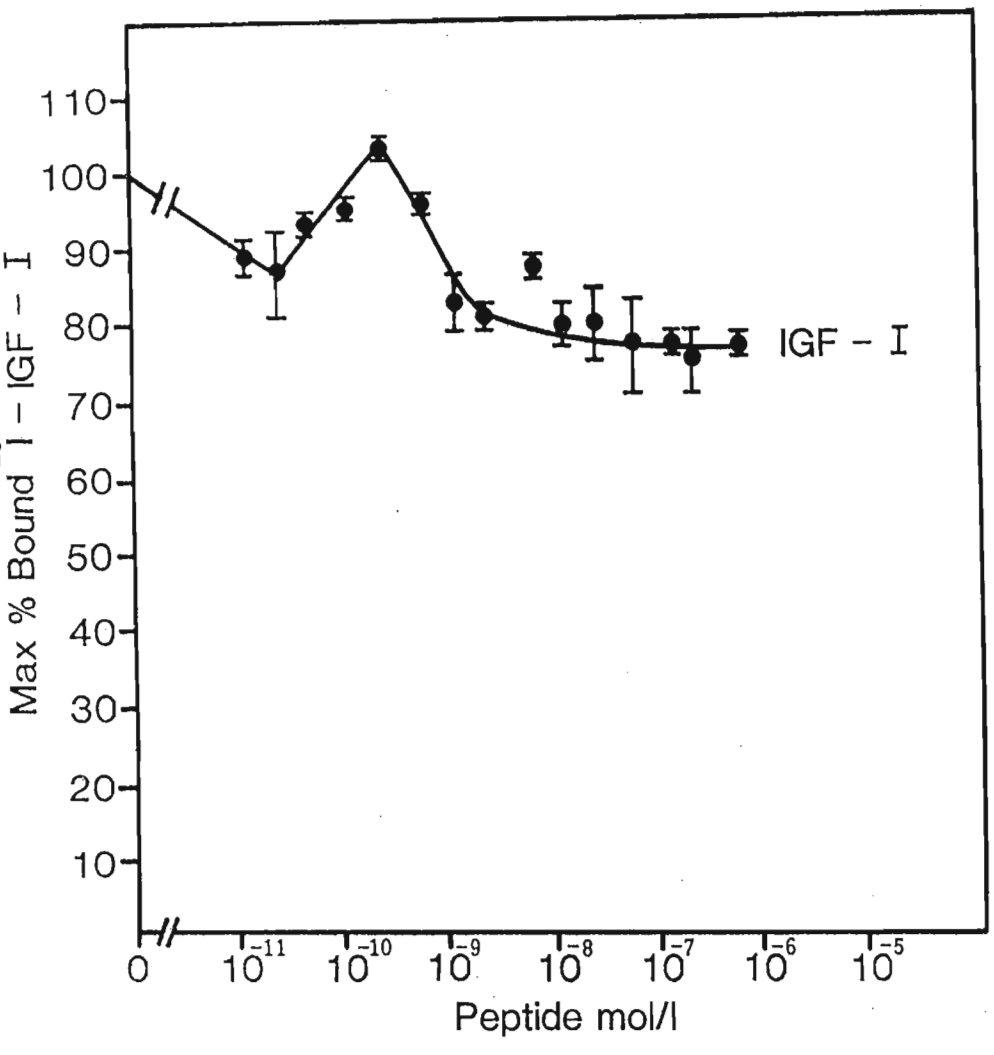


Fig. 7.3 (b) **Competitive binding of IGF-I on Raji cells grown in SFM.** ^{125}I-IGF-I (2,5x10^{-11} mol/l) binding was measured in the presence of increasing molar concentrations of unlabelled IGF-I. All determinations are the mean and one S.D. from duplicate samples.

In the presence of 10^{-6} mol/ℓ unlabelled IGF-I, about 78% of ^{125}I-IGF I was still bound to these cells. In this experiment the maximum amount of ^{125}I-IGF I bound was 5,7 ± 0,45% of total radioactivity and NSB was 4,6%.

The second peak in ^{125}I-IGF I binding which was found at 10^{-8} mol/ℓ unlabelled IGF-I in cells grown in 10% FCS (Fig. 7.2 b) was absent in these cells. However, the amount of labelled ligand bound in the presence of excess unlabelled IGF-I remained high.

7.4.3 Discussion

Insulin competition curves on Raji cells grown in SFM and medium supplemented with 10% FCS were identical (Figs. 7.4 a, and 7.3 a). The insulin receptor on Raji cells was thus unaffected by changes in hormones and other factors found in serum and the conclusion may be drawn that in this particular cell-line the insulin receptor was a stable component of the cell membrane and independent of receptor induction by exogenous factors. The implication is that the insulin receptor must play an important role in regulating cell metabolism and replication.

An interesting finding was that the increase in ^{125}I-IGF I binding in response to low concentrations of IGF-I on cells grown in serum supplemented medium was retained in cells grown in SFM. In contrast, the same effect at higher concentrations of IGF-I was absent in cells grown in SFM. Presumably therefore, factors present

in FCS are a pre-requisite for the stimulatory effect on receptor induction by high concentrations of IGF-I.

The failure to induce receptors at high concentrations of IGF-I would suggest that these receptors are not fundamental to Raji cell metabolism.

Nevertheless, the large amount of radiolabelled ligand bound in the presence of excess unlabelled IGF-I was also found in cells grown in SFM, which suggests that the labelled ligand was bound to a receptor from which it is not displaced by excess unlabelled IGF-I.

Thus, at least 70% of bound ^{125}I-IGF I was non-specific binding. As mentioned earlier, Rechler *et al.* (1980) found that ^{125}I-IGF II bound to human fibroblasts could not be completely displaced with unlabelled IGF-I. However, this binding pattern was not reproducible and was probably determined by the cell culture conditions. These observations highlight the fact that most growth factors cross-react with a number of related receptors and, therefore, the binding patterns are a composite of these interactions. Until all the relevant growth factors and their specific receptors are identified these binding assays will only provide a partial understanding of specific ligand-receptor interaction.

In an attempt to relate ligand-receptor binding to the biological effect of a particular hormone or growth factor, dose-response studies were therefore undertaken.

7.5 INSULIN AND IGF-I STIMULATION OF α-AMINOISOBUTYRIC ACID TRANSPORT IN RAJI CELLS

It was previously mentioned in section 1.3.1.2 that appropriate concentrations of IGF-I and insulin have a number of common biological effects. These biological actions can be divided into two types: acute metabolic effects which involve the rapid modulation of membrane-transport systems or enzyme activities, and long-term effects such as DNA synthesis (Zapf *et al*., 1981).

The acute effects of IGF I and insulin were investigated by measuring stimulation of amino acid transport across the cell membrane. Cellular uptake of radiolabelled α-aminoisobutyric (AIB), an analogue of alanine which is not metabolised, was measured in response to increasing concentrations of IGF I and insulin (Martin & Pohl, 1979).

7.5.1 **Materials**

α- methyl-^{3}H -Aminoisobutyric acid with a specific activity of 33,5 Ci/mmol was supplied by NEN Research Products, Boston, USA. Unlabelled α-aminoisobutyric acid was obtained from Sigma Chemical Company, St Louis, USA.

The incubation buffer was Earle's balanced salt solution (EBSS) pH 7,4 (5,4 mmol/ℓ KCl, 1,8 mmol/ℓ $CaCl_2$, 116 mmol/ℓ NaCl, 1 mmol/ℓ $NaHPO$, 25 mmol/ℓ Tris-HCl and 0,1% bovine serum albumin). 0,1% Sodium dodecylsulphate (SDS) was used to solubilise the cell preci-

pitate before addition to the scintillation cocktail. Instagel was purchased from Beckman Instruments, Illinois, USA.

Cells were gradually adapted to serum-free medium as described in seciton 7.4. Twenty four hours before use the cells were incubated with Iscoves modified Dulbecco's medium (IMDM), supplied by Flow Laboratories International.

7.5.2 Procedure

Twenty four hours after feeding Raji cell cultures, the cells were harvested by centrifugation at 200 g for 5 min. The cell pellet was resuspended in the same volume of EBSS and incubated at 37°C for 1 hour to facilitate the dissociation of any membrane bound hormones. Following incubation, cells were centrifuged at 200 g for 5 min and resuspended in the same volume of fresh EBSS. Duplicate portions of cell suspensions (450 $\mu\ell$) were pipetted into test tubes which contained 50 $\mu\ell$ of insulin or IGF-I, respectively (final concentrations: 0, 10^{-10} 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/ℓ). The incubation mixtures were maintained at 37°C for 4 hours, after which 10 $\mu\ell$ of ^{3}H-AIB and unlabelled AIB were added to each test tube to give a final concentration of 0,3 μCi ^{3}H-AIB and 0,1 mmol/ℓ AIB per tube, respectively. This was followed by a further incubation at 37°C for 25 min and three washes with EBSS at 4°C. The cell pellet was solubilised with 500 $\mu\ell$ of 0,1% SDS at 37°C. Portions (400 $\mu\ell$) were transferred to scintillation vials containing Instagel and counted in a Beckman LS 1800 Spectrophotometer which had a counting efficiency of about 51%.

7.5.3 Calculations

The number of disintegrations per minute (dpm), was calculated by dividing counts per minute (cpm) by the counting efficiency

$$\frac{cpm}{efficiency} = dpm.$$

Uptake of AIB (nmol) was calculated using the following formula where 1 nCi = 2,22 x 10^3 dpm (Thach & Newburger, 1972):

$$\text{nmol AIB} = \frac{\text{no. of dpm per tube}}{(2{,}22 \times 10^3)\ (\text{SA of AIB, nCi/nmol}).}$$

Results were expressed graphically as nmol AIB uptake per 10^8 cells versus hormone concentration (mol/ℓ).

7.5.4 Results

Stimulation of amino acid transport in Raji cells by increasing concentrations of IGF-I and insulin is shown in Fig. 7.4. The presence of increasing concentrations of insulin in the incubation medium resulted in a dose-related increase in ^{3}H-AIB uptake (0,34 - 0,48 nmol), which reached a plateau at 10^{-7} mol/ℓ insulin. IGF-I, on the other hand, did not stimulate ^{3}H-AIB transport in the Raji cells.

Half-maximal stimulation of amino acid uptake by insulin was about 5 x 10^{-10} mol/ℓ in Raji cells (Table 7.2)

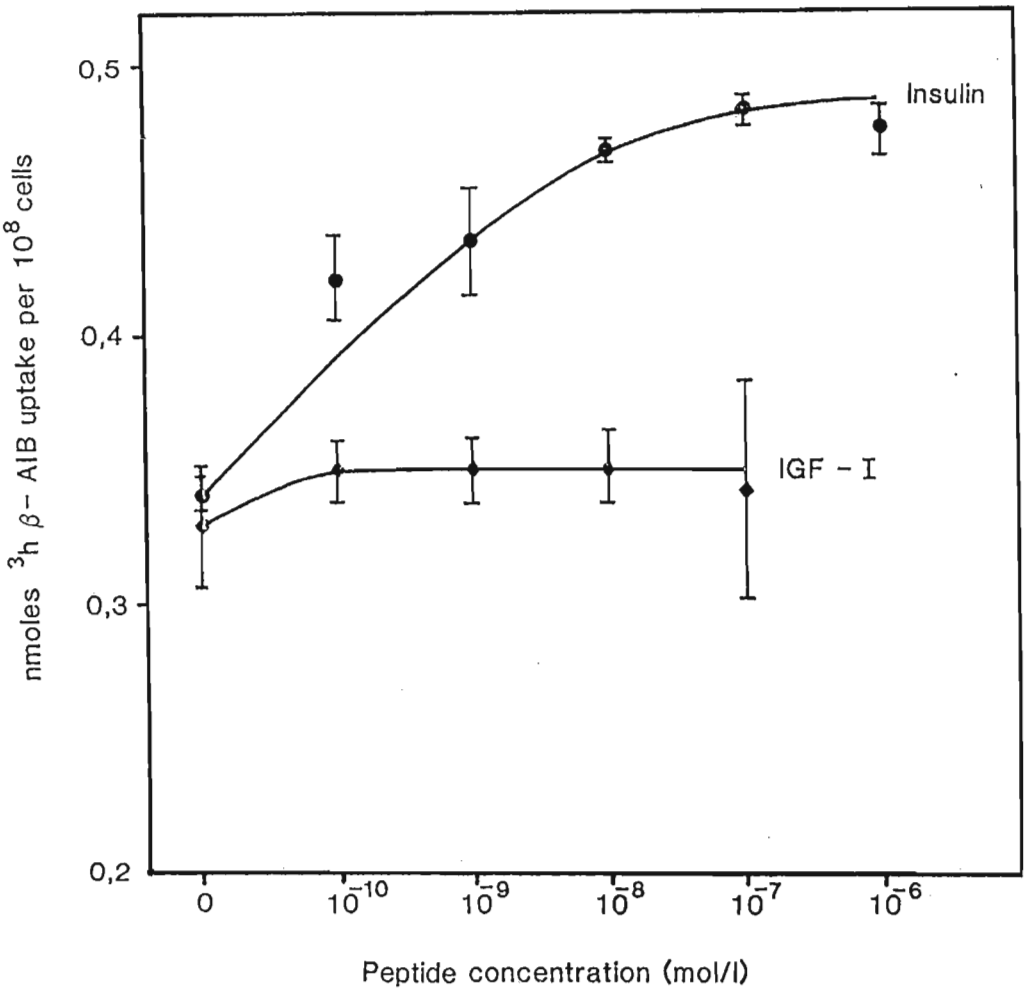


Fig. 7.4 **Insulin and IGF-I stimulation of α - aminoisobutyric acid uptake in Raji cells.** The effect of increasing molar concentrations of insulin and IGF-I on α -AIB transport in Raji cells was determined as described in section 7.5.2. Each point is the mean and one S.D. of quadruplicate measurements.

Table 7.2 Insulin and IGF-I Concentrations Eliciting Half-maximal Stimulation of α-AIB Uptake and DNA Synthesis

	Concentration of Unlabelled Peptide	
	Insulin (mol/ℓ)	IGF-I (mol/ℓ)
Half-maximal stimulation of α-AIB uptake	5×10^{-10}	0
Half-maximal stimulaiton of DNA synthesis	1×10^{-11}	2×10^{-11}

7.5.5 Discussion

It was concluded earlier (section 7.4.3), that the insulin receptor plays an important role in Raji cell metabolism. Support for this proposal was provided by the experimental stimulation of α-AIB uptake. Insulin stimulation of α-AIB transport was maximal at physiological concentrations of insulin. Half-maximal stimulation of α-AIB uptake corresponded with the ID_{50} of unlabelled insulin for the insulin receptor, 5×10^{-10} mol/ℓ and 3×10^{-10} mol/ℓ, respectively.

By comparison, increasing concentrations of IGF-I had no significant effect on amino acid transport in Raji cells. These results support the concept that the acute metabolic actions of insulin on these cells are mediated via the insulin receptor.

The longer term biological effects of IGF-I and insulin were next studied, to determine whether insulin or the type I receptor mediated the growth response.

7.6 *MEASUREMENT OF INSULIN AND IGF-I STIMULATION OF DNA SYNTHESIS IN RAJI CELLS*

To assess the relative potencies of insulin and IGF-I as stimulators of cell-proliferation, cells were exposed to increasing concentrations of each peptide and ^{3}H-thymidine uptake was estimated as a measure of cell proliferation (Rechler *et al.*, 1974).

If this biological response is mediated by ligand interaction with the insulin or type I IGF receptor, then the stimulatory effect should be reflected by receptor affinity for that ligand.

As mentioned earlier in section 7.4, the main function of serum in tissue culture is to provide essential hormones and growth factors.

The advantage of using serum-free medium (SFM), when measuring a proliferative response to specific growth factors, is the absence of unknown factors capable of blocking or facilitating cell growth.

For this reason Iscoves modified Dulbecco's medium (IMDM), which is a serum-free medium, was developed specifically for these studies (Gersten & Cohn, 1987). These authors have shown that IMDM optimally supports the proliferative response of T-lymphocytes to mitogen stimulation.

7.6.1 **Materials**

Methyl-^{3}H-Thymidine with a specific activity of 6,7 Ci/mmol was purchased from NEN Research Products, USA.

Microwell plates (96 wells) were obtained from NUNC, Denmark.

Glass fibre filters were supplied by Whatman, UK.

Iscoves modified Dulbecco's medium was obtained from Flow Laboratories, UK.

Suppliers of unlabelled insulin and IGF-I were as in section 7.2.1.

RPMI 1640 was purchased from Flow Laboratories, UK.

7.6.2 Procedure

Raji cells were grown in RPMI 1640 medium supplemented with decreasing amounts of FCS. Once the cells in RPMI 1640 and 1% FCS reached stationary growth phase, they were centrifuged at 200 *g* for 5 min. The cell pellet was then resuspended in the same volume of IMDM without FCS. The concentration of the cells was adjusted to 1-1,3 x 10^6 cells/mℓ and 150 µℓ of cell suspension (in sextuplicate) was added to each well of a 96 microwell plate. A further 50 µℓ of IMDM containing IGF-I and insulin in the concentration range 10^{-12} - 10^{-7} mol/ℓ was added to the cell suspension. Cells were grown for 72 hr at 37°C in a humidified 6% CO_2 atmosphere as described by Gordon *et al*. (1985).

After incubation 50 µℓ of ^{3}H-Thymidine was added to each well to give a final concentration of 2 µCi/mℓ; incubation was then continued for a further 4 hours at 37°C in 6% CO_2 and air.

Cells were harvested onto fibreglass filter paper using a multisample automatic harverster, washed with distilled water, dried and then dissolved in Instagel. Samples were counted in a Beckman LS 1800 spectrometer with a counting efficiency of about 47%.

7.6.3 Results

Results were presented as a plot of % maximal H-Thymidine uptake (y-axis), or cpm (opposite the y-axis), versus peptide concentration.

Stimulation of ^{3}H-Thymidine uptake by Raji cells 72 hrs after incubation with increasing concentrations of IGF-I and insulin is shown in Fig. 7.5.

Insulin and IGF-I were equipotent in stimulating ^{3}H-Thymidine uptake in Raji cells. Cell proliferation increased with increasing concentrations of both peptides and reached a peak at 10^{-9} mol/ℓ.

Maximal stimulation by IGF-I was maintained over a 100-fold concentration range (10^{-9} - 10^{-7} mol/ℓ). By comparison, there was a slight drop in insulin stimulation over the same concentration range. The ID_{50} values for insulin and IGF-I were very similar, 1×10^{-11} mol/ℓ and 2×10^{-11} mol/ℓ, respectively (Table 7.2).

7.6.4 Discussion

It is well known that pharmacological doses of insulin stimulate cell proliferation in tissue culture (Zapf *et al*, 1981). On the other hand, IGF-I was able to stimulate cell growth at molar concentrations, 50-fold lower than insulin. As discussed in section 1.3.1.2, the type I receptor has a greater affinity for IGF-I than insulin. Thus, it was initially thought that both IGF-I and insulin stimulated cell growth by acting on the type I IGF receptor. Although this hypothesis was true for human fibroblasts (King *et al.*, 1980) and chick embryo fibroblasts (Rechler *et al.*, 1980), a number of exceptions have since been reported (Rechler & Nissley, 1985).

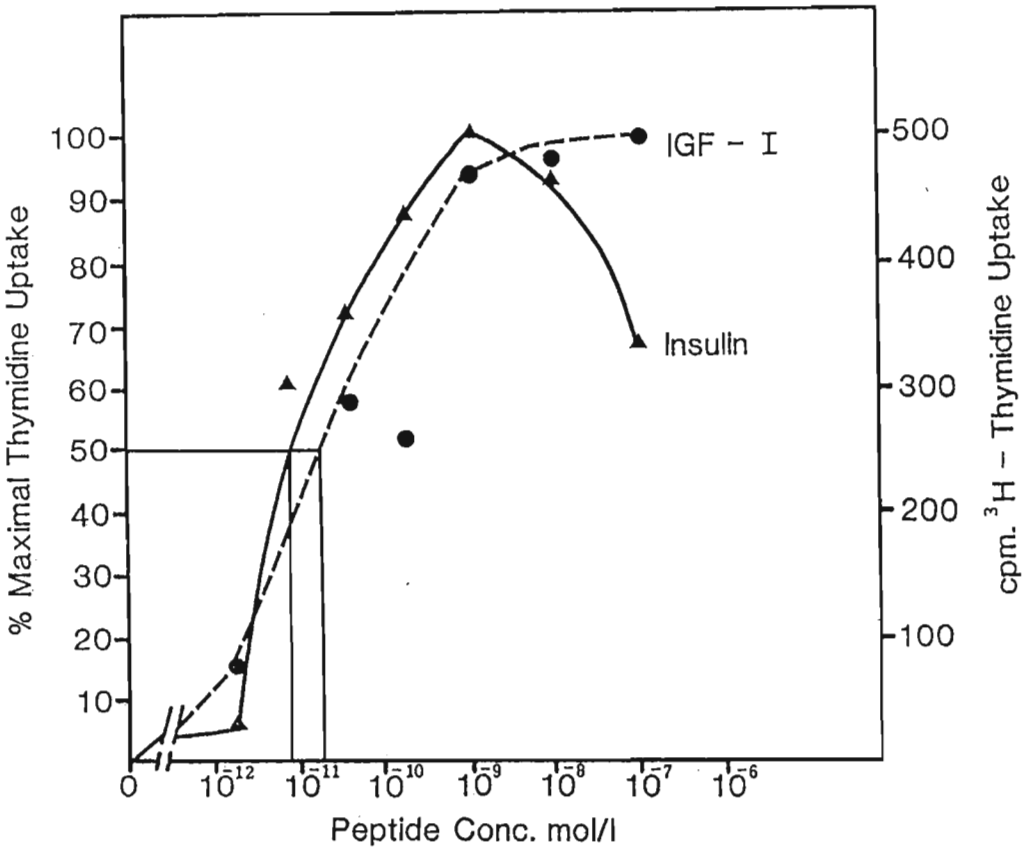


Fig. 7.5 **Stimulation of ^{3}H-thymidine uptake with increasing concentrations of insulin and IGF-I.** Raji cells were pre-incubated with increasing molar concentrations of insulin and IGF-I for 72 hours. After incubation, ^{3}H-thymidine was added to the cells and incubated for a further 4 hours. Cells were harvested, washed and the amount of ^{3}H-thymidine incorporated in the cells was determined. All measurements were done in sextuplicate.

In the Raji cells, concentrations of insulin in the physiological range (2×10^{-12} - 10^{-9} mol/ℓ) induced an increase in cell proliferation. These results implied that insulin action via the insulin receptor, stimulated cell growth. Convincing evidence that insulin acts as a mitogen through the insulin receptor has been reported in H-35 rat hepatoma cells (Koontz & Iwahashi, 1981) and F-9 teratocarcinoma cells (Nagarajan & Anderson, 1982).

In the Raji cells it was apparent that, if the dose - response curve for insulin had been continued with higher concentrations, it would probably result in a bell-shaped curve which is a typical lymphocyte response to mitogen stimulation (Di Sabato *et al.*, 1987). These observations indicate that in Raji cells, physiological concentrations of insulin stimulate cell growth through the insulin receptor.

However, the ID_{50} for insulin displacement of bound ^{125}I-insulin was ten-fold higher than the half-maximal stimulation of DNA synthesis. More importantly, these results have shown that IGF-I and insulin are equally potent in stimulating DNA synthesis. Equivalence in action may have been mediated by individual high affinity receptors or by a receptor which bound both ligands with the same affinity. Binding studies on Raji cells have established the presence of an atypical growth factor receptor which had a similar competitive binding profile for both unlabelled insulin and IGF-I. Low concentrations of both hormones (10^{-11} - 10^{-9} mol/ℓ) induced this receptor with which there was some cross-reactivity (section 7.3.2.2).

Because the binding profiles of insulin and IGF-I for this growth factor receptor were so similar, and because the mitogenic effects of these peptides were quantitatively identical, growth stimulation appeared likely to have been mediated by this induced receptor.

If this inference is correct, physiological levels of IGF-I and insulin would be capable of inducing a membrane receptor with which they subsequently interact with resultant stimulation of DNA synthesis.

In section 1.8 it was mentioned that Raji cells in early log phase produce a B-cell growth factor (BCGF) which can induce cell replication, presumably via a BCGF receptor (Gordon *et al.*, 1985). These authors suggested that the evolution of B-cell tumours represents a more efficient utilisation of BCGF or, alternatively, a growth factor-independent mechanism for autonomous growth. According to their model of B-cell lymphomagenesis the Raji cells are still responsive to an autologous growth factor. In view of the BCGF autocrine loop identified in Raji cells, it is quite conceivable that this growth factor might induce its own receptor, bind to it and thus stimulate cell growth. Indeed, this mechanism of action would result in extremely efficient use of low concentrations of autologous BCGF.

Manifestly, the Raji cells have a highly efficient growth factor receptor which is induced by low levels of IGF-I and insulin. Very low concentrations of these peptides stimulate DNA synthesis. As

the mechanism of action for IGF-I and insulin in Raji cells is similar to that postulated for BCGF (Gordon *et al.*, 1985) the two pathways could be the same.

It is suggested that this atypical growth factor receptor identified on these cells is in fact a BCGF receptor which cross-reacts with IGF-I and insulin.

7.7 CONCLUSION

Raji cells have a high affinity receptor which is specific for insulin. It is not induced by insulin or any growth factor.

In contrast, these cells have an unusual growth factor receptor which is induced by low concentrations of both insulin and IGF-I. This receptor has a relatively high affinity for IGF-I although about 70% of the binding to Raji cells is not displaceable, which suggests that IGF-I is not binding to a specific type I IGF receptor. A possible explanation for the high NSB is that IGF-I, and to a lesser extent insulin, bind to a specific B lymphocyte growth factor receptor, probably the BCGF receptor.

The acute metabolic effects of physiological concentrations of insulin on amino acid transport are mediated by the insulin receptor, whereas IGF-I does not demonstrate this response.

Growth promoting effects of insulin and IGF-I do not appear to be mediated via their specific receptors. A more likely mechanism is that these peptides induce a third receptor, the BCGF receptor, then bind to the induced receptor and through this binding mediate cell proliferation.

SUMMARY

This study has shown that the Raji cells have an insulin receptor on the cell membrane which is specific for insulin. Kinetic analysis of insulin binding data suggested the presence of two insulin binding sites, one of which had a high affinity for the ligand whereas the other had a low affinity for insulin. There was also some evidence of site-site interactions induced by a change in the conformation of the receptor. It was, therefore concluded, that the insulin receptor on these cells is best described by the receptor model which includes heterogeneous binding sites and negative co-operativity.

Physiological concentrations of insulin induced downregulation of the membrane receptor, which suggested that the insulin receptor is functional. However, degradation of internalised insulin did not occur in the lysosomes.

Affinity crosslinking of ^{125}I-insulin to the membrane receptor indicated the presence of a 130 kD insulin binding site and a non-reduced receptor with a molecular weight of about 300 kD. These findings support the structural model proposed by Czech, 1985.

Raji cells did not have a specific type I IGF receptor. Nevertheless, it was shown that low concentrations of insulin and IGF-I induced a growth factor receptor and both peptides appeared to cross-react with this receptor.

Insulin stimulation of amino acid transport in the Raji cells was mediated by the insulin receptor. In contrast, stimulation of cell proliferation by both insulin and IGF-I was probably mediated by regulation of the BCGF receptor.

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