THE MECHANISM OF D-GLUCOSE UPTAKE IN RAT ADIPOCYTES

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By

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To
  My Wife, Donna
    and My Mother, Theresa

About the Author

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No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other University or other institution of learning.

ABSTRACT

Insulin treatment of rat adipocytes effectively increased D-glucose uptake 3- to 7-fold. Cytochalasin B completely inhibited stereospecific D-glucose uptake in both basal and insulin stimulated adipocytes. Prolonged incubation of adipocytes at low temperatures (20^oC) resulted in enhancement of D-glucose uptake.

The role of calcium (Ca^{2+}) in the mechanism of insulin enhancement of hexose transport was investigated. Removal of extracellular Ca^{2+} from the incubation buffer resulted in -30% inhibition of insulin stimulation of transport activity, while addition of Ca^{2+} ionophore A23187 to basal cells resulted in a significant, but not maximal, increase in glucose uptake. Furthermore, adipocytes depleted of intracellular Ca^{2+} using A23187 in conjunction with EGTA failed to undergo stimulation of sugar transport upon insulin treatment. A possible role of Ca^{2+} in insulin activation of sugar transport is discussed.

The protein internalization inhibitor phenylarsine oxide (PhAsO) has been used to investigate the mechanism of D-glucose uptake. PhAsO rapidly inhibits the stereospecific uptake of D-glucose in both basal and insulin stimulated adipocytes. The inhibition is dose dependent and is partially reversed by dithiothreitol (DTT). The results are consistent with a direct interaction between the glucose transporter and PhAsO. By manipulating the sequence of exposure of cells to PhAsO and insulin it is possible to differentiate between the effects of PhAsO on transport into cells with receptor-rich and transporter-rich plasma membranes. PhAsO rapidly inhibits transport in insulin-stimulated adipocytes but at low concentrations inhibition is

transient and recovery of stereospecific uptake takes place after approximately 20 minutes. This transient inhibition of D-glucose uptake by low PhAsO concentrations also occurs when insulin stimulated adipocytes were treated with monensin, a drug reported to trap hormone-receptor complexes within endosomes.

$125I$-iodoinsulin binding experiments conducted on isolated rat adipocytes and plasma membrane preparations clarified that PhAsO ($10\mu M$) was a potent inhibitor of protein internalization and demonstrated that this arsenical did not inhibit insulin binding at concentrations where severe, permanent inhibition of D-glucose uptake occured. Scatchard analysis for the effect of PhAsO ($10-40\mu M$) on insulin binding did not reveal any significant alterations in the binding constant or in the total number of binding sites. Higher concentrations of PhAsO ($>50\mu M$) did however cause a marked decrease in specific binding. The presence of high "cold" insulin concentration did not prevent or reverse PhAsO inhibition of binding. DTT was able to effect a small reversal of PhAsO binding inhibition, however this reducing agent was itself found to markedly inhibit insulin binding in rat adipocytes.

A low pH (glycine/HCl) wash technique was used for removing surface bound insulin. Maximally stimulating insulin concentrations resulted in ~37% decrease of surface bound insulin. Monensin caused a significant accumulation of cell associated $125I$-iodoinsulin activity in a dose dependent fashion. Over short time periods this agent was quite effective in restricting ligand destruction, however with increasing time the ionophore was less efficient in preventing loss of label.

The data show that PhAsO is a potent inhibitor of stereospecific D-glucose transport and protein internalization. The effect of PhAsO on transport inhibition can be interpreted in terms of the recruitment mechanism of insulin stimulation of transport and demonstrate that a relatively large intracellular pool of transporters exists after insulin simulation. It also follows that sulphydryl groups probably play a critical role in the mechanism of glucose uptake and it is suggested that the internalized insulin receptor complexes may retain some biological activity.

CONTENTS

CHAPTER 2 - MATERIALS AND METHODS

CHAPTER 3

Section a: Activation of D-glucose Transport Activity

Section b: Role of Calcium (Ca^{2+}) in the Stimulation of Glucose Transport

CHAPTER 4

The Effect of Phenylarsine Oxide (PhAsO) on D-Glucose Uptake.

CHAPTER 5 - Insulin Binding to Rat Adipocytes

CHAPTER I

GENERAL INTRODUCTION

1.1   INTRODUCTION: THE DIABETIC PROBLEM

Endocrine systems function to deliver information that regulates the metabolism of target tissues. The net effect of this signal/response interaction is homeostasis. It is not surprising that many hormones exert their primary action via specific plasma membrane receptors since this organelle is the first cellular component encountered by any stimuli external to the cell. The peptide hormone insulin, exerts its action by binding to such specific plasma membrane receptors (Corin and Donner, 1982).

Insulin was first identified as a heat labile factor in pancreatic extracts by Banting and Best in 1921. It is secreted from the pancreas primarily under the stimulation of elevated blood glucose and it is a major regulator of mammalian metabolic homeostasis, acting as the body's signal of the fed or fasted state. However, more than fifty years after the discovery of insulin, its cellular mechanism still remains one of the major obstacles in cell biology (Van Obberghen and Gammeltoft, 1986).

Glucose enters cells via two routes:

(i) A non-saturable diffusion through the lipid bilayer dependent only on the concentration gradient and
(ii) A saturable carrier mediated diffusion which is specific for the D-glucose stereoisomer, and is under the influence of insulin.

The importance of glucose transport is well appreciated when one considers diabetes and the subsequent complications that arise in the diseased state.   Unfortunately neither the rate of appearance of

complications nor the diabetic's reduced life expectancy as compared to the general population has substantially changed in recent years (Olefsky and Sherwin, 1985).

Diabetes mellitus occurs because of an insufficiency of sensitivity to insulin resulting in hyperglycaemia. There is an increased production of glucose in the liver and failure of peripheral tissues such as muscle and fat to take up glucose, "starvation in the midst of plenty" (Hardy, 1981).

Hyperglycaemia is a consequence of a relative or absolute deficiency of insulin in the presence of a relative or absolute excess of glucagon (Unger and Foster, 1985). When the insulin deficiency is extreme, the hormonal abnormalities are responsible for the syndrome of diabetes mellitus. As the disease develops ketoacidosis, thirst, polyuria, tiredness, and muscle wasting are not uncommon. Later dehydration, decreased blood volume and circulatory failure occurs resulting in coma and death.

Diabetes mellitus can be divided into two major categories.

(a) Type 1 (Insulin-Dependent)

This diabetic state is characterized by low or absent endogenous insulin secretion, although insulin sensitivity in peripheral tissue is maintained. This state is due largely to pancreatic abnormalities i.e.

(i)   Absence of, or reduced β-cell mass.

(ii)   Defects of β-cell glucoreceptor sensitivity.

(iii) Defective conversion of proinsulin into insulin.

(iv)   Production of structurally defective insulin molecules.

(b) Type II (Non-Insulin-Dependent)

This is characterized by normal or increased insulin secretion in the earliest stages, however, in advanced cases insulin secretion is low. The β-cell mass is only moderately reduced and there is a tendency to insulin resistance in the peripheral tissues. This insulin resistance occurs at the cellular level and may be due to:

(i)   A reduced concentration of normal insulin receptors (type A insulin receptor defect syndrome).

(ii)   Reduced affinity of insulin receptors (type B insulin receptor defect syndrome).

(iii) Failure of receptor to couple insulin binding to signal generation i.e. defect distal to the hormone binding site.

(Hall *et al.*, 1980; Unger and Foster, 1985; Grunberger *et al.*, 1984; Stryer, 1981; Taylor *et al.*, 1984).

It is at the cellular level that this work is concerned. The interactions of insulin with its target tissues are extremely complex. Insulin binding to it's receptor manifests itself initially in structural and chemical modifications of the receptor which ultimately results in some as yet undefined biological signal. Hence a sound understanding of all the factors involved is required to elucidate the mechanism of insulin action and hopefully move closer to resolving the diabetic problem at the cellular level.

1.2   THE PHYSIOLOGICAL ROLE OF INSULIN

1.2.1   Structure of Insulin

Insulin is a small polypeptide of 51 amino acids arranged into two separate peptide chains, designated A and B, which are linked by disulphide bonds (Ryle et al., 1955; Sanger et al., 1959). High resolution x-ray crystallographic study of the secondary and tertiary structure have revealed three α-helical regions, A2-A8, A13-A19 and B9-B19; contained in a compact three dimensional globular structure (Blundell et al., 1972). Investigation of the amino acid sequence of 28 different insulins of vertebrate origin have revealed only minor structural variations (Blundell and Wood, 1975) and the molecule has been found to contain a highly conserved bioactive site incorporating residues from both A and B chains (Pullen et al., 1976).

1.2.2   Synthesis, Storage and Secretion

Insulin is synthesized in the β-cells or the endocrine pancreas, from where it is secreted in response to a variety of stimuli. It is believed that β-cells contain glucoreceptors sensitive to any rise in blood glucose above the normal fasting levels (Hall et al., 1980; Porte and Halter, 1981). The hormone is derived from a single chain precursor, proinsulin, which is synthesized in the rough endoplasmic reticulum. Once formed proinsulin is transferred to the golgi complex where proteases sequentially cleave the connecting peptide between the carboxyl- and amino-ends of B and A chains respectively, liberating the double chain insulin (Zuhlke et al., 1976; Steiner et al., 1972). The hormone is stored in β-granules in crystalline form. Glucose promotes the synthesis of proinsulin and favours calcium entry which

activates the microtubular system, facilitating granule migration and extrusion, a process called emiocytosis (Hall *et al.*, 1980; Hardy, 1981; Porte and Halter, 1981). Once secreted much of the insulin in the circulating plasma appears bound to a β-globulin.

1.2.3 *Physiological Effects of Insulin*

Insulin illicits a wide range of biological responses (Table 1.1). Insulin is primarily anticatabolic, i.e. antilipolytic and antiglycogenolytic, favouring glucose utilization for anabolic processes. Stimulation of membrane transport of sugars, amino acids, ions and nucleotide precursors, activation of membrane-bound and soluble enzymes, e.g. ATPase, cAMP-phosphodieterase, inhibition of protein degradation, stimulation of protein, RNA and DNA synthesis, e.g. in fibroblasts, are examples of the diverse cellular effects of insulin (Parman, 1979).

The major metabolic effect of insulin however, is to enhance glucose transport across cell membranes, thus increasing glucose utilization in most body tissues, particularly muscle and fat (Hall *et al.*, 1980). Though all tissues are capable of utilizing glucose, not all are sensitive to insulin. The metabolic needs of the brain, liver, renal medulla, leucocytes and peripheral nerves, necessitate a constant supply of glucose and hence sugar transport in these tissues is independent of insulin (Cahill, 1976).

Table 1.1 Metabolic Effects of Insulin

Metabolic Pathway	Effect	Tissues
Glucose transport	S	Muscle, adipose
Glycogen synthesis	S	Liver, muscle, adipose
Gluconeogenesis	I	Liver
Glycogenolysis	I	Liver, adipose
Amino acid transport	S	Liver, muscle, adipose
Protein synthesis	S	Liver
Proteolysis	I	Liver
Fatty acid transport	S	Adipose
Lipid synthesis	S	Liver, adipose
Lipolysis	I	Adipose
DNA synthesis	S	Cultured cells
Cell growth	S	Cultured cells

S = Stimulation; I = Inhibition

1.3   THE INSULIN RECEPTOR

1.3.1   Receptor Structure and Function

The insulin receptor is a specific device through which the hormone communicates with its target cells. It is thought that the receptor has the potential to initiate transmembrane signals that triggers off a range of cellular responses upon insulin binding. The native insulin receptor is an integral membrane glycoprotein comprising of different subunits held together by interchain disulphide bonds and strong non-covalent interactions (Massague et al., 1980; Jacobs et al., 1980a; Yip et al., 1981).

Receptors for insulin are present in all mammalian and vertebrate species studied (Shemer et al., 1986) with considerable conservation of structure (Czech et al., 1984). However, marked differences in the structure (including molecular weight, antigenic determinants and carbohydrate composition) and function exist between receptors found in neural tissue and those of the peripheral target tissues (Heidenreich et al., 1983; Hendricks et al., 1984; Ciaraldi et al., 1985). It appears though that the size of the protein backbone of the insulin receptor in adipocytes and brain is the same and that differences occur in the post-translational glycosylation of these receptors (Heidenreich and Brandenburg, 1986). Interestingly, the structural differences identified for brain and liver receptors in mammals has also been observed in reptiles, indicating strong evolutionary conservation (approx. 300 000 000 years) of receptor tissue specificity (Shemer et al., 1986).

Methods used to study the constituent polypeptides of the

receptor have included, affinity labelling techniques, immunoprecipitation with antibodies to the receptor, biosynthetic labelling of protein or carbohydrate constituents of the receptor, target size analysis and various methods of receptor purification (Baron and Sonksen, 1983). However, these wide range of techniques gave rise to discrepancies as to the number of stoichiometry of the subunits (Goren et al., 1983; Czech et al., 1981; Im et al., 1983; Yip and Moule, 1983; Baron and Sonksen, 1983).

Yip and Moule (1983), using photoaffinity labelling techniques have suggested that the receptor exists in one molecular weight species of 380 KDa but in three interconvertible redox forms, resolved electrophoretically as 380 KDa, 300 KDa and 230 KDa. Reduction of each of these separately, shows that disulphide linked subunits of 130 KDa, 90 KDa and 40 KDa are present in all three species. It is thought that a membrane factor or factors, sensitive to sulphydryl alkylating reagents, may be involved in regulating the redox state of these three receptor species through oxidation and reduction of the sulphydryl groups.

Subsequently, Yip and co-workers using the photoreactive analogue, N$^{\alpha B29}$-(monoazidobenzoyl) insulin have been able to label not only the major insulin binding subunit (130 KDa) but also four additional proteins of molecular masses 95, 85, 55 and 45 KDa indicating that a number of proteins may be participating in insulin binding (Helmerhorst et al., 1986). These researchers have proposed a high molecular weight species of 450 KDa for the insulin receptor. They concluded that a sulphydryl component of the membranes, which copurifies with the receptor, is involved in the generation of multiple high molecular weight forms of the receptor (Helmerhorst et al., 1986).

Fehlmann *et al.* (1985) have also predicted a high molecular weight receptor complex for the insulin receptor.

The most widely accepted model of the insulin receptor is, however, one in which the minimum subunit structure consists of two α- and two β-subunits, disulphide linked, to yield a β-s-s-α-s-s-α-s-s-β receptor complex of $M_r \approx 350\ 000$ (Massague *et al.*, 1980, 1981; Czech *et al.*, 1981). The α-subunit exhibits an apparent $M_r = 125\ 000$-$135\ 000$ while the β-subunit has a mass of about $90\ 000$-$95\ 000$ Da (Fig. 1.1). The isolation and characterization of the receptor subunits from purified receptors have provided further evidence that the $\alpha_2\beta_2$ complex is the basic structural unit of the insulin receptor (Fujita-Yamaguchi, 1984).

Treatment of native insulin receptor preparations with varying concentrations of exogenous reductant have led to the concept that at least two different classes of disulphide bonds exist in the insulin receptor complex (Massague and Czech, 1982). The disulphide bonds linking the two symmetrical $\alpha\beta$ receptor halves are relatively sensitive to reduction by dithiothreitol and are denoted class I. The disulphide bonds linking the α-subunit to the β-subunit are less sensitive to reduction by dithiothreitol and are termed class II.

Dithiothreitol alone is ineffective at releasing any of the subunits from the $\alpha_2\beta_2$ complex. However, if the receptor is unfolded with urea then dithiothreitol is able to release the α-subunits, thus demonstrating that the α-subunit is not a transmembrane protein (Grunfeld *et al.*, 1985). Both subunits are exposed to the extracellular environment, however, only the β-subunit traverses the membrane and has a portion of its chain exposed to the cytoplasm. Thus the spatial

Fig. 1.1

Schematic representation of several major features of the insulin receptor structure.
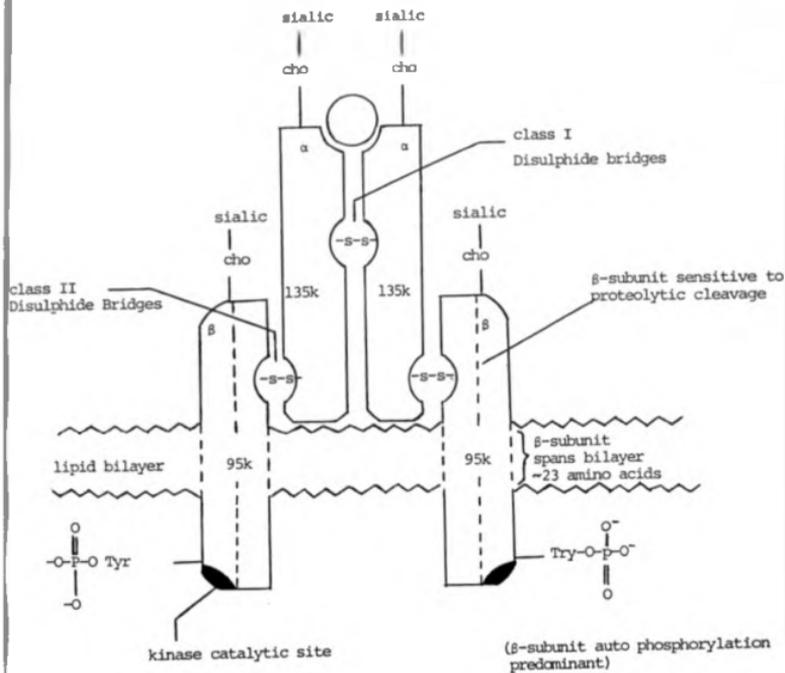
Modified from Czech, 1985 to embrace current ideas:

Ullrich *et al* 1985, Grunfled *et al.*, 1986, Hedo and Simpson, 1984; Van Obberghen and Gammeltoft, 1986).

orientation of the subunits in the membrane is asymmetric (Hedo and Simpson, 1985). Additionally, the β-subunit contains a site near the centre of its amino acid sequence that is exquisitely senstiive to elastase like proteases (Czech *et al.*, 1981). Hence specific proteolytic processing can excise a portion of each β-subunit leaving a fragment β_1 ($M_r = 49\ 000$) disulphide linked to the receptor.

Recently the complete amino acid sequence of the human insulin receptor was determined from a complementary DNA clone (Ullrich *et al.*, 1985; Ebina *et al.*, 1985) and the receptor structure deduced overwhelmingly supports earlier proposals of an $\alpha_2\beta_2$ complex. It was concluded that the α-subunit lacks any hydrophobic stretches to act as a membrane anchor and hence is almost exclusively in the extracellular domain. It contains sequences for asparagine N-linked glycosylation and a large number of cysteine residues and it has been suggested that in a compact structure these cysteine-rich clusters may embody the ligand binding region (Gammeltoft and Van Obberghen, 1986). The β-subunit contains a clearly well defined transmembrane region of 23-26 hydrophobic amino acids which divides this subunit into a shorter extracellular portion, which links the α-subunit through disulphide bridges, and a longer cytoplasmic region. In additon the deduced amino acid sequence of the β-subunit shows considerable homology with other tyrosine kinases such as the epidermal growth factor (EGF) receptor kinase and the tyrosine kinase domains of the family of retroviral protein tyrosine oncogenes (Ullrich *et al.*, 1985; Ebina *et al.*, 1985).

The insulin receptor is highly glycosylated (Cuatrecasas, 1972). The α and β subunits contain 15 and 6 potential N-glycosylation sites respectively; the latter has four of these exposed to the extracellular

medium (Ullrich *et al.*, 1985). Both subunits contain high mannose-type and complex-type N-linked glycosylation, as evidenced by biosynthetic incorporation of radioactive monosaccharides as well as surface labelling of galactose and sialic residues (Hedo *et al.*, 1981a; 1983). The carbohydrate chains are capped with sialic residues (Jacobs *et al.*, 1980a,b; Kasuga *et al.*, 1980) and contain mainly N-acetylglucosamine, galactose and mannose residues (Hedo *et al.*, 1981b). More terminal galactose and N-acetyl glucosamine residues are present on the α-subunit while the β-subunit has a higher degree of sialylation (Hedo *et al.*, 1981a). Characterization of the N-linked high mannose oligosaccharide have shown the predominant species in the α-subunit to be Man_8 Glc NAc_2 and that of the β-subunit is Man_7 Glc NAc_2 (McElduff *et al.*, 1986).

1.3.2   *Receptor Biosynthesis*

The α and β subunits are synthesized as a single polypeptide precursor, in the rough endoplasmic reticulum (Hedo *et al.*, 1983). The human insulin receptor precursor consists of 1,370 amino acids containing an initial 27-residue signal sequence followed by the α-subunit, 735 residues, then the β-subunit, 620 residues. Both subunits are separated by the tetrapeptide sequence Arg-Lys-Arg-Arg, which probably represents the recognition sequence for the precursor processing enzyme (Ullrich *et al.*, 1985). Synthesis of the primary proreceptor polypeptide backbone M_r–170 000-180 000 is accompanied by cotranslational N-linked glycosylation to yield a high mannose 190 000 M_r precursor (Forsayeth *et al.*, 1986; Lane *et al.*, 1985). The 190 000-M_r proreceptor contains exclusively asparagine-N-linked high mannose type carbohydrate chains (McElduff *et al.*, 1986; Hedo *et al.*, 1983).

This proreceptor molecule is rapidly transferred to the golgi where it is first proteolytically cleaved into low molecular weight forms of the α (120 000-M_r) and β (90 000-M_r) subunits. Each subunit then undergoes further processing which involves trimming of some mannose residues from high-mannose side chains and addition of distal sugars, (Galactose, N-acetylglucosamine, fucose and sialic acid), to form complex carbohydrate chains (Forsayeth et al., 1986; Ronnett et al., 1984). The degree of oligosaccharide processing in the α and β subunits is different (McElduff et al., 1986). The secondary glycolytic processing occurs just prior to insertion of the receptor in the plasma membrane, probably in a distal golgi vesicle (Hedo and Simpson, 1985 ; Hedo and Gorden, 1985).

The mature α (135 000-M_r) and β (95 000-M_r) subunits appear on the surface 2-3 hours after the synthesis of the newly-translated proreceptor (Marshall, 1983; Salzman et al., 1984). Immediately upon insertion, or shortly thereafter, receptors become funcitonal and are able to mediate the biological actions of insulin (Marshall, 1983). At least one research group (Ronnett et al., 1984) has suggested that the 190 000-M_r proreceptor is processed to yield a high molecular weight polypeptide M_r = 210 000, from which the α and β-subunits are generated. However, other workers have either failed to detect this 210 000-M_r polypeptide (Jacobs et al., 1983; Forsayeth et al., 1986) or have proposed that the small amounts of 210 000-M_r polypeptide present in the membrane is due to the failure of some 190 000-M_r proreceptor to undergo proteolytic cleavage but is still transformed through terminal glycosylation, hence the increase in molecular weight (Hedo et al., 1983; Hedo and Simpson, 1985).

N-linked glycosylation is believed to be important for the structural and functional maturation of the receptor and for its translocation from its intracellular site of synthesis to the cell surface (Forsayeth et al., 1986; Lane et al., 1985). However, Duronio et al. (1986) employing swainsonine (a drug which retards formation of complex N-linked oligosaccharides) have generated partially glycosylated insulin receptors which are apparently normal in all other respects suggesting that all the sugars normally added to complex type oligosaccharides are not required for normal processing and function of the receptor.

1.3.3 Receptor Organizational Structure

A fluorescent derivative of insulin, rhodamine-lactalbumin-insulin, used to visualise binding, aggregation and internalization of insulin on living fibroblastic cells have indicated that insulin receptors are initially homogeneously distributed on the cell surface but rapidly aggregate into patches on the cell membrane (Schlessinger et al., 1978). However, it has been reported that rhodamine-lactalbumin-insulin retains less than 2% of the activity of native insulin, (Lyen, 1983), revealing the difficulty with this technique to make accurate predictions of receptor binding and organisation. On the other hand, morphological studies of the distribution of insulin receptors on rat adipocyte plasma membranes, using monomeric ferritin insulin, have shown that the occupied insulin receptors occurred predominantly in natural groups of two to six receptor sites randomly distributed on the plasma membrane in association with an evenly distributed glycocalyx (Jarett and Smith, 1977). Further, neither microfilaments nor actin is involved in maintaining the grouped receptors in adipocytes (Jarett and Smith, 1983). In at least one cell type, IM-9

human lymphoblastoid cells insulin receptor capping occurs via a calmodulin regulated process (Majercik and Bourguigon, 1985).

Investigations involving the reducing agent dithiothreitol, N-ethylmaleimide (an alkylating agent), cytochalasin B (prevents formation of disulphide bonds due to its thiol binding properties) and oxidized glutathione (an oxidizing agent), have led to the proposal that two functionally different sets of disulphide bonds, which are not synonymous with Class I and Class II disulphide bonds, exist in the organization of insulin receptors on the adipocyte plasma membranes (Schweitzer *et al.*, 1980; Jarett and Smith, 1983). One set of disulphide bonds appears to be relatively unstable, spontaneously undergoing reduction to sulphydryl groups and reoxidation to the disulphide state with the disulphide bond favoured at equilibrium. These bonds are involved in maintaining the grouped arrangement of the insulin receptor. The second type of disulphide bond appears to be more stable and not susceptible to significant spontaneous reduction. These bonds are related to the ability of receptors to bind insulin and may correspond to the class I disulphide bond in the $\alpha_2\beta_2$ receptor complex (Jarrett and Smith, 1983).

Interestingly, it has been shown that although insulin receptors on adipocyte membranes occur predominantly in groups of 2-6 molecules, over 60% of insulin receptors on liver membranes occur as single molecules (Lyen *et al.*, 1983). Hence liver insulin receptors may contain only one functionally distinct disulphide bond as opposed to two for receptors in adipocyte plasma membrane. This observation has led to the proposal that the natural state of distribution of insulin receptors appears to be tissue specific (Lyen *et al.*, 1983 ; Jarrett and Smith, 1983).

1.4   INSULIN/RECEPTOR INTERACTIONS

1.4.1   Insulin Binding (Structural Requirements)

The binding of insulin to its receptor is the primary event in the hormone-receptor interaction which leads to the generation of a biological signal(s).   Insulin binds rapidly and reversibly (Sonne, 1986) to the α-subunit, which is the major binding component of the receptor (Pilch and Czech, 1980).   The amino acid residues of the insulin which participates in binding has been carefully studied and reveals that the important residues which are involved either directly or indirectly are Gly^{A1}, Leu^{A2}, Val^{A3}, Glu^{A4}, Gln^{A5}, Tyr^{A19}, Asn^{A21} and Val^{B12}, Tyr^{B16}, Phe^{B24}, Phe^{B25}, Tyr^{B26} (Nakagawa and Tager, 1986; Kitagawa et al., 1984a.b; Kobayashi et al., 1986).

The predominantly hydrophobic C terminus of the B chain (Tatnell et al., 1983; Nakagawa and Tager, 1986) and the N-terminal region of the A-chain extending to A3 (Kobayashi et al., 1986) both play key roles in the binding event while van der Waals contact between isoleucine A^2 and tyrosine A^{19} stabilises the helical segment A^2-A^8 and appears to be critical for high biological activity of insulin (Kitagawa et al., 1984a).   Although the actual insulin binding domain of the receptor has not been characterized (Ng and Yip, 1985), it has been proposed that binding involves hydrophobic interaction between receptor and hormone stabilized by hydrogen bonding between these two species (Pullen et al., 1976).   Moreover an insulin receptor histidine residue appears to be critical for hormone binding (Pilch, 1982).

Both insulin and its receptor undergo early structural modifications upon formation of the insulin receptor complex (Juul *et al.*, 1986a). A small peptide is proteolytically cleaved from the C terminus of insulin's B chain after binding (Juul *et al.*, 1986a) while the occupied receptor exhibits greater sensitivity to trypsin digestion than the unoccupied receptor alluding to receptor conformational change (Pilch and Czech, 1980; Czech *et al.*, 1981). Interestingly, even after substantial cleavage of its binding subunit, by trypsin, the receptor is fully activatable by insulin (Brandenburg *et al.*, 1985). It has recently been reported that the 350 000 Da intact $\alpha_2\beta_2$ tetramer apparently undergoes substantial proteolytic processing subsequent to insulin binding to produce a 140 000 Da product which can be reduced to yield species of 100 000 Da and 68 000 Da (Juul *et al.*, 1986b). However, these researchers have not ascertained the significance of these products.

The $\alpha_2\beta_2$ receptor complex can be reduced, upon treatment with dithiothreitol, to yield $\alpha\beta$ monomers which are capable of binding insulin (Massague and Czech, 1982; Velicelebi and Aiyer, 1984). However, the receptor molecule, $\alpha_2\beta_2$ is monovalent (Pang and Shafer, 1983). The latter also suggest that if insulin receptors contain more than one insulin-binding subunit then a high degree of negative cooperativity will be associated with the binding of insulin to its receptor.

1.4.2 *Kinetics of Insulin Binding*

The interaction of insulin with its receptor is very complex. Insulin binds rapidly and reversibly to its receptor with the maximal effects of insulin occuring when only a small percentage of the

receptors are occupied (Gammeltoft and Gliemann, 1973; Kono and Barham, 1971). Insulin binding to its receptor is frequently analysed by Scatchard graphs (Scatchard, 1949). The general Scatchard equation used to represent the interaction of hormones with their receptors is given by:

$$\frac{[B]}{[F]} = K(n-B)$$

where [B] and [F] denote the concentration of bound and unbound (free) hormone respectively, K represents the binding affinity constant and n is equal to the toal number of binding sites. Consequently a plot of the concentration ratio of bound to free hormone, [B]/[F] should be a linear function of the bound hormone concentration [B]. This linearity holds true provided all the binding sites are independent and identical.

Theoretically the gradient of linear Scatchard plots should yield the affinity constant while the total number of binding sites is obtained where the slope and the abscissa intersect. This analysis can only hold if the Scatchard plot unambiguously intercepts both the [B]/[F] and [B] axes (Jones et al., 1986). In practice Scatchard plots do not meet the requirements. Analysis of insulin binding data by several research groups using different tissues have frequently yielded curvilinear Scatchard plots with varying degrees of upward concavity (De Meyts et al., 1973,1976; Etherton and Walker, 1982; Calderwood and Hahn, 1983; McElduff and Eastman, 1981) and though some researchers did not observe this curvilinear behaviour (Gliemann et al., 1975; Pollet et al., 1977) recent studies have continued to demonstrate curvature (Freidenberg et al., 1987; Koch et al., 1986; Haring et al., 1986; Wang, 1986). Researchers therefore, are forced to extrapolate

from the curvilinear plots to predict the desired parameters, the affinity constant and the number of binding sites, hence creating doubts as to the veracity of the results. It is not surprising then that the Scatchard analysis has been criticised as being misleading (Klotz, 1982; Klotz *et al.*, 1984; Baulieu and Raynaud, 1970).

Various groups have tried to find better ways to express the binding data. De Meyts and Roth (1975) have used a graphical analysis of cruvilinear Scatchard plots based on a model in which receptor affinity varies with occupancy to yield affinity constants for "empty" and "filled" binding sites. Klotz (1982,1984) have suggested the use of a semilogarithmic graph in which the moles of bound ligands, B, are plotted against the concentration of the free ligand, F, on a logarithmic scale. He purports that for an ideal situation in which every binding site is equivalent the graph should produce an S-shaped curve symmetrical about an inflection point. The plateau of this sigmoidal curve should correspond to the total number of binding sites. However, this approach has its shortcomings in that the expected inflection point and plateau is not always attained (Klotz, 1982).

More recently, two more rigorous lines of approach have been adopted for treatment of binding data: Jones *et al.* (1986) have attempted to correlate the binding isotherm obtained for Klotz analysis with the binding potential concept for linked sites as proposed by Wyman (1965). These researchers derived a relation between binding potential for insulin, π_{In}, and the free energy of binding, ΔG^0. A plot of ΔG against the bound concentration produced smooth curves which when extrapolated to zero bound concentration presumably yields the high affinity constant for isolated receptors. This analysis

though is unable to supply the total number of binding sites.

Lipkin *et al.* (1986a), have suggested the use of observation equations; the amount of insulin in the cell pellet, the dependent variable, plotted as a function of the total added insulin concentration, the independent variable. They determined that 99.7% of the binding sites are homogenous while approximately 0.3% had extra high affinity for insulin. This analysis was suitably supported by relevant kinetic experiments (Lipkin *et al.*, 1986b) suggesting that the use of appropriate observation equations might prove quite useful.

It is clear therefore that the relationships of mathematical parameters of an equation to molecular features of the interacting species are generally complex (Klotz and Hunston, 1984). However, it has been argued that binding studies present a particularly challenging problem of negotiation in the uneasy truce between data and theory, for which statistical analysis represents an objective way of estimating the nature and amount of receptor capacity and is indispensible (Feldman, 1983).

The curvilinear Scatchard plot can be interpreted as evidence for the presence of multiple classes of binding sites that have diferent but fixed affinities or the existence of site-site interactions in which the affinity of the receptors is not fixed but decreases as the occupancy increases; i.e. negative cooperativity (De Meyts *et al.*, 1976). The former interpretation has been supported by some researchers (Olefsky and Chang, 1979; Etherton and Walker, 1982; Bonen *et al.*, 1984). However, De Meyts *et al.* (1973), have shown that when receptor occupancy is increased with unlabelled insulin the dissociation rate of previously bound [I^{125}]-insulin is greatly enhanced thus

implicating negative cooperativity in insulin receptor interactions.

Additionally insulin induced tracer dissociation occurs in the isolated $\alpha_2\beta_2$ tetramer, but not in the isolated $\alpha\beta$ dimer, thus implying that the presence of the two α-subunits in the receptor structure may be responsible for negative cooperativity (Deger *et al.*, 1986). This is supported by the observation that the $\alpha\beta$ dimer yields linear Scatchard plots unlike the curvlinear plots obtained for the $\alpha_2\beta_2$ complex (Koch *et al.*, 1986). Also anti-receptor antibodies which bind to the α-subunit at sites distinct from the insulin binding site impairs binding in the tetramer but not in the dimer. Hence it is probable that a conformational change within the receptor complex leads to a subsequent decrease in the binding affinity of both α-subunits while the dimer which lacks the second α-subunit avoids the decrease in binding due to the non-interaction of two α-subunits (Deger *et al.*, 1986). This evidence strongly suggests that interactions of the two α-subunit is involved in negative cooperativity. Interestingly, Pang and Shafer (1983,1984) demonstrated that the receptor has a high affinity for only one molecule of insulin (kd – 0.6nM), the affinity of the receptor for a second molecule of insulin is approximately 100 times less than the first (kd$_2$ – 80nM).

Apart from the aparent negative cooperativity for insulin binding to its receptor, Marsh *et al.* (1984) have indicated that at physiological (low) concentrations of insulin, binding curves exhibits sigmoidicity consistent with positive cooperativity. This property appears to be intrinsic to the receptor and may be due to conformational changes in the oligomeric structure.

The entire situation is compounded by the observation that on prolonged incubation of ^{125}I-insulin with rat liver plasma membranes the tracer does not readily dissociate from the receptor on exposure to high cold insulin concentrations, implying that insulin induces a time-dependent increase of a receptor high affinity state (Donner and Corin, 1980; Corin and Donner, 1982). This process is temperature dependent, occuring at 15^oC, 23^oC and 37^oC but not at 4^oC (McCaleb and Donner, 1981; Donner and Corin, 1980). It is felt that hormone binding alters the equilibrium between the two states favouring the formation of the higher affinity state (Corin and Donner, 1982).

Insulin dissociates from its receptor in a biphasic manner which is described as consisting of two kinetic components (rapidly and slowly dissociating hormone) corresponding to the different states of receptor affinity (Donner and Corin, 1980). On this basis a two state model for the insulin receptor was proposed (Corin and Donner, 1982):

(1)

$$
\begin{array}{ccc}
 & K_1 \text{ [Rapid]} & \\
H + R \rightleftarrows & & H \cdot R \\
 & K_{-1} & \\
 & & (2) \\
(4)\,K_4 \quad K_{-4} & & K_{-2} \;\; K_2 \;\text{slow (time} \\
 & & \text{-dependent} \\
 & & \text{conversion)} \\
 & K_{-3} & \\
H + R^{\circ} \rightleftarrows & & H \cdot R^{\circ} \\
 & K_3 & \\
 & (3) &
\end{array}
$$
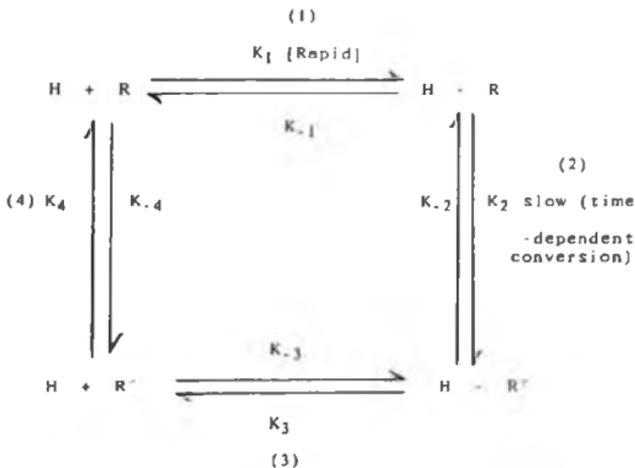
The hormone (H) interacts with the receptor (R) to yield a complex (H-R) from which the hormone dissociates rapidly. In a time-dependent manner the complex becomes stabilized through conversion (K_2) of the receptor state from which hormone dissociates slowly (H-R'). The accumulation of H-R' is non-cooperative. Steps 3 and 4 are speculative. Lipkin *et al.* (1986) have proposed a similar model, suggesting that the initial insulin receptor complex, RI, can change its state or cellular location reversibly to form a new complex, R'I, which is not in direct equilibrium with free insulin. At equilibrium the R'I state constitutes 63% of the occupied receptor.

Alterations of tryptic lability during hormone binding, observed by Donner and Yonkers (1983) established that binding produces two conformational changes in the receptor; the initial conformational change induced by insulin binding and also observed by others (Pilch and Czech, 1980) is followed by a second slower conformational change which renders the receptor less sensitive to trypsin. Hormone binding to liver membranes induces degradation of the 135 000-Da α-subunit of the receptor to a fragment of molecular mass 120 000-Da which represent the low affinity and high affinity α-subunits respectively (Donner and Yonkers, 1983).

Inhibition of proteolytic degradation of the α-subunit from 135 000-Da to 120 000-Da by serine proteinases did not prevent or alter the time course of conversion between the receptor affinity states (Lipson *et al.*, 1986). Hence initial binding yields a low affinity complex, which is not a substrate for proteolysis, but which can convert to a high affinity state (through the intermediacy of a slow conformational change) which is selectively degraded to form

complexes in which the hormone remains bound with high affinity (Lipson *et al.*, 1986). Additionally the curves obtained by Jones *et al.* (1986) for the free energy of binding (G^0) versus the bound insulin concentration, in which there is a smooth transition from high negative ΔG^0 values at low binding concentrations to smaller negative ΔG^0 values at high binding concentrations indicates a gradual alteration of receptors from high to low affinity states.

A two state model has been used to represent a number of hormone interactions (Corin and Donner, 1982), hence receptor conversion between affinity states may be a generalized phenomenon, although it may have different functions in different receptor systems. Further, it is possible that conversion of occupied receptors to a high affinity state may be a precursor step for internalization of the hormone (Donner and Yonkers, 1983).

The observed transition of affinity states within the receptor appears to be ATP-dependent (Steinfelder and Joost, 1983) and may be due to alterations of sulphydryl groups within the receptor (McElduff, 1981). A component which is part of, or closely associated with, the insulin receptor on its cytoplasmic side has been implicated in regulating receptor affinity (Haynes *et al.*, 1986) while the carboxy-terminal B-chain (of the insulin molecule), which is required for solidifying insulin-receptor interactions is also believed to be involved in inducing receptor affinity changes. In particular, the Phe B^{25} residue appears to play a significant role in the receptor conversion to a high affinity state (Nakagawa and Tager, 1986).

It is apparent therefore that the association of insulin with its receptor is extremely complex. However, although there is evidence for receptor negative cooperativity, positive cooperativity and non-cooperative affinity changes these proposals are all significant since they were observed under different experimental conditions, high excess cold insulin, low (physiological) levels of insulin and prolonged binding respectively. These observations then may accurately reflect the behaviour of the insulin receptor under specific experimental conditions.

1.4.3   Role of Glycosylation

Digestion of fat cells by glycosidases results in impairment of insulin binding (Hedo *et al.*, 1981). Within the hormone recognition area glycosylated peptide chains are strictly required for insulin-receptor interaction. Blockade or removal of certain carbohydrate groups will almost terminate the high affinity insulin binding process suggesting that change in receptor affinity is related to the role of carbohydrates in insulin binding (Caron *et al.*, 1983). Sialyated glycosidic moieties containing D-galactose, D-mannose and N-acetyl D-glucosamine units are involved in both processes of insulin high affinity binding and activation of glucose transport but are not implicated in the degradation of the hormone (Cherqui *et al.*, 1982).

1.5   POST BINDING EVENTS

1.5.1   Phosphorylation

Kasuga *et al.* (1982a,b) demonstrated that insulin induced phosphorylation of the β-subunit of its own receptor in intact cells and cell free systems. In the absence of insulin phosphorylation occured slowly, but addition of insulin caused rapid incorporation of ^{32}P into the β-subunit. Phosphoamino acid analysis of the β-subunit from the cell-free system revealed the presence of mainly phosphotyrosine with a small amount of phosphoserine. In intact cells phosphoamino acid analysis of the phosphorylated β-subunit contained phosphoserine, phosphothreonine and phosphotyrosine residues under basal conditions (Kasuga *et al.*, 1982a ;Yu and Czech, 1984).

However, insulin induces a rapid, several fold, increase in phosphorylation of tyrosine followed by a slower rise in phosphoserine residues (Ballotti *et al.*, 1987; Gazzano *et al.*, 1983). These observations suggested that the insulin receptor complex consist of two different insulin-stimulatable kinase activities: a tyrosine-specific kinase that is a constituent of the receptor and whose activation is likely to be the first post-binding event in insulin's action, and a serine specific kinase closely associated with the receptor in the cell membrane but not an integral part of the receptor structure (Gazzano *et al.*, 1983; Ballotti *et al.*, 1986).

In fact the serine kinase can copurify with the insulin receptor after wheatgerm lectin chromatography, presumably due to its glycoprotein nature, but is lost after purification by anti-receptor-antibodies (Ballotti *et al.*, 1986). This demonstrates that the serine kinase is closely associated with the $\alpha_2\beta_2$ tetramer. Further, although insulin leads to the appearance of a signifcant amount of

phosphotyrosine-containing receptors not all receptors undergo this tyrosine phosphorylation, since a distinct receptor population, which cannot be precipitated by anti-phosphotyrosine antibodies, contains exclusively phosphoserine residues indicating that at least two different classes of phosphorylated insulin receptors may coexist on membranes (Balloti *et al.*, 1987).

The relationship of the two receptor kinases is not very clear. It has been suggested that both kinases may serve separate cellular functions or one kinase may serve to activate the other, i.e. sequential activation (Gammeltoft and Van Obberghen, 1986). However, it has been demonstrated that receptors which contain phosphoserine in the basal state show markedly reduced tyrosine phosphorylation after addition of insulin (Pang *et al.*, 1985). Additionally, Haring *et al.* (1986) observed that isoprenaline caused a cyclic-AMP-kinase-dependent phosphorylation of the insulin receptor at serine residues which inhibited tyrosine kinase activity. Further, protein kinase C and cyclic-AMP kinase act directly on the receptor to induce phosphorylation on serine residues resulting in reduced kinase activity (Bollag *et al.*, 1986; Roth and Beaudoin, 1986). Taken together it appears that serine kinase may play an important role by regulating the tyrosine kinase.

Phosphorylation sites on the insulin receptor β-subunits can be resolved into several phosphopeptide fractions by high pressure liquid chromatography (HPLC) (White *et al.*, 1984; Yu and Czech, 1984). White and co-workers have determined that the β-subunit contains at least five sites for autophosphorylation, which phosphorylate at different rates suggesting that autophosphorylation of one site may stimulate the autophosphorylation of other sites. Yu and Czech have

determined that at least seven sites for autophosphorylation exist on the β-subunit, which are divided into three main domains of phosphorylation, designated peaks one, two and three. All three peaks were found to contain phosphotyrosine and phosphoserine while peak two also contained phosphothreonine. The extent of phosphorylation of tyrosine residues in receptor domain two closely parallels the receptor kinase activity state, suggesting phosphorylation of this domain may play a key role in regulating the insulin-receptor tyrosine kinase.

The insulin receptor tyrosine kinase has been shown to phosphorylate exogenous substrates, such as histones and casein, in cell-free systems (Petruzzelli *et al.*, 1982). It is believed that the insulin-bound receptor self phosphorylates altering its conformation so that the oligomer gains the capacity to catalyse the phosphorylation of exogenous substrates (Rosen *et al.*, 1983). Recently a number of intracellular substrates have been found to be phosphorylated by the β-subunit tyrosine kinase which include the guanine nucleotide binding protein (G-protein) transducin (TD), hence suggesting the involvement of G proteins in insulin action (Zick *et al.*, 1986). Also two independent research groups have identified a cellular protein substrate M_r - 110 000-112 000 for the insulin receptor kinase in wheatgerm agglutinin purified glycoproteins from rat liver and rabbit brown adipose tissue (Sadoul *et al.*, 1985; Rees-Jones and Taylor, 1985). The role of this substrate is as yet undefined. Also the phosphorylation of a 46 KDa membrane protein has been suggested as possibly, an early step in insulin signal transmission (Haring *et al.*, 1987).

The elucidation of the amino acid sequence of the insulin receptor (Ullrich *et al.*, 1985; Ebina *et al.*, 1985) has allowed researchers the opportunity to investigate phosphorylation of peptides synthesized to

correspond to potential autophosphorylation sites. Immunological studies have shown that tyrosine-1150 is critical in the sequence of phosphorylation events elicited by insulin since this residue is preferentially phosphorylated by the purified receptor kinase (Stadtmauer and Rosen, 1986; Herrera and Rosen, 1986). Ellis *et al.* (1986) employing site directed mutagenesis have found that replacement of one or both of twin tyrosines (residues 1162 and 1163) with phenylalanine results in a dramatic reduction in, or loss of insulin-activated autophosphorylation and kinase activity *in vitro*. In fact the two tyrosine residues mentioned above, tyrosine-1150 and tyrosine-1162 are identical since Rosen and colleagues used Ullrich's proposal of 1370 amino acids in the $\alpha\beta$ dimer while Ellis and coworkers used Ebina's proposal of 1382 amino acids for the $\alpha\beta$ dimer. The work from these two independent laboratories correlates well, implicating the identical tyrosine moeity as being essential for autophosphorylation.

Treatment of receptor preparations with elastase-like proteases generates receptors proteolytically "nicked" in the β-subunit resulting in the abolition of insulin dependent autophosphorylation without affecting the insulin binding capacity of the α-subunits (Roth *et al.*, 1983a; Shia *et al.*, 1983). Subsequently the carboxy terminal end of the β-subunit was shown to be crucial for the kinase activity of the receptor (Ellis *et al.*, 1986; Kathuria *et al.*, 1986). An interesting observation that the activity of the purified receptor kinase is rapidly lost under conditions where insulin binding function is preserved, led to the discovery that the 90-KDa β-subunit is degraded first to an 88-KDa form and then to a 50-KDa β_1-subunit form by proteolysis, even after purification, when stored at $4^{\circ}C$ (Kathuria *et al.*, 1986). The 88-KDa β-subunit, which lack the carboxyl-terminal ~ 2-KDa portion exhibits almost no

autophosphorylatin activity, nor does insulin stimulate autophosphorylation. The 2-KDa peptide is separate from the kinase domain and further emphasizes the importance of the carboxyl-terminal in autophosphorylation and also suggests that kinase inactivation may be facilitated by selective proteolytic digestion at the carboxyl-terminal region of the β-subunit.

The way in which the observed insulin induced conformational changes in the receptor may be related to the activation of the insulin receptor kinase is not known. However, the rate of insulin stimulated autophosphorylation of the insulin receptor is independent of the receptor concentration and thus proceeds via an intramolecular process (the receptor acting on itself) rather than an intermolecular process (one receptor acting on another receptor) (Shia et al., 1983).

Examination of the disulphide bonds within the receptor has shown that insulin activation of the receptor/kinase involves alterations of intramolecular disulphides (Wilden et al., 1986). In addition class I disulphide bonds are required for insulin binding and subsequent autophosphorylation although these class I disulphides are not necessary for the maintenance of the high kinase activity (Pike et al., 1986). This is supported by the observations that only the intact receptor, $\alpha_2\beta_2$, is competent to both bind insulin and undergo phosphorylation (Pang et al., 1984), and $\alpha\beta$ dimer-dimer interaction appears to be critical for the insulin-dependent activation of the receptor's intrinsic kinase activity (Boni-Schenetzler et al., 1986). Further, since the β-subunit contains only one membrane spanning domain some degree of flexibility might exist whereby the receptor conformational change generated on insulin binding might facilitate interaction of the two $\alpha\beta$ dimers such that the β-subunits would interact to allow enhanced

autophosphorylation (Boni-Schnetzler *et al.*, 1986).

Insulin receptor phosphorylation is temperature dependent, as one would expect for an enzymatic reaction, and occurs in a time and dose-dependent manner (Shia and Pilch, 1983). The β-subunit contains an ATP binding site (Shia and Pilch, 1983) and ATP appears to be the sole substrate of the kinase, since no other nucleoside triphosphate competed detectably with ATP (Nemenoff *et al.*, 1984).

Certain metal ions have been found to be closely associated with receptor phosphorylation. Ca^{2+} together with calmodulin, enhances insulin-stimulated phosphorylation of the β-subunit (Graves *et al.*, 1986; Plehwe *et al.*, 1983). Moreover, autophosphorylation of the insulin receptor appears to be under dual regulation since insulin stimulates autophosphorylation by increasing the V_{max} while Mn^{2+} activates this reaction by decreasing the K_m for ATP. Mg^{2+} apparently has no effect on the reaction (White *et al.*, 1984). The concentrations of Mn^{2+} needed to permit optimal receptor phosphorylation indicate that this requirement for Mn^{2+} is not only related to its ability to form a complex with ATP; Mn^{2+} appears to act as a cofactor as well (Czech, 1985). Nemenoff *et al.* (1984) also found that insulin stimulated autophosphorylation by increasing the V_{max} with the reaction being supported by Mn^{2+} at low ATP concentrations and Mg^{2+} at high ATP concentrations. The reaction thus appears to be under dual regulation between insulin and divalent ion(s) (Mn^{2+} and/or Mg^{2+}). Together with this, the maximal activity of the receptor kinase seems to require a reduced sulphydryl group at or near the active site (Shia *et al.*, 1983).

Once the receptor is phosphorylated the kinase activity becomes

independent of insulin. Hence enzymatic dephosphorylation and not simply dissociation of bound insulin may be involved in terminating the insulin signal (Haring *et al.*, 1984). Alkaline phosphatase has been shown to dephosphorylate the β-subunit *in vitro* (Klein *et al.*, 1986). Kowalski *et al.* (1983) speculated that the phosphatases involved in β-subunit dephosphorylation are cytosolic while Haring *et al.* (1984) reasoned that the phosphatase activity is a glycoprotein associated with, but not an integral part of the receptor. Recently though it has been proposed that dephosphorylation occurs through an insulin receptor directed protein phosphatase which may involve GTP binding proteins (Horn *et al.*, 1986).

Simpson and Hedo (1984), found that polyclonal antireceptor anti-serum B-10, obtained from a patient with severe insulin resistance had clear insulin-mimetic properties but did not induce phosphorylation of the β-subunit. Zick and co-workers (1984) found that four different antisera B-d, B-8, B-2 and B-10, had insulin-mimetic properties. However, only B-d and B-8 stimulated autophosphorylation of the β-subunit while B-2 and B-10 failed to do so. Taken together, the work of these two research groups indicate that the activation of the tyrosine specific protein kinase might not be an obligatory step in coupling insulin binding to insulin action.

On the other hand insulin action was blocked by a monoclonal antibody that inhibited the insulin receptor kinase (Morgan *et al.*, 1986). Also phosphorylation and dephosphorylation of the insulin receptor parallels the onset and termination of the insulin action (Haring *et al.*, 1984). Further, analysis of solubilized insulin receptor from a patient with type A insulin resistance revealed a defect in insulin stimulated tyrosine kinase activity (Grunberger *et al.*, 1984).

Typically in type A insulin resistance insulin binding is markedly diminished. Grunberger *et al.* (1984) studied a patient with insulin resistance and other phenotypic features of the type A syndrome but with normal insulin binding. Their findings indicated that the receptors had a defect distal to the insulin-binding site. The defect was located in the β-subunit, which contains the ATP binding site and the receptor kinase. Failure of the tyrosine specific kinase to phosphorylate the receptor in the presence of insulin thereby bringing about insulin-resistance demonstrates that the receptor phosphorylation might play a significant role in signal transduction.

Subsequently it has been demonstrated that rats made diabetic with streptozotocin showed decreased autophosphorylation and kinase activity (Okamoto *et al.*, 1986). Also a decrease in kinase activity was observed in non-insulin dependent diabetics, due to a reduction in coupling efficiency between insulin binding and activation of receptor kinase (Freindenberg *et al.*, 1987). Finally human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin (Chou *et al.*, 1987).

It is apparent therefore that insulin stimulated phosphorylation of the β-subunit is an essential intermediary in the mechanism of insulin action. It is not surprising that most of insulin's known biological effects require β-subunit-phosphorylation (Chou *et al.*, 1987) including D-glucose transport (Kohanski *et al.*, 1986; Ellis *et al.*, 1986). The contradictory evidence for the importance of autophosphorylation presented above by Zick *et al.* (1984) and Simpson and Hedo (1984) suggests that the mechanism by which polyclonal anti-receptor antisera mimics insulin bioactivity may differ from the mechanism of action of insulin itself.

1.5.2   Insulin-Receptor Complex Internalization

Morphological and biochemical studies have identified a host of receptors for bioactive polypeptides in numerous tissue types, including a wide variety of cultured cells. Cellular uptake of polypeptides via these receptors, which involves concentration of ligands in the plane of the plasma membrane, can proceed up to 10,000 times more rapidly than uptake of fluid phase markers and initiate a cascade of receptor-specific functional and degradative processes (Dickson, 1985).

Fluorescence studies have demonstrated that insulin receptors are initially diffusely distributed on the plasma membrane (Pastan and Willingham, 1981). Binding of insulin to its receptor results in a time-dependent lateral migration of the insulin-receptor complex into clathrin coated pits, which serve to concentrate hormone-ligand complexes prior to internalization in rat hepatocytes (Carpentier et al., 1986a,b), cultured fibroblasts (Pastan and Willingham, 1981) and human fibroblasts (Gorden et al., 1982). Degradation of insulin and recycling of receptors follows internalization of the insulin-receptor complex (Gorden et al., 1982; Bridges et al., 1982). Receptor recycling is energy dependent and protein synthesis independent (Suzuki and Kono, 1979). The entire, intact receptor complex, $\alpha_2\beta_2$, is internalized and not merely a part of the receptor (Simpson et al., 1984).

Quantitative ultrastructural analysis of receptor-mediated insulin uptake into adipocytes using ferritin-insulin failed to reveal any significant amount of insulin within coated pits (Smith and Jarett, 1983). In fact the relative paucity of coated pits found in adipocytes compared with most other cell types, 0.3% and 1-2% of the cell surface area respectively, suggests that these specialized structures may

have a relatively insignificant role in insulin uptake in fat cells (Smith and Jarrett, 1983; Stahl and Schwartz, 1986). Additionally insulin simultaneously increase internalization of its receptor and accelerate fluid phase pinocytosis in U-937 monocytes. However, in rat adipocytes insulin– stimulated internalization was not accompanied by increased fluid phase pinocytosis (Oefelin et al., 1986). It appears therefore that the lack of involvement of coated pits in receptor-mediated endocytosis in adipocytes cannot be explained simply by increased fluid phase pinocytosis suggesting that more complex events are occuring.

However, coated pits occur in essentially all cell types and although its role in adipocytes membranes may be limited, it has been shown to be essential for the uptake of a large number of hormones, including insulin, in a variety of cell types and hence may represent a generalized phenomenon for receptor ligand internalization (Reviews: Carpentier et al., 1986a; Wileman et al., 1985). Morphological evidence suggests that coated pits are not specific for a particular hormone-receptor complex and a variety of ligand-receptor complexes may accumulate in the same coated pit (Geuze et al., 1984). Moreover, coated pits not only concentrate receptor molecules but selectively exclude other plasma membrane proteins. Subsequently the neck of the coated pits fuse giving rise to clathrin coated vesicles (Pastan and Willingham, 1981). The clathrin coat consists of a basic protein structure, a triskelion, which can spontaneously polymerize to form polygonal clathrin coated cages (Harrison and Kirchhausen, 1983; Kirchhausen and Harrison, 1984).

The α-subunit undergoes a conformational change during the early phase of insulin-receptor internalization which is distinct from the initial conformational change observed upon insulin binding (Berhanu et al., 1987). The significance of this change has not been ascertained. Shortly after internalization uncoating of the vesicles occur by way of a 70 KDa uncoating ATPase (Braell et al., 1984; Schlossman et al., 1984) resulting in uncoated vesicles which rapidly associates with a heterogenous complex set of vacuoles located in the peripheral and perinuclear cytoplasm termed endosomes (Helenius et al., 1983).

Gueze et al. (1983) using double-label immunoelectron microscopy on ultrathin cryosections of rat liver have been able to show that coated vesicles transfer hormone-receptor complexes to an elaborate system of anastomosing tubules and vesicles, in which uncoupling of the complexes occur. This organelle system they termed CURL (Compartment of Uncoupling of Receptors and Ligand). Previously Pastan and Willingham (1981) identified receptosomes; organelles intimately involved in hormone-receptor endocytosis. Recently it has been suggested that receptosomes, endosomes and CURL are just different terms for the same organelle system (Willingham and Pastan, 1985).

Acidification to pH 5.0 occurs almost immediately after endocytosis within endosomes (Tycko and Maxfield, 1982) which facilitates ligand-receptor uncoupling (Harford et al., 1983a; Wolkoff et al., 1984; Van Resenwoude et al., 1982). In fact, even before entering the endosomes ligand-receptor dissociation commences within the clathrin coated vesicles which contain an ATP-dependent proton pump capable of acidifying the vesicles interior (Forgac, 1983). Endosomes

contain an ATP-driven electrogenic proton pump along with an anion channel or transporter which is responsible for the generation and maintenance of acidity (Stahl and Schwartz, 1986; Mellman et al., 1986). The pH is not uniform throughout the various elements of the endosomal compartment with ligands encountering decreasing pH with time from an initial pH of about 5.5-6.0 in peripheral endosomes to a minimum of 4.5-5.0 in the lysosomal region (Mellman et al., 1986).

Gueze et al. (1984) proposed that once dissociated, the ligands accumulate in the aqueous dilatations of CURL while the receptors migrate along the tubular projections. It is envisaged that receptor-rich membrane vesicles bud from the tubular projections and are recycled. A small fraction associates with the Golgi-complex, while the ligand-rich vesicles are delivered to lysosomes for degradation (Fig. 1.2). CURL plays a central role in the sorting mechanisms that occur during receptor-mediated endocytosis: ligand is sorted from receptor, and various types of receptors are segregated from one another.

Uncoupling may take place in the vesicular portion of CURL, after which the receptor molecules are transported laterally into the connected tubules or in the tubules or at the tubulo-vesicle junction, with CURL functioning as a sieve for ligand, whereby receptor escapes degradation by remaining behind in the tubular structures (Geuze et al., 1983). Rome (1985) has suggested that segregation of receptor into the membraneous compartment may occur spontaneously, since aproximately 90% of the membrane is associated with the tubules then receptors will rapidly distribute throughout the membrane and be found on average 90% enriched within the tubules. However, the mechanism for receptor-ligand uncoupling and processing is unclear.

Fig. 1.2

Model of receptor and ligand traffic (modified from Geuze *et al.*, , 1984).
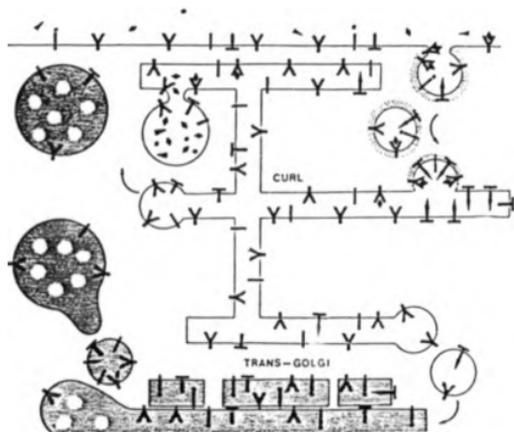
Receptors ($\perp$, $\mid$, Y) on the cell surface are available for ligand (•, ♦, ➤) binding. Internalization of ligand-receptor complexes, via clathrin coated pits, leads to fusion of coated vesicles with CURL. Receptors and ligands are separated in this organelle system resulting in recycling of receptor rich vesicles while ligands, which are localized within aqueous dilatations, subsequently undergo lysosomic degradation. It is envisaged that a small amount of receptors are processed through the Golgi and may also undergo degradation in lysosomes. A small fraction of the surface receptors also arise through the secretory pathway (shade).

It is envisaged that receptor internalization may form part of a continuously occuring internalization process independent of receptor occupancy (constitutive) or ligand binding may trigger endocytosis (inducible). Evidence for constitutive recycling comes from the observation that weak bases and carboxylic ionophores can inhibit recycling of asialoglycoprotein receptors on HepG2 hepatoma cells and deplete greater than 50% of the cell-surface receptors within 20 minutes even in the absence of ligand (Schwartz *et al.*, 1984). Unoccupied insulin receptors were shown to be continuously internalized in mouse fibroblasts (Kadle *et al.*, 1983) and Chvatchko *et al.* (1984) have suggested that insulin does not regulate the rate of internalization of its receptor in FaO hepatoma cells.

However, the evidence for insulin induced receptor internalization is overwhelming. In the absence of insulin 90% of the total cell receptors, in rat adipocytes, reside on the cell surface with 10% being intracellular (Arsenis *et al.*, 1985; Marshall, 1985a). Insulin caused a rapid redistribution of receptors by translocating surface receptors to the cell's interior in a temperature and energy dependent process with receptors accumulating intracellularly at a rate of approximately 20 000 per minute at 37°C (Fig. 1.3). After 6 minutes the size of the intracellular pool plateaus for up to 2 hours with 35% of the surface receptors residing within the cell (Marshall, 1985a). Similar insulin induced receptor redistribution has been observed in 3T3 L1 adipocytes (Reed *et al.*, 1984), hepatocytes (Draznin *et al.*, 1984) and fibroblasts (Knutson *et al.*, 1983).

Fig. 1.3.

Proposed itinerary of insulin and insulin receptors after endocytotic uptake in rat adipocytes (from Marshall, 1985b).
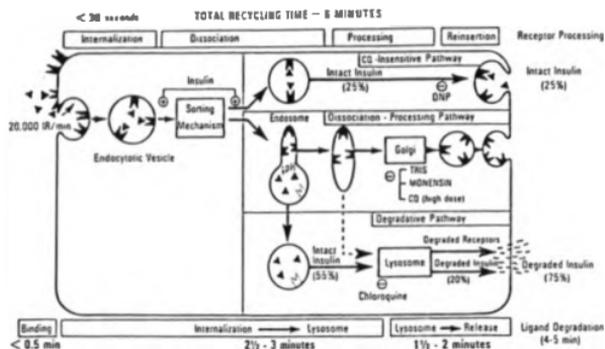
Insulin-receptor complexes are rapidly internalized where upon the complexes may enter distinct pathways:

A reteroenodocytic pathway which is insensitive to acidtropic agents but sensitive to to energy depletors and which delivers intact insulin back to the extracellular medium.

Dissociation-processing pathway:

Separate ligands and receptors, recycles receptors and sensitive to acidtropic agents

Degradative pathway:

Allows degradation of previously separated receptors and ligands in lysosomes. Degraded fragments are extruded from the cell. Sensitive to acidtropic drugs.

The mechanism by which insulin induces endocytosis of its own receptor is unclear. However, Iacopetta *et al.* (1986) have indicated that protein kinase C may play a part in modulating insulin-receptor internalization in HL 60 cells. This is curious since protein kinase C has also been implicated in the regulation of insulin receptor β-subunit phosphorylation (Bollag *et al.*, 1986). Recently Marshall and Garvey (1986) have suggested that the phosphorylated insulin receptor complex may play a role in the intracellular sorting of the endocytotic vesicles which carry insulin-receptor complexes into the cell. Endocytosis of epidermal growth factor (EGF) receptor is associated with the phosphorylation of a onine residue, on the receptor, which may directly interact with clathrin coated pits (Hannover and Dickson, 1985). Whether such an arrangement exists for the insulin receptor remains to be determined.

Insulin induces receptor internalization in mature human erythrocyte ghosts and since these cells lack intracellular organelles and protein synthesizing components, this suggests that insulin receptor internalization may require only the plasma membrane and underlying cytoskeletal elements (Keller *et al.*, 1987; Peterson *et al.*, 1983). Also vinblastine, a microtubule disruptive drug, significantly inhibit intracellular translocation of insulin receptors from and to the plasma membrane in isolated cardiocytes thus implicating microtubule involvement in endocytosis. Further the plasma membrane bound Na^+/H^+ antiport may play a role in ligand uptake through Na^+ influx and intracellular pH regulation (Hwang *et al.*, 1986).

Subcellular fractionation has led to the identification of several receptor enriched compartments participating in the concentrative

receptor-mediated endocytosis of insulin (Bergeron *et al.*, 1985). Peptide hormones have been found localized within golgi associated clathrin destined for delivery to lysosomes (Hanover *et al.*, 1984). Additionally golgi-associated ligands and receptors enter a proposed golgi subregion termed GERL (Golgi Endoplasmic Reticulum Lysosomes); an acid phosphatase-rich membrane network with a lysosomal terminus (Dickson, 1985). However, although internalized insulin-receptor complexes have been found associated with highly purified golgi-rich fractions (Khan *et al*, 1982; Sonne and Simpson, 1984), other researchers have failed to identify these complexes within the golgi cisternae (Carpentier, 1986a).

After dissociation of the insulin-receptor complexes in CURL, or some specialized golgi/GERL region the hormone is often associated with multivesicular bodies (MVB) in transit to lysosomes (Carpentier *et al.*, 1986a; Gorden *et al.*, 1982). Receptor rich vesicles bud off and recycle from CURL (Geuze *et al.*, 1983, 1984). Dissociation of the ligand from its receptor seems to be required for receptor recycling since weak bases and acidtropic agents, which cause deacidification of endocytic vesicles and hence prevents ligand-receptor separation, results in the accumulation of intracellular receptors and inhibition of lysosomal hormone degradation (Whittaker *et al.*, 1986; Harford *et al.*, 1983a,b; Smith and Jarett, 1982). Acidtropic agents may even impair cycling of unoccupied receptors suggesting that receptor exocytosis may also require acid compartments (Mellman *et al.*, 1986; Wileman *et al.*, 1984).

It is also apparent that adipocytes may process insulin through either of two separate pathways: a retroendocytic pathway that culminates in the release of intact insulin, and a degradative pathway

that terminates in the intracellular catabolism and release of degraded insulin. Further, once insulin enters the degradative compartments it is committed to catabolism and cannot exit the cell through the retroendocytic route (Fig. 1.3) (Marshall, 1985b; Levy and Olefsky, 1986). The retroendocytic pathway does not involve vesicular acidification or dissociation of insulin receptor complexes, hence this pathway is insensitive to acidtropic agents (Marshall, 1985b). The higher the insulin levels the greater the proportion of internalized insulin which traverses the retroendocytic pathway, indicating that retroendocytosis is under insulin regulation (Levy, 1986). The intricacy of insulin-receptor endocytic processing is further complicated by the observation that insulin covalently linked to its receptor may recycle slowly through the lysosomal compartment (Carpentier *et al.*, 1986b). What determines which pathway the complexes will follow is at present unknown, however, it has been suggested that the state of receptor aggregation in endosome membranes determines the fate of the receptors (Mellman *et al.*, 1984).

Acute treatment of adipocytes with a high concentration of insulin leads to a time, temperature and energy dependent loss of surface receptors (Marshall *et al.*, 1984). The initial insulin induced redistribution of receptors to intracellular endocytic compartments is followed by an increased rate of receptor degradation leading to marked alterations in receptor content and receptor distribution in these down regulated cells (Bergeron *et al.*, 1985). In chick liver cells down regulation may occur solely through receptor redistribution and intracellular sequestration without any loss to the total cell receptor number (Krupp and Lane, 1982). A protein factor appears to be involved in the inactivation of internalized receptors, which preceeds receptor degradation (Knutson *et al.*, 1985). Further, degradation of

insulin and its receptor are distinct, separable processes occuring at different rates, and probably in different subcellular locations (Knutson *et al.*, 1985). Enzymes which participate in insulin degradation exists both in the cytosol and in lysosomes (Yonezawa *et al.*, 1986; Shii *et al.*, 1986; Misbin *et al.*, 1983).

Internalized insulin receptors gradually pass from a recyclable pool to a non-recyclable pool leading ultimately to degradation and a lower steady state cellular receptor level. Twelve hours after addition of insulin, fall of total receptor level ceases and the down regulated steady-state is achieved. At this stage the intracellular receptor pool is approximately 16% of the total initial receptor (Knutson *et al.*, 1983). Initially the rate of degradation is 13-17 fold slower than the rate of internalization, indicating that the initial rate of internalization of the insulin-receptor complex is not the rate-limitng step in their degradation (Reed *et al.*, 1984).

In contrast to the depletion of surface receptors during chronic insulin stimulation, receptor-mediated endocytosis is impaired in hypoinsulinemic diabetic patients (Carpentier *et al.*, 1986c). These researchers suggest that the delayed or reduced internalization, of the insulin receptor complexes, may serve to amplify the muted signal caused by deficient hormone secretion in this diabetic state.

The significance of insulin mediated receptor internalization:

1. Ligand destruction, which prevents ligands dissociating and interacting with other receptors (Yonezawa *et al.*, 1986).

2. Down-regulation ensures desensitization of target cells until high circulatory levels of hormone drops to negligible levels. It is ironic that although down regulation protects against overstimulation a mechanistic defect in receptor reinsertion into the plasma membrane may lead to insulin resistance in obesity and non-insulin dependent diabetes (Garvey *et al.*, 1985; Begum *et al.*, 1985).

3. Bioresponse: potential for trans-membrane signalling and amplification of hormone signals. 3T3-L1 adipocytes accumulate potentially significant amounts of insulin in nuclei by an insulin receptor-mediated process which suggests that the transport of insulin or the insulin-receptor complex to nuclei may be directly involved in the long term biological effects of insulin, particularly in the control of DNA and RNA synthesis (Smith and Jarett, 1987; Jarett and Smith, 1986).

1.5.3   *Receptor Regulation*

A large number of agents and altered physiological conditons are known to affect insulin binding to target cells. For example, addition of catecholamines, glucagon or glucocorticoids to cells, as well as the conditions of diabetes and obesity, modify insulin-receptor activity (Reviews: Czech, 1985; Stadtmauer, 1986). Negative cooperativity (De Meyts, 1973; 1976), positive cooperativity (Marsh, 1984) and affinity changes (Corin and Donner, 1982; Lipson *et al.*, 1986) have all been observed for insulin receptor interaction. It has been suggested that alteration of receptor oligomeric forms may regulate changes in receptor affinity (Crettaz *et al.*, 1984).

However, it is intriguing that phorbol esters, catecholamines and insulin itself, all of which appear to modify insulin binding activity in intact cells also appear to cause concomitant phosphorylation of the insulin receptor or other proteins that may affect the insulin receptor (Czech, 1985). These effects are summarized in Fig. 1.4. β-Adrenergic agonists which activate cAMP kinase through elevation of cytoplasmic cAMP, appear to induce receptor phosphorylation on serine/threonine residues, inhibiting receptor tyrosine specific phosphorylation (Roth and Beaudoin, 1986) and inhibiting insulin binding (Pessin et al., 1983). Hence catecholamines counter-regulate basal and insulin stimulated glucose transport in rat adipose cells (Arsenis and Livingston, 1986; Smith et al., 1984).

Phorbol ester activation of protein kinase C results in the inhibition of insulin binding (Jacobs et al., 1983) and deactivates the tyrosine specific kinase in a fashion similar to cAMP kinase. Fig. 1.4. (Bollag et al., 1986). Both catecholamines and phorbol esters modulate the receptor kinase by increasing its K_m for ATP which may lead to insulin resistance (Obermaier et al., 1986). Insulin might modulate its own kinase activity through an insulin-receptor directed protein phosphatase (Horn et al., 1986) and also by stimulating protein kinase C activation through increased production of diacylglycerol and intracellular calcium (Discussed further in Section 1.6) (Saltiel et al., 1986; Houslay, 1987).

Fig. 1.4.

Hypothetical pathways whereby the insulin receptor kinase may be regulated by β-adrenergic agonists, phorbol esters, and insulin in intact cells (from Czech, 1985).
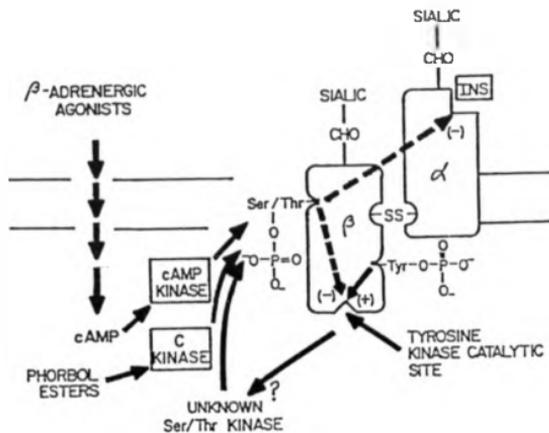
It is proposed that a complex network of serine/threonine and tyrosine phosphorylations might modulate insulin-receptor activity in an antagonistic manner.

1.6   SECOND MESSENGERS IN INSULIN ACTION

1.6.1   Evidence for Second Messengers

The evidence for the existence of second messengers comes from the observation that a number of substances can mimic insulin action, apparently by a mechanism similar to that of insulin itself (Cheng and Larner, 1985).   Treatment of rat adipocytes with low levels of the proteolytic enzyme trypsin, results in rapid stimulation of glucose transport, glucose oxidation, glycogen synthase activity state and pyruvate dehydrogenase activity state (Kikuchi et al., 1981).   Trypsin has also been shown, like insulin, to stimulate phosphorylation of the β-subunit of the insulin receptor suggesting that in intact cells trypsin acts directly on the receptor (Tamura et al., 1983) and that proteolytic action might be involved in the generation of insulin mediator (Kikuchi et al., 1981).

Antibodies to the insulin receptor, obtained from patients with type B insulin resistance, stimulated glucose transport and metabolism in isolated adipocytes (Kahn et al., 1977) and concanavalin A and wheat germ agglutinin also exhibit insulin mimetic properties (Roth et al., 1983b). Both concanavalin A and antireceptor antibody stimulated pyruvate dehydrogenase activity in a subcellular system consisting of plasma membrane and mitochondria.   Removal of plasma membrane from the system resulted in abolition of the stimulatory effect, suggesting that a chemical mediator was being generated by the plasma membrane or the receptors (Seals and Jarett, 1980).   It is envisaged that such a mediator would be generated directly from the plasma membrane-bound insulin-receptor complexes since the process of internalization appears to be too slow to explain rapid insulin effects

on membrane transport processes as well as on enzyme activation and inactivation, both of which are observed within seconds after hormone addition (Cheng and Larner, 1985).

However, if a mediator was released from the insulin receptor itself, one might expect receptors to become rapidly inactivated, which does not appear to happen (Houslay and Heyworth, 1983). In addition, vanadate, which mimics insulin, causes an 8-fold increase in glucose transport by an effect distal to the insulin receptor (Green, 1986). Hence implying the production of an insulin mediator that is distinct from the receptor. Further, since concanavalin A and antireceptor antibody could mimic insulin action, then the mediator must be part of the plasma membrane and not a derivative of the insulin molecule (Seals and Jarett, 1980). The ability of lysosomotropic agents to inhibit insulin degradation without affecting insulin's metabolic events also demonstrates that it is insulin binding rather than the biological degradation that is required for biological activity (Sonne and Gliemann, 1980).

Although endocytosis of the insulin-receptor complexes may not be required to initiate mediator production, the internalized complexes may continue signal transduction (Ueda et al., 1985; Douen and Jones, 1986). Also internalization of insulin may be required to elicit its inhibitory effect on endogenous protein degradation (Trowbridge and Draznin, 1986; Draznin and Trowbridge, 1982) and also to elicit the long term mitogenic effects of insulin (Smith and Jarett, 1987).

1.6.2   Failure of Classical Second Messengers: Cyclic AMP (cAMP), Cyclic GMP (cGMP) and Hydrogen Peroxide (H₂O₃)

Insulin induces a transient increase in cGMP concentration in adipocytes, however, other agents such as norepinephrine can elevate cGMP levels without characteristic effects of insulin. Extracellularly added H_2O_2 mimics the effects of insulin, however, H_2O_2 cannot stimulate receptor phosphorylation and the effect of insulin on adipocyte peroxide production may be the result of changes in free fatty acid concentration. cAMP can account for some but not all of insulin's actions. It seems therefore that direct involvement of these three established second messengers of hormonal action therefore fail to satisfactorily explain insulin action (Review: Cheng and Larner, 1985).

1.6.3   Protein Mediators

Insulin has been found to regulate its intracellular metabolic events by promoting phosphorylation and dephosphorylation of enzymes (Cheng and Larner, 1985). Larner *et al.* (1979) and Jarett and Seals (1979), isolated a chemical mediator of insulin action, with an approximate M_r of 1000-1500, which was found to be heat stable, water soluble, stable at neutral pH and in cell free systems was able to regulate phosphorylation of major metabolic enzymes by activating phosphatases and inhibiting cAMP dependent kinases while having no effect on cAMP independent kinase (Kiechle *et al.*, 1981). Extensive work from a number of laboratories has implicated this new family of mediators in the physiological role of insulin (Reviews: Cheng *et al.*, 1985; Jarett *et al.*, 1985 b; Cheng and Larner, 1985). Insulin mediator induces receptor down regulation (Caro *et al.*, 1983) and is

capable of acting externally on intact adipocytes, to mimic insulin action, as well as in a cell free system (Zhang *et al.*, 1983). Further, mediator prepared from skeletal muscle of insulin treated rats stimulated pyruvate dehydrogenase activity in intact adipocytes and the mediator was degraded with time (Jarett *et al.*, 1985a).

The actual mode of generation of mediator is unclear, however the observation that trypsin stimulates production of insulin-like but not insulin-identical mediators from the plasma membrane (Kikuchi *et al.*, 1981) and that mediator generation is inhibited by several protease inhibitors (Seals and Czech, 1980) implies that limited proteolysis is a key event in mediator production (Larner, 1984). The exact structure of insulin mediator is not known, however, since it can be destoryed by proteases (Seals and Czech, 1980; Zhang *et al.*, 1983) and is sensitive to neuraminidase (Begum *et al.*, 1983) suggests that it might be a glycopeptide.

Insulin elicits antagonizing biological actions at high and low concentrations indicating the existence of more than one type of mediator and indeed separate mediators which activate and deactivate glycogen synthase phosphoprotein phosphatase (Cheng *et al.*, 1980) and pyruvate dehydrogenase (Saltiel *et al.*, 1982) have been isolated. Hence it appears that mediator and antimediator may be involved in signal transduction in insulin action. Several distinct antagonistic mediators have been isolated which are rapidly generated *in vivo* as a result of insulin action (Larner, 1984). However, insulin-generated mediators that work on various intracellular insulin-sensitive enzyme systems cannot account for all pleiotropic effects of insulin on target cells and accordingly mediators appear to have no effect on glucose transport or oxidation under conditions where certain enzyme processes

were activated (Jarett *et al.*, 1985a,b).

On the other hand, a large protein macromolecule may be involved in the enhancement of transport activity. Schoenle *et al.* (1984), observed that incubation of adipocytes with insulin caused a dramatic quantitative decrease in the plasma membrane of a 90 KDa protein. The insulin effect was dose-dependent, with a half-maximal effect at 9.5 microunits/ml, time-dependent ($t_{\frac{1}{2}}$ < 20 sec) and was not due to proteolysis since presence or absence of protease inhibitors caused no change in the 90 KDa protein concentration. These researchers propose that the 90 KDa protein is involved in the transmission system of insulin in the plasma membrane. They suggest that this protein while in the membrane inhibits the hexose carrier system. Upon stimulation of glucose transport by insulin, this inhibitory protein is removed from the membrane. Divalent chelating agents caused a similar reduction in the concentration of the 90 KDa protein which was overcome by adding equivalent amounts of calcium, suggesting the involvement of calcium. However, since endocytosis of the insulin-receptor complex is thought to be too slow to explain insulin enhancement of membrane transport (Cheng and Larner, 1985), it is questionable whether the decrease of the membrane bound 90 KDa protein would be rapid enough to account for activation of transport.

1.6.4. *Guanine Nucleotide Binding (G-) Proteins*

It is now firmly established that receptors for a large number of hormones and neurotransmitters, as well as for light in the case of rhodopsin, are linked in the plasma membrane to G-proteins (Rodbell, 1985a). The family of G proteins are characterized by their ability

to selectively bind GTP with high affinity and degrade the nucleotide to GDP. They are composed of at least 3 distinct subunits (α,β,γ) and they can be ADP ribosylated by chlorea toxin and pertusis toxin (Rodbell, 1985a; Hughes, 1983; Heyworth *et al.*, 1985; Elks *et al.*, 1983). The interaction of G-proteins with adenylate cyclase has received considerable attention (Gilman, 1984; Houslay and Heyworth, 1983), presumably because of the role of cAMP as a "second messenger". However, there is growing evidence for the involvement of G-proteins in other systems including inositol phosphate hydrolysis (Michell and Kirk, 1986; Rapiejko *et al.*, 1986) and glucose transport (Baker *et al.*, 1983).

There are several different types of G-proteins, which are described in terms of their function as G_s (stimulatory for adenylate cyclase), G_i (inhibitory for adenylate cyclase), G_o (unknown function) and G_t (transducin, in retinal photoreceptors) (Gilman, 1984; Barnes, 1986). Various G-proteins share a great deal of structural homology (Rodbell, 1985a,b). Only the α-subunit binds GTP and the type of α unit coupled depends on the type of G-unit (and associated receptor) to which it is attached, while the β and γ units are highly conserved proteins having similar if not identical structures irrespective of the type of α-unit attached (Rodbell, 1985b). The β/γ complex probably serves as an anchor to the cytoplasmic face of the plasma membrane (Bourne, 1986).

Interaction of hormone-receptor complexes with G-units occupied by GTP, causes the oligomer to dissociate into monomers and receptors are thought to be transformed from high to low affinity (Rodbell, 1985b). Free α-GTP is discharged into the cytoplasm where it activates and regulates various systems, e.g. adenylate cyclase (Houslay,

Fig. 1.5

Molecular interactions of G proteins during signal transduction.
(from: Gilman, 1987.)
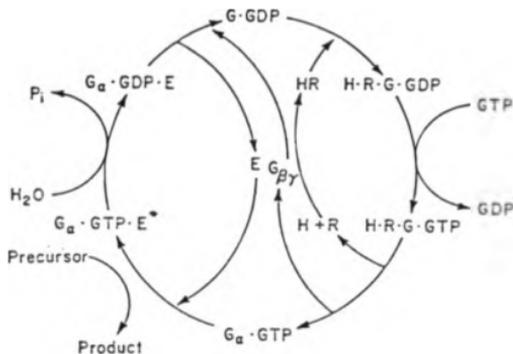
Hormone (H) binding to its receptor (R) causes GTP to bind to the Gα
subunit. This activates effector (E) molecules inside the cell to make
a product. The Gα.GTP.E complex is inactivated by removing a phosphate
group (P_i) and reforming the unstimulated G.GDP complex.

1986), insulin receptor kinase (Horn, 1986) (Fig. 1.5). Conversion of G_α-GTP to G_α-GDP by GTPases facilitates the inactivation of the signal (Barnes, 1986). A 25 KDa protein has been isolated, from hepatocyte plasma membranes, which contain a high affinity GTP binding site, undergoes cholera toxin ADP-ribosylation and can interact with the insulin receptor. It has been proposed that this is the G^α_{ins} which mediate some of insulins action (Houslay, 1986; Heyworth *et al.*, 1985). Another research group have shown the existence of, 41 000-M_r (G_i) and 39 000-M_r (G_0) proteins in rat fat cells which either together or separately may mediate hormonal regulation of adenylate cyclase and phospholipase C (Rapiejko *et al.*, 1986).

The advantage of a large protein molecule (G_α) as a second messenger, as opposed to a small molecule (such as cAMP), is the malleability of the former (Rodbell, 1985a). Proteins can undergo a large number of covalent and non-covalent modifications i.e. phosphorylation, methylation, oxidation, degradation and attachment of other proteins via disulphide bonds. Indeed it has been speculated that tyrosine phosphorylation of G_α-units may be linked with the regulation of their interaction with effector enzymes (Espinal, 1986), and the G_t-α (transduction) unit has been shown to undergo multisite phosphory- lation on tyrosine residues by insulin receptor kinase (Zick *et al.*, 1986).

The pluripotency of protein molecules forms the basis of Rodbell's programmable messenger theory (Rodbell, 1985b) in which the α-subunits are available for modification (or programming) upon release into the cytosol hence rendering different regulatory structures:

$$H.R + G_{\beta\gamma}$$

$$\begin{array}{ccccccc}
 & & M^0 & \rightarrow \alpha^0 & \rightarrow E^0 & \rightarrow S^0 & \rightarrow \text{Response} \\
\overset{\alpha}{\underset{GTP}{}} & \Big\langle & M^1 & \rightarrow \alpha^1 & \rightarrow E^1 & \rightarrow S^1 & \rightarrow \text{Response} \\
 & & M^2 & \rightarrow \alpha^2 & \rightarrow E^2 & \rightarrow S^2 & \rightarrow \text{Response}
\end{array}$$

$$H.R.G.GTP$$
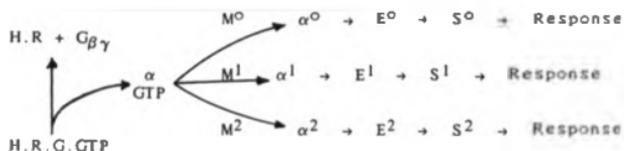
The released α-subunit is exposed to modifying enzymes (M) that transform α into new structures having affinities for different effector (E) units which, when activated, yield signals (S), the combination of which give the pleiotypical responses of the target cell.

1.6.5 *Calcium (Ca^{2+}), Inositol Phospholipids, Protein Kinase C*

The plasma membrane is a regulatory barrier separating high extracellular calcium ($Ca_c{}^{2+}$) concentrations from low intracellular cytoplasmic calcium ($Ca_i{}^{2+}$) hence this ion enters the cell by moving passively down its' steep concentration gradient (McDonald *et al.*, 1982). Ca^{2+} is energetically extruded from the cell by two mechanisms: a Na^+/Ca^{2+} antiport system and a unidirectional export via a high affinity Ca^{2+} extrusion pump. The latter is active at submicromolar concentrations and may be responsible for normal physiological control of Ca^{2+} (Pershadsingh *et al.*, 1980). The electrochemical gradient of Ca^{2+} represents a large available energy source that could serve as an ideal trigger mechanism in response to a small input of energy, such as hormone receptor binding (McDonald *et al.*, 1982).

Since it is believed that the glucose transporters are held in intracellular vesicles lying just beneath the plasma membrane as pictured by Kono (1983) then conceivably only a trigger mechanism with a low threshold is all that is needed to allow juxtaposed

membranes (of the vesicles and the plasma membrane) to fuse. Ca^{2+} may facilitate membrane fusion by forming an ionic bridge between the heads of phospholipids of opposing membranes. However, no formal evidence exist in favour of such a hypothesis and much controversy surrounds the role of Ca^{2+} in the mechanism of insulin action (Review: Klip, 1984).

Some laboratories have failed to observe any dependence for Ca_e^{2+} or Ca_i^{2+} in the stimulation of glucose transport both in adipcotyes (Williams et al., 1986) and muscle cells (Klip et al., 1984 ; Hall et al., 1982). In addition it has been suggested that magnesium (Mg^{2+}), rather than Ca^{2+}, is essential in the process of insulin-stimulated sugar transport (Hall et al., 1982; Eckel et al., 1983). On the other hand insulin has been found to inhibit a high affinity, plasma membrane bound, ($Ca^{2+} + Mg^{2+}$)-ATPase, in adipocytes, causing an increase in intracellular calcium (Pershadsingh and McDonald, 1979) and removal of Ca_e^{2+} by employing a Ca^{2+}-free buffer or the Ca^{2+} chelator, EGTA, markedly reduced insulin stimulation of glucose transport in myocytes (Bihler et al., (1985), 3T3 mouse fibroblasts (Yamanishi et al., 1983) and rat adipocytes (Taylor et al., 1979). McDonald et al. (1981) have isolated an insulin mediator which mimics insulin action in many ways but unlike insulin it does not inhibit ($Ca^{2+} + Mg^{2+}$)-ATPase. These researchers propose that insulin binding to the receptor may generate an antimediator which inhibits the ($Ca^{2+} + Mg^{2+}$)-ATPase resulting in accumulation of Ca^{2+} in the cell.

The ($Ca^{2+} + Mg^{2+}$)-ATPase can be stimulated 3-fold by the ubiquitous Ca^{2+}-dependent regulatory protein calmodulin (Pershadsingh et al., 1980) which suggests that intracellular redistribution of calmodulin may play an important part in the early regulatory events

directed by insulin (Goewert *et al.*, 1983). In addition the insulin receptor contains a calmodulin binding site (Graves *et al.*, 1985) and calmodulin enhances insulin-mediated receptor phosphorylation of the β-subunit and exogenous substrates by a Ca^{2+}-dependent process while insulin in turn, stimulates phosphorylation of calmodulin (Graves *et al.*, 1986; Plehwe *et al.*, 1983).

Receptor mediated activation of a plasma membrane bound phospholipase C, which catalyses the hydrolysis of inositol phospholipids (IPLs) into diacylglycerol (DAG) and inositol-phosphates [mono-(IP), di-(IP$_2$), tri-(IP$_3$)] is recognized as a common mechanism for transducing various extracellular signals into the cell (Nishizuka, 1984, 1986; Berridge, 1984). The IP$_3$ branch and the DAG branch of the phosphoinositide signal cascade appear to act synergistically to phosphorylate proteins, the former by elevating Ca^{2+} to activate calmodulin-dependent protein phosphorylation and the latter to activate C-kinase, which phosphorylates a different set of proteins (Hokin, 1985).

Insulin transiently activates phospholipase C (Farese *et al.*, 1986), possibly through a specific G-protein (Houslay, 1986; Rapiejko *et al.*, 1986), resulting in the hydrolysis of IPLs into DAG and inositol phosphates. In addition insulin rapidly increases *de novo* phospholipid synthesis and production of DAG directly from phosphatidic acid (Farese *et al.*, 1985). Insulin activation of rat adipocyte phospholipase C correlates with the stimulation of hexose transport suggesting the involvement of this enzyme's products in the mechanism of insulin stimulation of sugar transport (Goldman *et al.*, 1986). It has also been proposed that insulin activation of phospholipase C hydrolyzes a novel glycolipid resulting in the generation of DAG and an inositol

phosphate- glycan (containing glucosamine and other carbohydrates), the latter product being able to regulate cAMP phosphodiesterase activity (Saltiel *et al.*, 1986). It is possible then, that a unique pool of inositol-containing phospho/glycolipid is acted upon by insulin (Houslay, 1986).

IP_3, generated from the breakdown of IPLs, can provoke the release of Ca^{2+} from intracellular stores (located within the smooth endoplasmic reticulum) (Berridge and Irvine, 1984) by a process apparently involving a specific G-protein (Dawson, 1985). Ca^{2+} efflux from rat liver microsomes can be promoted by GTP alone or by a GTP enhanced IP_3-promoted Ca^{2+} release (Dawson, 1985; Dawson *et al.*, 1986). Similar observations were found for Ca^{2+} efflux from the sarcoplasmic reticulum of smooth muscle (Saida and van Breen, 1987) and the endoplasmic reticulum of islets (Wolf *et al.*, 1987). Dawson *et al.* (1987) purports a mechanism involving GTP-induced vesicle fusion for the effects of GTP on Ca^{2+} efflux from rat liver microsomes.

The rise in Ca^{2+} triggered by this route is transient but may suffice to illicit rapid metabolic changes (Houslay, 1987). Indeed when rat adipocytes were loaded with the Ca^{2+} chelator quin 2 the stimulation of both glucose transport and oxidation were inhibited (Pershadsingh *et al.*, 1986a). In addition the identification of a Ca^{2+}-binding site in the insulin receptor (Williams and Turtle, 1984) and the adeptness of Ca^{2+} to enhance the ability of calmodulin to stimulate insulin-mediated phosphorylation of the β-subunit further illustrate the importance of Ca^{2+} in insulin action (Graves *et al.*, 1986).

IP$_3$ can be converted to inositol (1,3,4,5) tetraphosphate (IP$_4$) by a specific kinase in a Ca^{2+}-dependent reaction and this IP$_3$/IP$_4$ pathway may serve to deactivate the Ca$_i^{2+}$ mobilizing power of IP$_3$ (Irvine et al., 1986). In addition IP$_4$ might be capable of opening Ca^{2+} gates located within the plasma membrane thus allowing entry of Ca$_e^{2+}$ (Houslay, 1987). This may represent a crucial step in hormonal action since Ca$_i^{2+}$ stores are only of limited size (Berridge and Irvine, 1984). It might be envisaged that this flood of Ca$_e^{2+}$ could be sufficient to allow fusion of transporter vesicles with the plasma membrane, however, this remains to be determined. The action of IP$_3$ is curtailed by inositol triphosphatase which facilitates dephosphorylation of IP$_3$ to IP$_2$ (Berridge and Irvine, 1984).

The enzyme protein kinase C (pk-C) can be activated by the synergistic action of an increase in Ca$_i^{2+}$ concentration and the formation of DAG (Nishizuka, 1986). pk-C also has a requirement for phosphatidylserine and once activated may serve to phosphorylate various intracellular substrates (Nishizuka, 1984, 1986; Hokin, 1985) including the insulin receptor (Jacobs et al., 1983) and G-proteins (Zick et al., 1986; Williams et al., 1987) and pk-C is believed to be involved in the modulation of insulin and transferrin receptor internalization (Iacopetta et al., 1986). pk-C activity has been found to be enhanced by GTP-binding proteins in rat peritoneal neutrophils (Huang et al., 1987). Phorbol esters exhibit insulin like properties, to some extent, presumably through the activation of pk-C (Cherqui et al., 1986). This "insulin-like" effect (stimulation of hexose transport) of phorbol esters has been observed in L6 muscle cells, with a corresponding alteration of the subcellular distribution of pk-C, however in contrast, insulin activation of hexose transport failed to result in any detectable movement of this enzyme suggesting that

insulin stimulation of sugar transport in these cells may not involve pk-C (Klip and Ramlal, 1987).

On the other hand, phorbol ester activation of pk-C in rat adipocytes stimulates both glucose transport and metabolism, (Pershadsingh *et al.*, 1986a; Cherqui *et al.*, 1986) suggesting the possible involvement of pk-C in insulin action, in this cell type. Additionally phorbol esters enhances basal glucose transport activity while inhibiting insulin-stimulated transport in rat adipocytes (Kirsch *et al.*, 1985). However, pk-C is capable of phosphorylating the insulin receptor specifically on serine/threonine residues which has an inhibitory effect on the receptor tyrosine specific kinase resulting in desensitization of the cells (Bollag *et al.*, 1986). pk-C therefore may act not only as a mediator of insulin action but may also provide a feed-back mechanism for controlling insulin action (Nishizuka, 1986). DAG, which is required for the activation of this kinase, can itself by hydrolysed into phosphatidic acid and arachidonic acid, the latter being able to function as a second messenger (Nishizuka, 1984; Berridge and Irvine, 1984).

The entire sequence of events described for the activation of phospholipase C by a specific G-protein, the generation of DAG and IP_3 and the subsequent release of Ca^{2+} from intracellular stores and opening of plasma membrane Ca^{2+} gates is depicted in Figure 1.6.

Fig. 1.6. One possible sequence of events that may be involved in insulin stimulation of hexose transport.

References: Houslay et al., 1987; Houslay, 1986; Rapiejko et al., 1986; Goldman et al., 1986; Dawson et al., 1987; Delfert et al., 1986.
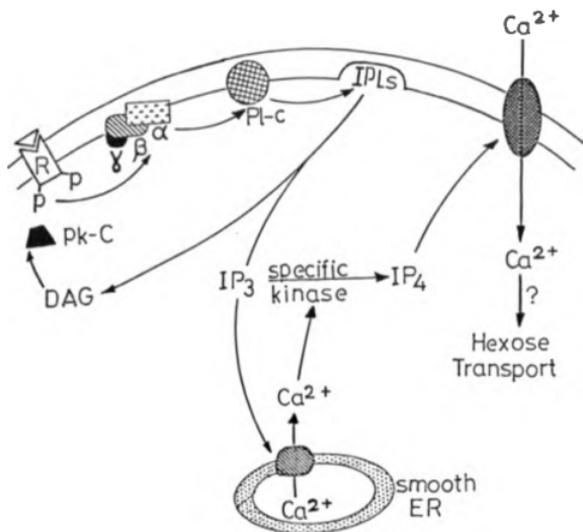
Insulin induced inositol phospholipid (IPLs) hydrolysis gives rise to diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 induces Ca^{2+} release from intracellular stores. The possible conversion of IP_3 to inositol tetraphosphate (IP_4) facilitates the opening of plasma membrane Ca^{2+} gates. Both DAG and Ca^{2+} activates protein kinase-C (pK-C).

1.7 D-GLUCOSE TRANSPORTER

1.7.1 Structure and Synthesis

Virtually all mammalian plasma membranes contain a system for the stereospecific transport of glucose. In some tissues such as kidney and small intestine the transporter is a sodium-dependent active carrier, while in other non-epithelial tissue, a facilitated transport system is evident (Birnbaum et al., 1986). The most extensively studied glucose transport protein is that of the human erythrocyte, primarily because in this cell type the plasma membrane has a relatively simple composition, it is easy to isolate and is not contaminated by intracellular organelles (Klip et al., 1986). Nevertheless, the glucose carrier of several cell types, including rat adipocytes (Lienhard et al., 1982; Shanahan et al., 1982), 3T3-L1 adipocytes (Schroer et al., 1986) rat skeletal muscle (Klip et al., 1983) and hepatocytes (Mueckler et al., 1985) have been identified or isolated by a number of different techniques involving separation by SDS-PAGE followed by cytochalasin B binding and immunological analysis (Klip et al., 1986; Klip and Walker, 1983).

The D-glucose transporter is an integral membrane glycoprotein containing about 15% carbohydrate by weight and with a M_r – 46 000 (Baldwin and Lienhard, 1981; Wheeler et al., 1982). Proteolysis sutdies reveal that this protein is transmembrane in nature, with an extracellular domain that is susceptible to trypsin cleavage (Simpson and Cushman, 1986). In addition exofacial sulphydryl groups on the molecule appear to be important for both the function and regulation of hexose transport (May, 1985). The amino acid sequence of the glucose transport protein from human Hep G2 hepatoma cells

deduced from analysis of a complementary DNA (cDNA) clone revealed a 492-residue polypeptide (Mueckler *et al.*, 1985). Further, hydropathy analysis indicates the existence of 12 membrane spanning segments, several of which could potentially form amphipathic α-helices containing numerous hydroxyl and amide side chains and thus provide either a glucose binding site and/or an appropriate lining for a pore through the membrane (Simpson and Cushman, 1986; Mueckler *et al.*, 1985). The transporter contains at least one N-linked oligosaccharide localized on asparagine 45, and both the amino- and carboxy-terminal regions are situated within the cytoplasm (Mueckler *et al.*, 1985).

It has been postulated that ribosomes engaged in the synthesis of glucose transporter never interact directly with the rough endoplasmic reticulum membrane (Mueckler and Lodish, 1986). These researchers have suggested that the transporter contains at least two distinct signal sequences. It is envisaged that polypeptide chain elongation continues until the first signal sequence emerges from the ribosomes where upon a signal recognition particle binds to the sequence and facilitates its insertion into the membrane as a loop structure through the interaction of the complex with docking protein. Chain elongation continues until a subsequent sequence emerges from the ribosome after which the cycle repeats itself.

Isoelectric focusing of human (Mathaei *et al.*, 1987) and rat (Ciaraldi *et al.*, 1986) glucose transporters photolabelled with [^{3}H] cytochalasin B revealed two distinct glucose transporter isoforms in low density microsomes focussing at pH 5.6 and pH 6.4, however, in the plasma membrane only the pH 5.6 isoform was detectable, while the pH 6.4 form was found in the high density microsomes. Hence it was proposed that the pH 6.4 species represents an immature form

which migrates to the low density microsomes where it matures, converting to the pH 5.6 species, and becomes available for translocation to the plasma membrane (Matthaei *et al.*, 1987). The observation that only the pH 5.6 isoform of the transporter is susceptible to neraminidase digestion (which removes sialic acid moieties from terminally glycosylated proteins) generating a pH 6.0 species whereas the pH 6.4 isoform is unaltered by nuraminidase, further supports the proposal that the pH 6.4 species may represent a biosynthetic precursor (Simpson and Cushman, 1986).

1.7.2 *Activation on the Transport Activity*

The entry of glucose across the plasma membrane represents the rate limiting step for the intracellular metabolism of glucose; in fat cells transport remains rate limiting for glucose uptake at concentrations upto 80mM (Crofford and Renold, 1965). Further, the translocation of bound glucose across the membrane rather than carrier substrate dissociation represents the rate limiting step in the overall transport cycle (Wang *et al.*, 1986; May and Mikulecky, 1982). The transport process is under stringent regulation by insulin and is an important means of controlling glucose flux (Horuk and Olefsky, 1985). The effect of insulin upon transport is evident within one minute (Whitesell and Gliemann, 1979) and maximal in about five minutes (More and Jones, 1983). Insulin stimulates glucose transport in rat adipocytes 10-30 fold (Gliemann and Rees, 1983; Simpson and Cushman, 1986). It is envisaged that insulin induced activation of transporter activity might occur either by an increase in transporter affinity for the sugar or through an increase in the number of functional transporters in the plasma membrane.

Work done independently in the laboratories of Kono and Cushman gave strong support to the recruitment hypothesis. Suzuki and Kono (1980) using a reconstitution technique for preparing liposomes from subcellular membrane fractions of isolated rat adipocytes and measuring cytochalasin B-sensitive glucose transport activity directly in these synthetic vesicles determined that insulin increases the glucose transport activity in the plasma membrane-rich fraction while decreasing the activity in the Golgi-rich fraction, thus indicating that insulin sequestered transporters to the plasma membrane from an intracellular storage site specifically associated with the low density microsomal fraction. This effect of insulin is reversible and can be blocked by ATP inhibitors (1mM 2,4-DNP) but is not inhibited by protein synthesis inhibitors (0.1mM puromycin) (Kono *et al.*, 1981). Hence these researchers proposed: (i) insulin induces translocation of the glucose transport mechanism from the Golgi rich fraction to the plasma membrane-rich fraction (ii) this reaction is reversible (iii) the glucose transport activity is recycled between the two subcellular fractions by an energy-dependent and protein synthesis-independent process.

Cushman and Wardzala (1980) observed an increase in the D-glucose-inhibitable cytochalasin B binding activity in plasma membranes prepared from isolated rat adipocytes preincubated with insulin for 15 minutes relative to plasma membranes prepared from basal cells and hence also proposed that transporters were being translocated from an intracellular pool to the plasma membrane. In a subsequent publication from the same laboratory, Karnieli *et al.* (1981) localized the transporter pool in the low density microsomal fraction, corresponding to the Golgi-rich fraction identified by Kono, and showed that removal of insulin resulted in decreased transport activity

due to the redistribution of transporters from the surface membrane to the low density microsomes. In the basal state approximately 90% of the cells glucose transporters are associated with the Golgi-rich fraction. Insulin increases the number of transporters in the plasma membrane 4-5 fold with a corresponding decrease in the intracellular pool (Simpson *et al.*, 1983; Cushman and Wardzala (1980).

Oka and Czech (1984), using the essentially impermeant glucose analogue ethylidene glucose have demonstrated the translocation phenomenon in intact cells. Recently Matthaei *et al.* (1987) demonstrated the existence of heterogenous species of glucose transporters in human adipocytes and showed that one of these, isoform pH 5.6, can be sequestered to the plasma membrane. It is interesting that in rat liver, a tissue in which glucose transport is independent of insulin, only one isoform of the carrier exist (pH 6.05) compared with two isoforms in rat adipocytes (pH 6.4; pH 5.6) (Ciaraldi *et al.*, 1986).

Kinetic studies of the rat adipocyte hexose transport system performed under equilibrium exchange (Whitesell and Gliemann, 1979; Taylor and Holman, 1981) or under zero-trans (Taylor and Holman, 1981) conditions reported K_m values in the range of 3-6mM and most importantly insulin appeared to dramatically increase the V_{max} while having no significant effect on the K_m. These observations gave further support to the recruitment hypothesis in that the increase in V_{max} corresponds to an increase in the number of transporters in the plasma membrane.

However, Whitesell and Abumrad (1985) observed that insulin decreased the K_m for glucose transport to one-tenth the value measured in basal cells while doubling the V_{max}, suggesting that the intrinsic transporter activity of the carriers are being modified by insulin. In addition insulin has been found to significantly decrease the K_m value for D-glucose transport in human erythrocytes without any change in the V_{max} (Dustin et al., 1984). Hyslop et al. (1985) examined the effects of the membrane-impermeant amino group-modifying agent fluorescein isothiocyanate (FITC) on the basal and insulin-stimulated hexose transport activity of isolated rat adipocytes and determined insulin activates the transporter activity within the plasma membrane and suggest that recruitment may not be the major locus of acceleration of hexose transport by the hormone. It has also been suggested that isoproterenol and adenosine can modulate the transporter intrinsic activity (Joost et al., 1986).

It has been shown that cell agitation for 10 minutes prior to the transport assay decreased the basal K_m from 35 to 12mM while deprivation of metabolic substrate produced a further reduction down to 2mM suggesting that experimental conditions can produce large changes in the K_m of basal glucose carriers (Whitesell and Abumrad, 1986) and may help to explain discrepancies of K_m values, for the transporter, observed by various researchers. It is proposed that insulin might activate carriers already in the plasma membrane by overriding metabolic factors which keep the K_m high or activation might accompany the recruitment of carriers from intracellular stores (Whitesell and Abumrad, 1985,1986). Interestingly, Karnieli et al. (1981) observed that the rise in membrane transporter number (as measured by D-glucose-inhibitable cytochalasin B binding sites) preceded increase in transport activity in intact cells by about 1-2

minutes. This lag phase may represent the time needed to activate the transporters and would mean the transporters are activated in the membrane.

Simpson et $al.$ (1983) observed that insulin did not influence the K_m of the glucose transporters in the plasma membrane fraction for cytochalasin B binding (98 nM) but lowers that in the intracellular pool (from 141 nM to 93 nM). They suggested that intracellular glucose transporters may undergo conformational changes during their cycling to the plasma membrane in response to insulin and transporter translocation may represent only one component in the mechanism through which insulin regulates glucose transport in intact cells.

Martz et $al.$ (1986) in a kinetic study of the equilibrium exchange flux of 3-O-methylglucose in isolated rat adipocytes using the technique described by Whitesell and Abumrad (1985) failed to observe any decrease in K_m upon insulin treatment but instead obtained a 21-fold increase in V_{max} thereby creating doubt as to the possible modulation of the transport K_m by insulin. However, a reassessment of insulin effects on the K_m and V_{max} values of hexose transport in rat adipocytes, from Kono's laboratory (Toyoda et $al.$, 1987) revealed only an approximately 2-fold change in K_m upon insulin stimulation with a corresponding 16-18-fold increase in V_{max}. Hence they concluded that the major effect of insulin on hexose transport is to increase the V_{max} value, but the hormone has the additional effect of lowering the apparent K_m value.

The putative mediator(s) involved in the insulin stimulation of the transport activity is unknown, however, insulin receptor phosphorylation, G-proteins, protein kinase-C and Ca^{2+} may all play a

critical role in this process (Discussed in Section 1.6). In addition the lipid bilayer represents a major factor in determining the activity of various membrane proteins (Carruthers and Melchior, 1986) and the hexose transporter activity has been shown to be governed by bilayer lipid composition in reconstituted vesicles (Tefft *et al.*, 1986; Carruthers and Melchoir, 1984; Sandra *et al.*, 1984; Wheeler and Hinkle, 1985). Incorporation of unsaturated fatty acids into plasma membranes increased basal transport activity concomitant with the increased membrane fluidity (Pilch *et al.*, 1980). It is probable that insulin might cause fluidization of the plasma membrane and hence enhance the function of the carrier protein (Melchior and Czech, 1979). However, a direct demonstration of interrelation between insulin binding, hexose transport and membrane fluidity remains to be evaluated.

1.7.3   *Transport Mechanism*

The exact molecular description of the solute translocation pathway within the protein remains elusive, however, it appears intuitively correct to assume that the transporter is an "inside out" protein having a hydrophobic surface to keep the protein firmly anchored in the membrane lipid bilayer with an internal hydrophilic domain accommodating a channel of water (Chin *et al.*, 1986).

It is felt that D-glucose transport is facilitated through a conformational change within the molecule (Rees and Holman, 1981). This proposal is supported by recent studies involving circular dichroism measurements which indicated that α-helices comprises 70% of the protein with small amounts of β-turn (20%) and random coil (10%) and that the α-helical content further increases in the presence

of D-glucose at the expense of β-turn (Chin *et al.*, 1986). Further, linear dichroism data indicated that the α-helices are preferentially oriented perpendicular to the lipid bilayer plant forming an effective tilt of less than 30° from the membrane normal and D-glucose reduces this effective tilt angle (Chin *et al.*, 1986).

Only one substrate site exists on the transporter molecule at any time (Baldwin and Lienhard, 1981). D-Glucose transport in adipocytes is stereospecific and orientation specific with the molecule being transported in the pyranose ring form (Rees and Holman, 1981; Holman *et al.*, 1981). Glucose molecules in the external solution approaches the transporter with C-1 facing the inside while C-4 faces the external solution (Holman *et al.*, 1981). The important hydrogen bonding positions involved in sugar interaction with the insulin-stimulated adipocyte transporter are the ring oxygen, C-1 and C-3 with possible weaker hydrogen bonding to C-6 (Rees and Holman, 1981).

Hydrogen exchange studies have revealed that the erythrocyte transporter contains a large amount of free amide hydrogens, suggesting the existence of an intramolecular aqueous channel (Jung *et al.*, 1986), in agreement with Mueckler *et al.* (1985). A proton NMR study of the mechanism of the erythrocyte glucose transporter has led to the proposal of a sliding-barrier model for the translocation of bound glucose across the membrane, in which a transport unit containing a channel surrounded by six or more transmembrane α-helices possesses a sliding helical barrier that prevents free diffusion through the channel. The D-glucose molecule binds to a transport site in a cleft between two the channel-forming helices so that C-2, 3 and 4 lie within the cleft, while C-1, 5, 6 lie outside the cleft. During

transport the sliding barrier migrates from one side of the membrane, past the transport site to the other side of the membrane (Wang *et al.*, 1986).

1.7.4 *Transport Activity/Insulin Resistance*

It has been suggested that insulin resistance of adipocytes from obese subjects may involve post-receptor sites (Livingston *et al.*, 1984). Indeed depletion in the number of intracellular glucose transporters resulting in a corresponding reduction in the translocation of glucose carriers from the intracellular stores to the plasma membrane has been observed in insulin resistant adipocytes (Horuk *et al.*, 1983). A similar depletion of intracellular glucose transport system occurs in streptozotocin-induced diabetes mellitus (Karnieli *et al.*, 1981b). In a recent study by Karnieli *et al.* (1986), insulin was able to induce transporter translocation 30-50% in insulin resistant (obesed) rat cells without significantly promoting glucose transport, suggesting that a reduction in the intrinsic activity of the transporter, at the plasma membrane level, is occuring. Hence the down regulation of glucose transporters found in plasma membrane in basal and insulin-stimulated conditions (in the obesed state) may represent a negative feedback mechanism regulating the rate of cellular growth in such a way that, as the cell enlarges, glucose uptake is correspondingly reduced and further growth is controlled (Karnieli *et al.*, 1986).

1.8 SUMMARY

The insulin receptor is an integral membrane glycoprotein (M_r – 350 000) composed of two α-subunits (M_r – 130 000) and two β-subunits (M_r – 95 000) linked by disulphide bonds to yield a symmetrical $\beta\alpha$-$\alpha\beta$ structure. The amino acid sequence deduced from cDNA of the single polypeptide chain precursor of human placental insulin receptor revealed that α- and β-subunits consist of 735 and 620 residues, respectively. Synthesis of the primary proreceptor polypeptide backbone, M_r – 170 000 is accompanied by cotranslational N-linked glycosylation to yield a high mannose 190 000-M_r proreceptor which is subsequently cleaved into low molecular weight forms of the α and β-subunits. Mature α and β units appear in the plasma membrane 2-3 hours after synthesis.

The α-subunit, which contains the insulin binding domain, is hydrophilic, disulphide-bonded, glycosylated and lacks any hydrophobic stretches to act as a membrane anchor and is almost exclusively in the extracellular domain. The β-subunit is also glycosylated and consists of a short extracellular region linking the α-subunit through disulphide bridges, a hydrophobic transmembrane region and a longer cytoplasmic region containing the insulin tyrosine specific kinase. The insulin receptors of adipocytes occur predominantly in groups of 2-6 molecules randomly distributed on the plasma membrane.

Scatchard analysis of insulin binding data by several research groups using different tissues have frequently yielded curvilinear Scatchard plots suggesting the existence of negative cooperativity in insulin receptor interactions. It has been speculated that the presence of two α-subunits in the receptor structure may be responsible for the

proposed negative cooperative effect. However, a positively cooperative effect occuring at physiological concentrations of insulin and may be due to receptor affinity changes has also been observed. In addition, prolonged incubation of ^{125}I-insulin with rat liver plasma membranes induces a time-dependent increase of a receptor high affinity state, suggesting the interconversion of low and high affinity receptor states is regulated by insulin.

Both insulin and its receptor undergo early structural modifications upon formation of the insulin-receptor complex. It is this receptor conformational change that is believed to induce transfer of signal from extracellular to cytoplasmic receptor domains leading to the activation of the β-subunit-associated tyrosine kinase. The deduced amino acid sequence of the β-subunit is homologous to the EGF receptor tyrosine kinase and the tyrosine kinase domains of the family of retroviral protein tyrosine oncogenes and in addition the deduced sequence contains the ATP binding sequence conserved in all protein kinases. The purified insulin receptor autophosphorylates its own β-subunit and other exogenous protein substrates in an insulin-sensitive, nucleotide (ATP)-specific, Mn^{2+}-dependent reaction.

The receptor in the intact cell contains multiple sites of phosphorylation, including serine residues. It is felt that the insulin receptor serine kinase may play a significant role in the regulation of the tyrosine kinase activity. Dephosphorylation, and hence deactivation, of the receptor kinases may occur through an insulin receptor directed protein phosphatase which may involve GTP binding protein. The importance of insulin stimulated phosphorylation of the β-subunit in insulin action was demonstrated by Grunberger et al. (1984) who studied a type A insulin resistant patient, with normal

insulin binding and discovered a defect in the insulin receptors, distal to the insulin binding site, which was localized in the β-subunit. Subsequently, a number of researchers also observed decreased tyrosine kinase activity of the insulin receptor in diabetics, thereby corroborating Grunberger's initial discovery. It is now believed that β-subunit phosphorylation is an absolute requirement for most of insulin's known biological effects including glucose transport.

The cellular function of insulin receptors is dual: transmembrane signalling and endocytosis of hormone. In some cell types (hepatocytes, fibroblasts) the insulin-receptor complexes are found localized within clathrin coated pits prior to internalization. In adipocytes however, much doubt remains as to the involvement of coated pits in the internalization process in these cells. Nevertheless, in the absence of insulin 90% of the total cell receptors, in rat adipocytes, reside on the cell surface and insulin incites a rapid redistribution of surface receptors to intracellular sites within minutes. A small proportion of the internalized complexes undergoes retroendocytosis and returns to the surface intact, however, the majority of complexes are found associated with specialized acidic compartments, termed endosomes or CURL, which promotes the dissociation of the complexes. Once detached, receptors are recycled from the tubular projections of CURL while the hormone molecules bud off in aqueous vesicles and are delivered to lysosomes where they are subsequently degraded. Acute treatment of adipocytes with high insulin concentrations leads to a time, temperature and energy dependent loss of surface receptors. Degradation of internalized receptors follows resulting in a new, down-regulated, steady-state receptor level.

Insulin receptors from different tissues and animal species are homologues in their structure and function, but also show significant differences regarding size of α-subunits, binding kinetics, insulin specificity and receptor mediated degradation. It is suggested that the heterogeneity of receptors may be linked to the diversity of insulin effects on metabolism and growth in various cell types (Gammeltoft and Van Obberghen, 1986). One of insulins major biological effects is its ability to enhance glucose transport and metabolism in a wide range of tissues (Table 1.1).

Glucose transpoort in adipose tissue occurs via a facilitated transport system which is highly specific for the D-glucose isomer. The amino acid sequence deduced from cDNA revealed a 492 residue polypeptide containing 12 potential membrane spanning segments. It is conceivable that these segments may form a hydrophilic proe through which D-glucose might be transported. Translocation of the glucose molecule is stereo- and orientation-specific and is thought to be facilitated via a conformational change within the transporter. A sliding barrier model for the transition of glucose through the carrier has been proposed from proton NMR studies; it is suggested that glucose is held in a cleft between two channels forming helices and during transport a sliding barrier migrates from one end of the membrane to the other side. The activation of glucose transport systems by insulin is largely due to the sequestration of hexose carriers from cytoplasmic stores, located within the golgi, to the plasma membrane. However, a direct enhancement of the transporter intrinsic activity might also be occuring.

The question remains however, as to the manner by which insulin enhances transport activity. Despite the vast amount of work done in the area of plausible mechanism for insulin activation of the transport system remains elusive. Traditional second messengers and putative protein mediators can only manage to explain a some of insulin's intracellular metabolic events. Insulin receptor regulation of plasma membrane G-proteins can activate adenylate cyclase and phospholipase C in rat adipocytes. The latter enzyme can in turn hydrolyze plasma membrane associated IPLs resulting in the generation of DAG (involved in activation of pk-C) and IP_3 (capable of mobilizing Ca^{2+} from intracellular stores). The subsequent metabolism of IP_3 to IP_4 may result in the opening of plasma membrane Ca^{2+} gates (Fig. 1.5). Both pk-C and Ca^{2+} have been implicated as the possible second messengers involved in the activation of glucose transport. However, much controversy exists in the literature as to the role of these two candidates. So that in the final analysis a mechanism for insulin stimulation of glucose transport in mammalian cells remains unestablished. The basic concepts involved in insulin action is summarised in Fig. 1.7.

1.7

Model demonstrating: A - Transporter activity

B   Fate of ligand and receptors
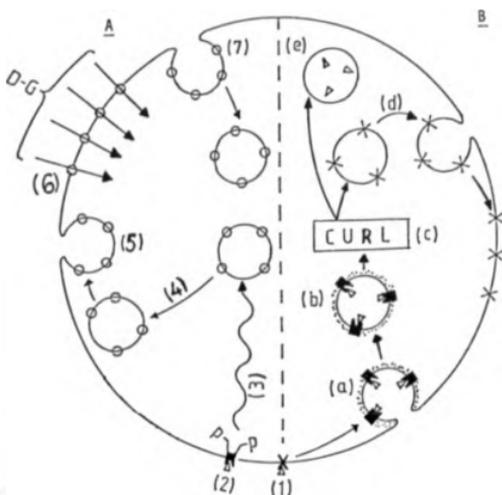
Transporter Activity:

Receptor binds insulin and undergoes a conformational change and autophosphorylation (1 and 2). Biological signal generated (3). Translocation of the carriers (4). Fusion of transporter vesicles with plasma membrane (5). Stereospecific D-glucose uptake (6). Transporters are endocytosed upon termination of signal (7).

Fate of Receptors:

Hormone-receptor complexes accumulate in clathrin coated pits (a), which gives rise to clathrin coated vesicles (b). Ligands and receptors are uncoupled in CURL (c). Receptors are recycled (d). Ligands undergo lysosomic degradation (e).

CHAPTER   2

Materials   and   Methods

MATERIALS

Labelled D-[U-^{14}C] glucose was obtained from Amersham International (Amersham, Bucks., U.K.); L-[1-^{3}H] glucose was obtained from New England Nuclear (Dreieich, Germany). Tyrosine A14 [^{125}I]monoiodoinsulin was prepared from bovine insulin according to the procedure of Linde *et al.*, 1983 and had an initial specific activity of 1.6Ci μmole^{-1} or purchased from Amersham International, specific activity 2.55 Ci μmole^{-1}. Highly purified beef insulin (Weddel Pharmaceuticals Ltd) was obtained from the Manchester Royal Infirmary. Bovine insulin (crystalline), cytochalasin B, phloretin, Hepes, phenylarsine oxide, dithiothreitol, monensin and A23187 were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.).

Crude bacterial collagenase (batch nos. 14443357, 10439322-68, 10388928-67, 10479230-70 and 1204458) was obtained from Boehringer Corporation (London) Ltd. (Lewes, East Sussex, U.K.). Fatty acid free bovine serum albumin (fraction V) was obtained from both Sigma and Boehringer. Male *S*prague Dawley rats were purchased from the Animal Unit within the Medical School. Quin 2 was kindly supplied by Dr. T. Ansah (Physiology Department). All other chemicals were obtained from B.D.H. Chemicals Ltd. (Poole, England) and were of analytical grade.

METHODS

Preparation of Rat Adipocytes

Buffer(s) required.

Krebs-Henseleit Buffer:

NaCl (120mM); KCl (4.8mM); KH$_2$PO$_4$ (1.2mM)

MgSO$_4$ (1.2mM); NaHCO$_3$ (24mM); CaCl$_2$ (2.5mM)

Appropriate concentrations of the individual salts were prepared separately and the buffer was routinely prepared by mixing the solutions at a fixed ratio. Only freshly prepared buffer was used for a given experiment.

For the preparation of adipocytes, 2mM D-glucose and 1% bovine serum albumin (BSA) was added to the Krebs-Henseleit buffer. The buffer was then filtered twice, first through Whatman 42 filter paper and secondly through a millipore ($0.22\mu m$). Since suction was used, filtering was carried out carefully with only minimum foaming. The final solution was then adjusted to pH 7.4 by gasing; CO_2/O_2 (95% of 5%) mixture. The pH of this buffer tended to drift slightly on standing hence once prepared the buffer was checked periodically and the pH readjusted if necessary.

Adipocytes were prepared by collagenase digestion of epididymal and perirenal fat pads as described by Rodbell (1964). All steps were carried out at 37^oC and plastic vessels were used througout. All containers used in the preparation of adipocytes, including those used for buffer preparation, were washed with lipsol detergent and then acid (2M HCl) soaked to further assist cleansing. The vessels were then throughly rinsed with distilled water prior to cell preparation. Routinely 3-4 male Sprague-Dawley rats of approximately 150-180g were used. The fat was excised, chopped with scissors and put into the freshly made buffer.

The adipose tissue was put into a 60ml plastic bottle, containing 5ml of buffer per rat used, and collagenase added to a final concentration of 1.50mg/ml. The bottle was capped and its contents incubated, with gentle shaking, for 60 min. at 37^oC. The partially

digested adipocyte suspension was filtered through a $212\mu m$ nylon mesh and cells were then washed three times with a collagenase-free buffer. During the washing centrifugation was avoided as a means of isolating the cells, instead on each occasion the suspension was allowed to stand, at 37^0C, until a clearly defined cell layer was attained (usually 5-10 min). Typically, the fat from one rat yielded 2-3ml suspension. The cell suspensions were used directly in glucose uptake or insulin binding studies. To avoid errors due to changing pH in the cell medium, just prior to using the adipocytes the cell suspension was washed free of "old" buffer and resuspended in a newly adjusted (pH 7.4) Krebs-Henseleit buffer.

D-glucose Uptake Measurement

Buffer(s) required.

Stopping Buffer:

a) Mercuric chloride ($HgCl_2$):

10mM Hepes containing 250mM sucrose and 2mM $HgCl_2$, pH 7.4

b) Phloretin:

This substance was dissolved directly into 1ml of ethanol and then made up to 100mls with Hepes/sucrose solution such that the final stopping mixture contained 0.15M phloretin, 250mM sucrose, 1% ethanol and 10mM Hepes, pH 7.4.

Mercuric chloride has previously been used as an inhibitor of sugar transport (More & Jones, 1983). Additionally compared to 0.15mM phloretin, a concentration previously used to effectively inhibit sugar transport in erythrocytes (Lowe & Walmsley, 1986), no significant difference was observed in the stopping efficiency between these materials and at zero time, $HgCl_2$ (2mM) buffer completely inhibited stereospecific uptake (Table 2.1).

Table 2.1

Comparison of the ability of $HgCl_2$ (2mM) and Phloretin (0.15M) to inhibit hexose transport.

Stopping Buffer	Uptake Time (sec)	nmole glucose uptake/mg prot.		
		D-glucose	L-glucose	Stereospecific (D-L)
$HgCl_2$ (2mM)	0	0.236	0.286	0.000
	60	2.70	0.182	2.52
		3.06	0.284	2.78
		2.73	0.277	2.45
				2.58 ± .17
Phloretin (0.15M)	60	3.15	0.223	2.93
		3.28	0.244	3.04
		3.06	0.199	2.86
				2.94 ± .09

D-glucose Uptake Assay

Stereospecific carrier mediated uptake of D-glucose was taken to be the difference in the amounts of D- and L-glucose taken up by adipocytes. All assays were performed in Eppendorf tubes pre-incubated at 37°C (or 20°C for low temperature experiments).

Adipocytes were maintained throughout all incubations and assays at the required temperature. The transport assay was started by putting 45μl of adipocytes suspension into an Eppendorf tube containing 5μl of labelled glucose solution; 10mM Hepes pH 7.4 containing 5mM D-[U-^{14}C] glucose (25μCi/ml) and 5mM L-[1-^{3}H] glucose (50μCi/ml). The reaction mixture was gently agitated (manually) for the prescribed time and the incubation terminated by the addition of 1ml of ice-cold stopping buffer.

Adipocytes were separated from the medium by an oil flotation technique as described by Gliemann *et al.*. (1972). Dinonyl phthalate (0.3ml) was layered over the stopping buffer and the tube centrifuged in an Eppendorf microfuge (12,000g) for 40 seconds. The adipocytes were then removed by using small strips of filter paper, which were then placed in plastic scintillation vials with 4.0ml of scintillation fluid (Scintron Cocktail T; B.D.H Chemicals). Radioactivity was determined using a Beckman LS9800 liquid scintillation counter.

Radioactivity Standards: 10μl of a 100 fold dilution of the labelled glucose stock solution was put into a vial. A strip of filter paper was added followed by scintillation cocktail (4.0 mls).

D-glucose uptake measurements, for any given incubation time, was always done at least in dulpicate and the mean of these were used in the representation of the data.

Preparation of Reagents used in the investigation of Insulin Action

1) A23187, Monensin and Cytochalasin B

These agents were dissolved in absolute ethanol to yield solutions of desired concentrations. A23187 was stored at -80°C, while cytochalasin B and monensin was stored at 0°-4°C. These stock solutions were used in adipocytes suspension at a rate of 10μl per ml of suspension. Final ethanol concentration ~1% ($^v/v$).

2) Phenylarsine Oxide (PhAsO)

16mg of the arsenical was dissolved in 1.7 mls of absolute ethanol, which was then made up to 100mls with 0.15M NaCl, so that the stock solution (1mM PhAsO) contained 1.7% (v/v) enthanol. This solution was stored 0-4°C. The final ethanol concentration in the experiments was in the range 0.0017 to 0.017% (v/v).

3) EGTA

This substance was not readily soluble in distilled water but dissolve relatively easily in slightly alkaline solution. Stock solution contained 0.1M EGTA in 0.5M NaOH and was stored at 0-4°C

4) Dithiothreitol

The cyrstaline solid was dissolved directly into saline and stored at 0-4°C.

The ethanol concentrations present in the adipocyte suspension in these experiments ($\leqslant$ 1%) have already been shown not to interfere with sugar transport (Jones *et al.*, 1985; More & Jones 1983). However as an additional precaution ethanol was routinely included in control systems to correspond to the amounts present in the given experiment.

Protein Concentration Measurements

The concentration of solubilised proteins was measured using a Coomassie Blue Dye binding method described by Bradford (1976) and using bovine serum albumin as standard.

Preparation of protein reagent

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50ml 95% ethanol. To this solution 100ml (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

Protein assay (standard curve)

Protein solution containing 10 to 100μg protein in a volume up to 0.1ml was pipetted into 10 test tubes. The volume in the test tube was adjusted to 0.1ml with Krebs/Henseleit buffer (free of bovine serum albumin). 5.0mls of protein reagent was added to the test tube and the contents mixed by vortexing. The absorbance at 595nm was measured after 2 min. and before 1 hour. in 3ml cuvettes against a reagent blank prepared from 0.1ml of buffer and 5ml of protein reagent. The absorbance values were plotted against weight of protein.

Fig. 2.1

Protein standard curve. Each point is the mean of duplicate determinations.

Fig 2.1
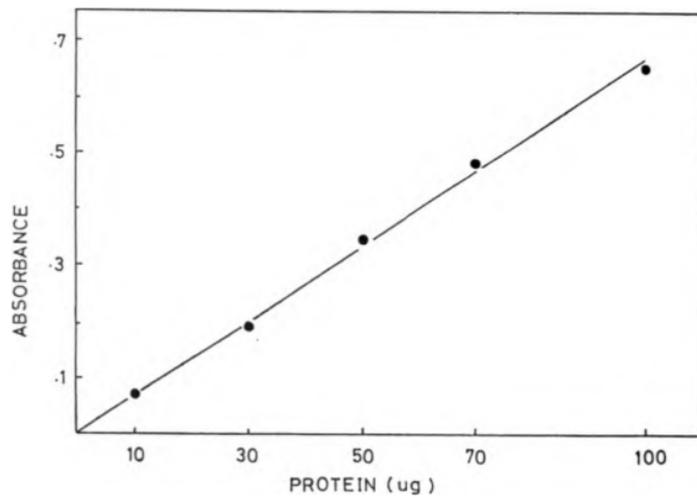
The Coomassie reagent was prepared monthly and a new standard curve was obtained for each new solution. Fig. 2.1 shows a typical standard protein curve.

Protein Determination of Adipocyte Suspension

For each adipocyte preparation 1ml of cells was washed three times in BSA-free Krebs-Henseleit buffer (pH 7.4). $50\mu l$ portions of this washed suspension were pipetted into plastic disposable 10ml test tubes and were sonicated for 3-5 min using a probe sonicator (Soniprep 150 MSE) $50\mu l$ of buffer (BSA-free) was added to the ruptured cells, followed by 5.0mls of Coomassie Blue reagent. The absorbance was read after 2 min and the protein content determined from the standard curve.

Cell Counts

Adipocyte suspensions were counted using a Coulter Counter, model ZB, containing a $100\mu m$ orifice. The settings used on the Coulter Counter were as follows:

Aperture size during counting: 0.5 mls

$$\frac{1}{A} = 8 \qquad \text{where } A = \text{Amplification}$$

$$\frac{1}{I} = 1 \qquad \text{where } I = \text{Aperture current}$$

Threshold values:

Lower = 5

Upper = Maximum anticlockwise position.

Fig 2.2

Determination of cell number.

Adipocytes were diluted in saline and counted on a Coulter Counter. The number of cells was plotted against mls of adipocyte suspension. Each point is the mean of at least five determinations.

Fig 2.2
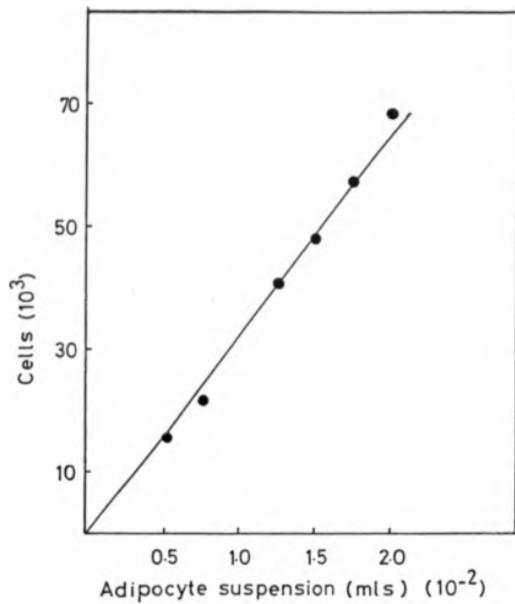
Typically a series of dilutions of the adipocyte cell suspension were prepared for counting. 0.15M saline that had been filtered through a millipore (0.22μm) was used to dilute the cell suspension. Aproximately 5 readings were obtained for any given dilution and the mean of these readings was used to determine the quantity of cells per ml. By plotting the number of cells against the volume of adipocyte suspension a linear graph was obtained and the number of cells per ml was derived from the slope (Fig. 2.2).

Relation between Protein Concentration and Cell Counts

Two samples of adipocytes were prepared as previously described and washed free of BSA. The number of cells per ml and the protein concentration (μg) per ml was determined. The protein content of one cell, for each sample of adipocytes, was derived by equating the number of cells per ml with the number of μg protein per ml.:

$$\text{sample } 1 = 5.1 \times 10^{-4} \text{ μg protein/cell}$$
$$\text{sample } 2 = 5.8 \times 10^{-4} \text{ μg protein/cell}$$
$$\text{Mean} = 5.45 \pm .49 \times 10^{-4} \text{ μg protein/cell.}$$

Hence for any given cell preparation both the number of cells/ml and the number of μg protein/ml could be determined if any one of these factors are known.

Quin 2 Fluoresence Measurements

The procedure is a modification of that used by Tsien *et al.*, (1982). To measure changes in $[Ca^{2+}]_i$, adipocytes (~10^6 cells) were

Fig 2.3

Equilibration of adipocytes were quin 2 at 37°C.

Adipocytes were incubated with $100\mu M$ quin 2/AM. After various time intervals the cells were washed and resuspended in fresh buffer (without quin 2/AM). Quin 2 fluoresence was monitored in 2ml aliquots of cell suspension at 480nm.

Fig 2·3
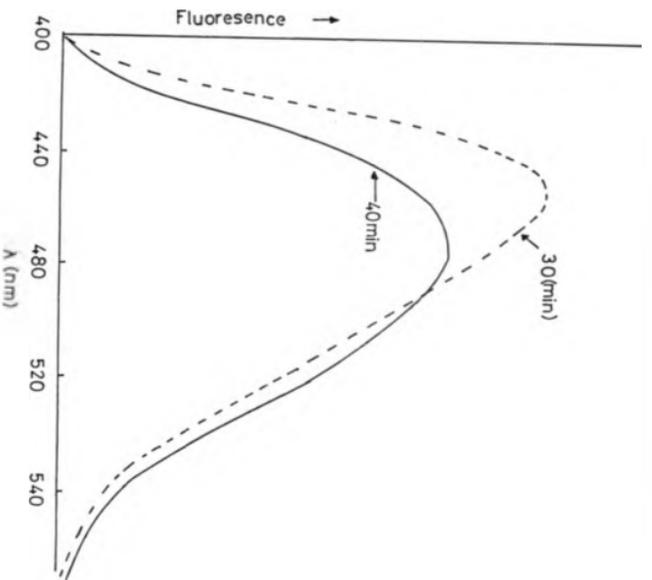
suspended in a Krebs-Henseleit calcium free ($CaCl_2$ omitted) buffer containing 0.1% BSA, 2mM D-glucose and 100μM quin 2/AM (acetoxymethyl ester) in dimethyl sulfoxide (DMSO) at 37°C. The final concentration of DMSO from the quin 2/AM stock did not exceed 0.2% (v/v). Quin 2/AM readily permeates the membrane and is hydrolysed in the cytoplasm, thus trapping the impermeant quin 2 there. The time taken for loading was ~ 40 min (Fig 2.3).

For measurements of fluorescence cells were washed twice, resuspended in buffer omitting quin 2/AM and transfered to the cuvette. Quin 2 fluorescence was monitored in 2ml aliquots of cell suspension in a Perkin-Elmer spectrofluorometer (LS-3) at an excitation wavelength of 339nm (5nm slit) and an emission wavelength of 480nm (20nm slit). Samples were continously stirred and maintained at 37°C.

Preparation of Plasma Membrane from Isolated Rat Adipocytes

Solutions Required:

1) Homogenization Buffer:
   20mM Tris-HCl containing 1mM EDTA
   and 255mM Sucrose, pH 7.4

2) Sucrose Cushion:
   20mM Tris-HCl containing 1.12M Sucrose and 1mM EDTA.

Preparation of plasma membranes

The method employed was a modification of that used by Simpson *et al.*, (1983). Adipocytes were prepared from 20-25 male Sprague-Dawley rats (200-250g) as discribed above. The adipocytes

were divided equally into two portions and one of these was stimulated with insulin (100nM for 20 min). The cells from both basal and insulin states were then subjected to the following steps. Adipocytes were washed twice with homogenizing buffer, resuspended in ~20 mls of this same buffer and homogenized at room temperature in a glass homogenizing tube fitted with a Teflon pestle. The homogenate was cooled on ice and all subsequent operations carried out a 4°C. Homogenization buffer was used throughout the preparation.

The original homogenate was spun at 16 000 x g_{max} for 15 min, using a Beckman J2-21 centrifuge. The solid fat cake was carefully removed and discarded, and the supernatant was saved for the preparations of the microsomal fraction. The initial pellet was resuspended in 5mls of buffer, applied to a 1.12M sucrose cushion and centrifuged at 101 000 x g_{ave} on a Prepspin 65 ultracentrifuge. The plasma membranes were collected at the interface and resuspended in ~35ml and centrifuged at 48 000 x g_{max} for 45 min. The pellet was resuspended in 10ml buffer, repelleted and finally resuspended to ~1.6mg protein/ml.

The initial supernatant was centrifuged at 200 000 x g_{ave} in heat sealed tubes using a Beckman L7-55 ultracentrifuge to obtain the microsomal membrane fraction. The pellet was resuspended and repelleted before final resuspension to ~1.2mg protein/ml. The membrane fractions were stored as follows: 100µl aliquots from each suspension were pipetted into separate Eppendorf tubes and each vial was flushed with a stream of nitrogen. Samples were stored at -20°C. Once thawed the samples were not reused.

Marker Enzyme Analysis

5' Nucleotidase:

This enzyme has frequently been used as a plasma membrane marker and relies upon detection of liberated inorganic phosphate (P_i) The method of Atkinson *et al.*, (1973) was used.

Solutions required:

A. 5% (w/v) cirrasol (lubrol)

B. Acid molybdate

2% (w/v) ammonium molybdate in 1.8M H_2SO_4.

Stored in dark at 4°C.

C. 10 vols. solution A

25 vols. solution B

65 vols. Distilled water

(prepared fresh and kept at 4°C)

D. 150mM Tris–HCl buffer containing 15mM $MgCl_2 6H_2O$, pH 8.5.

E. Standard P_i

Prepared from KH_2PO_4 in concentrations up to 0.6 μmoles P_i per ml.

Standard Pi Curve

2mls of solution C was added to 1ml aqueous sample containing up to 0.6μmole P_i. The mixture was vortexed and allowed to stand at ambient temperature for 10 min. The extinction was read at 390nm against a reagent blank. A typical standard curve is shown in Fig 2.4.

Fig 2.4

Inorganic phosphate (P_i) standard curve. Each point is a mean of duplicate determinations.

Fig 2·4
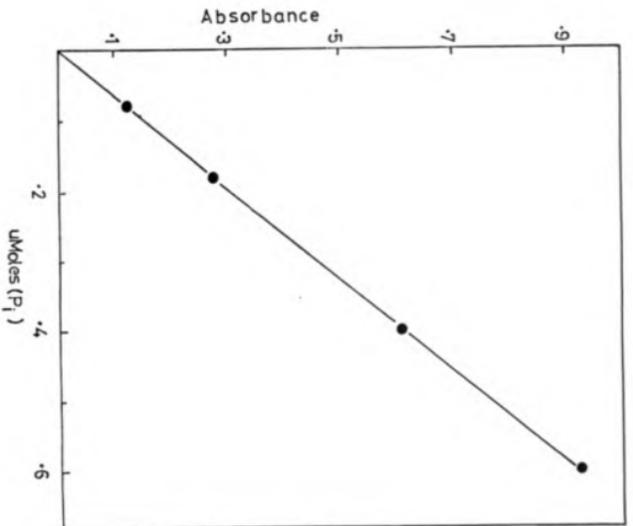
Procedure for Enzyme Incubation

5' AMP was added to buffer D to give a final molarity of 15mM. 0.2ml aliquots of this solution were placed into separate vials and preincubated at 37°C for 30 min. 0.1ml aliquots of membrane suspension was added and the reaction was allowed to proceed for 15 min. The reaction was stopped by the addition of 2ml of ice-cold cirrasol-moybdate reagent (solution C). The vials were left to stand at room temperature for five min and then centrifuged (~ 5000g) for 5 min to sediment any particulate matter that may persist in the membrane suspensions. The absorbance of the samples was read at 390nm against distilled water after which the zero time values were subtracted.

Results

The mean specific activity of this enzyme in plasma membrane and microsomal fractions were 0.914 μmoles hr^{-1} mg protein^{-1} and 0.3μmoles μr^{-1} mg protein^{-1} respectively based on at least 4 separate samples per membrane fraction. This 3-fold increase in activity in plasma membrane over microsomal membrane compares well with previous reports (Simpson *et al.*, 1983; More and Jones, 1983).

Preparation of Tyrosine A^{14} (^{125}I) Monoiodoinsulin and Isolation by Polyacrylamide Gel Electrophoresis (PAGE)

Solutions Required.

Sodium Citrate Buffer:

Citric acid- sodium buffer solution pH5.6

21.0 mls of 0.1M - citric acid plus 79.0 mls of 0.1M

Tri-sodium citrate.

Solution F:	30g acrylamide
	1.6g bisacrylamide
Solution G:	Upper reservoir buffer
	5mM Tris containing 0.04M
	Glycine pH 8.3
Solution H:	0.06M Tris-HCl, pH 6.7
Solution I:	Lower Gel Buffer (running gel)
	0.38M Tris-HCl, pH 9.15

Lower Running gel (10% gel)

1.	Solution F	=	6.7 mls
2.	Solution I	=	5.0 mls
3.	TEMED	=	0.02 mls
4.	Tween 80 (10%) solution	=	0.02 mls
5.	Ammonium persulphate	=	0.08 mls
	(1g/4mls freshly prepared)		
6.	Distilled water	=	8.18 mls

Solution F, I and distilled water were mixed and degassed for about 2-3 min. Next Tween 80, TEMED and ammonium persulphate were added. The mixture was then quickly placed into 22cm long glass tubes. Polymerization occured within minutes.

Stacking gel (4% gel)

1.	Solution F	=	1.35 mls
2.	Solution H	=	2.5 mls
3.	TEMED	=	0.01 mls
4.	Tween 80 (10% solution)	=	0.01 mls
5.	Ammonium persulphate		
	(as above)	=	0.04 mls
6.	Distilled water	=	6.0 mls

Solution F, H and distilled water were mixed and degassed for 2-3 min minutes. Tween 80, TEMED and ammonium persulphate were added. This gel was layered directly unto the previously polymerised running gel.

Reservoirs: Upper: Solution G

Lower: Solution I (4-fold dilution)

Preparation of ^{125}I-Iodoinsulin

The method used for the preparation of tyrosine A^{14} [I^{125}] monoiodoinsulin was a modification of that used by Linde *et al.*, (1981), 10mls of highly purified beef insulin (Weddel brand), 100IU/ml in 0.36% sodium phosphate B.P. containing 0.06% phenol B.P. and 0.16% m-cresol, was dialysed overnight against 0.05 M HCl. The dialysis solution was changed 4 times. The resulting insulin solution was used directly for iodination.

To $20\mu l$ of 2mCi (^{125}I) iodine contained in 0.1M NaOH, $40\mu l$ of insulin solution was added followed by $4\mu l$ of sodium citrate buffer pH 5.6. To the resulting mixture $2\mu l$ of hydrogen peroxide (1mM in distilled water) was added for 1 min after which time $2\mu l$ of lactoperoxidase (0.74mg/ml) was added for 1 min. The addition of H_2O_2 and lactoperoxidase was repeated once again. The reaction was terminated by the addition of $\sim30\mu l$ of 0.1M NaOH; final pH ~9. $58\mu l$ of 40% sucrose was added prior to isolating insulin by PAGE.

The samples were placed onto the gels and $-2\mu l$ of bromophenol blue tracer dye was added. The samples were run at 2.5mA per tube for the first 15 min and then 4mA per tube thereafter. The run was near completion after -3.5 hrs. After termination, a small amount of Tween 80 (20% solution) was injected along the sides of the gels which were forced out into an ice bath. After a few minutes the rigid gels were sliced into 1mm portions using a gel slicer. The first one third portion of the gel slices were placed in pairs into vials. For the second two thirds portion, slices were placed individually into separate vials. 0.5mls of ammonium bicarbonate buffer (0.1M NH_4HCO_3 adjusted to pH 8.0) containing 0.5% human serum albumin was added to each vial and the slices were eluted for at least 24 hrs at 4^0C. $5\mu l$ samples were then pipetted from each vial and the activity of each sample determined on a Wlij Model 2001D γ-counter.

Characteristically two peaks were almost always obtained from this procedure (Fig 2.5). However the eluate from the second peak was incapable of binding to cells. It is possible that this peak might represent excess ^{125}I iodine on the gel or possibly diiodoinsulins, which may not have full biological activity (Maceda *et al.*, 1982). On the other hand, the eluates from the first peak was capable of binding to adipocytes and these fractions were pooled to form the stock solution and had an initial specific activity of 1.6Ci $\mu mole^{-1}$. It should be noted that extreme care was taken during the preparation and lead shields were used throughout the entire procedure.

Small μl quantities of the stock ^{125}I-iodoinsulin solution, which were roughly enough for one experiment, were pipetted into Eppendorf

Fig 2.5

Elution profile for the isolation of Tyrosine A14[125I]-monoiodoinsulin by PAGE.

Fig 2.5
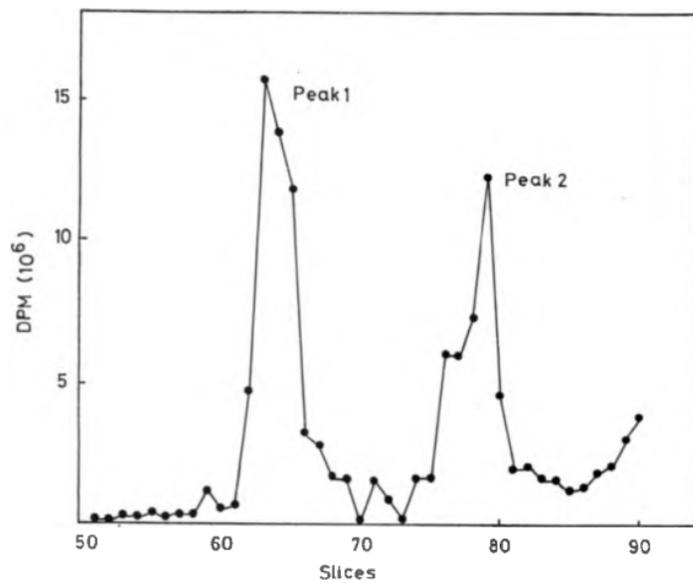
tubes.   A stream of nitrogen was passed into the vials prior to
storage at -20°C.

Small aliquots were used for storage in an effort to avoid
continuous thawing and refreezing.   A quantity of tyrosine A[14]
[125I]-iodoinsulin was also purchased from Amersham.   This sample
was reconstituted in 500μl of distilled water and aliquots were stored
as described above.   For insulin binding experiments the sample in
one of the vials was thawed and made up to the desired activity
(usually 0.5-1μCi/ml) using Krebs-Henseleit buffer containing 1% BSA
and 2mM D-glucose, pH 7.4.

Insulin Binding Assays

Adipocytes were prepared from 180-200g rats as described above
and the suspensions maintained in Krebs-Henseleit buffer (pH 7.4)
containing 1% (w/v) bovine serum albumin plus 2mM glucose. Aliquots
(0.2ml) of adipocyte suspension were pipetted into 1.16mls of buffer
and the suspension allowed to equilibrate at the required temperature
for at least one hour.   The binding assay was started by the addition
of 0.17ml of "hot" insulin (0.5-1.0μCi/ml).   Non-specific binding was
assessed by addition of "cold" insulin at a concentration of 10μM.
The assay was terminated at the required time intervals by oil
flotation of the cells by centrifugation in an Eppendorf microfuge
(12,000g) for 10 seconds. Cells were retrieved by using short strips of
pipe cleaner and the cell associated activity determined using a Wlij
Model 2001D γ -counter.

Solutions

1. 0.2M acetic acid in 0.5M NaCl, pH 2.5 or
2. 50mM glycine/HCl buffer in saline, pH 3.5

0.4ml aliquots of adipocyte suspension were pipetted into 2.32 mls of Krebs-Henseleit buffer and the suspensions equilibrated at 15°C for one hour. The binding assay was started by the addtion of 0.34mls of "hot" insulin (0.5 - 1μCi/ml). Acid washing was carried out by carefully withdrawing the buffer from the cells and replacing it with the acid medium. Termination of this low pH wash was effected by withdrawing the acid solution followed by two consecutive washings with Krebs-Henseleit buffer; final pH of the cell suspension ~7.3.

CHAPTER 3

<u>Section a:</u>   Insulin stimulation of glucose transport activity.

RESULTS

It has previously been determined in our laboratory that maximum insulin stimulation of D-glucose transport, at 37°C, occurs after exposure of adipocytes to 100nM insulin after a period in excess of 5 min. (More and Jones, 1983) and a protocol for insulin stimulation was established whereby adipocytes were treated with 100nM-insulin for 20 min. Fig 3.1a shows the time course for total and stereospecific (taken to be the difference between the D- and L-isomers) D-glucose uptake measured in the presence or absence (basal state) of insulin, over a 120 sec. time interval. Routinely a 3- to 7-fold stimulation was observed in accordance with previous data (More and Jones, 1983). The levels of passive, non-specific uptake was consistently low (Fig 3.1b). In 8 independent experiments the non-specific uptake (total L uptake, calculated as a percentage of the total D-glucose (D_{tot}) uptake) for both basal and insulin stimulated state was $10.04 \pm 4.62\%$ and $4.0 \pm 2.16\%$ respectively (Fig. 3.1b).

The effect of cytochalasin-B ($25\mu M$) is shown in Figs 3.2 and 3.3. Cytochalasin B is known to disrupt the cytoskeletal elements of the cells, in particular it inhibits microfilament formation as well as monosaccharide transport (Poste et al., 1975), however the inhibitory effect of cytochalasin B on the glucose transport system is believed to be predominantly on the monosaccharide transporter itself and not an indirect effect arising from the action of cytochalasin B on the cytoskeletal system (More and Jones, 1983). Cytochalasin B treatment of both basal (Fig. 3.2b) and insulin stimulated cells (Fig. 3.2a) caused marked inhibition of D-glucose uptake. The level of D_{tot} falling to the level of total L-glucose (L_{tot}) uptake resulting in almost total abolition, > 96%, of the stereospecific transport, Fig 3.3.

The effects of prolonged incubation of adipocytes at low temperatures is shown in Fig. 3.4. A 3hr incubation at 20°C caused a significant increase in D-glucose uptake as compared to basal levels. This observed "insulin-like" effect of low temperatures is in agreement with previously published data (Vega and Kono, 1979; Ezaki & Kono, 1982). Higher levels of simulation were attained for a 3hr incubation at 20°C than for a 1hr incubation at the same temperature thus indicating that the longer the incubation period the greater the levels of enhancement. The observed stimulatory levels for this insulin-like effect was however well below that observed for insulin treatment.

The effect of different batches of collagenase is seen by comparing Fig. 3.1, where a 7-fold stimulation was obtained, with Fig. 3.5, where an approximately 30-fold stimulation was observed.

Fig 3:1.a.   Time course of stereospecific D-glucose uptake in adipocytes at 37°C, represented as a percentage of the stereospecific basal state.

Basal levels of total □, and stereospecific, ■, D-glucose uptake; Insulin-stimulated (100nM for 20 min) total, 0, and stereospecific, ●, D-glucose uptake.

Fig 3.1.b.   D-glucose uptake (open bars) and L-glucose uptake (shaded bars) measured for 120 sec.   Uptake presented as a percentage of control (D_{tot}) for basal and insulin stimulated states.   Results are the mean of 8 independent experiments.

Fig 3.1

(a)

D-Glucose Uptake (% of Basal)

(b)

D-Glucose Uptake (% of D_tot)
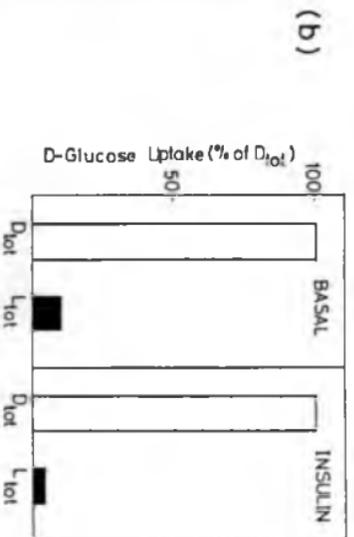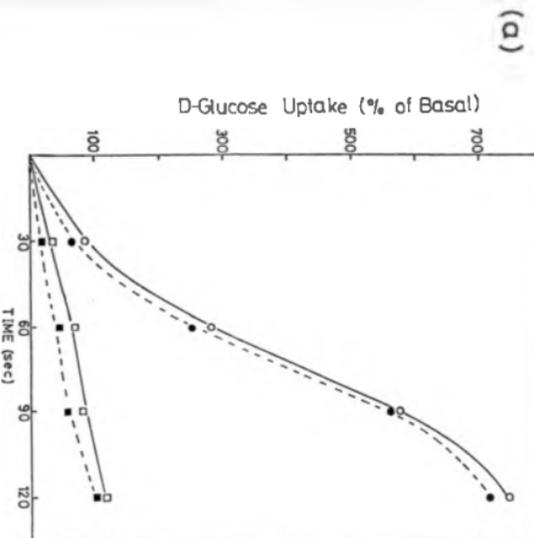
Fig 3.2   Time course for the effects of cytochalasin-B on the stereospecific uptake of D-glucose in isolated rat adipocytes at 37°C.

a)   Shows the effect of cytochalasin B on the insulin-stimulated state (100nM for 20 min). ●, D-glucose uptake in insulin-stimulated adipocytes. ■, cytochalasin B (25μM for 5 min) treated insulin-stimulated adipocytes; O, total-L-glucose uptake.

b)   Shows the effect of cytochalasin B on the basal state.
     ●, D-glucose uptake in basal adipocytes.
     ■, cytochalasin-B (25μM for 5 min) treated basal adipocytes.

Fig 3·2
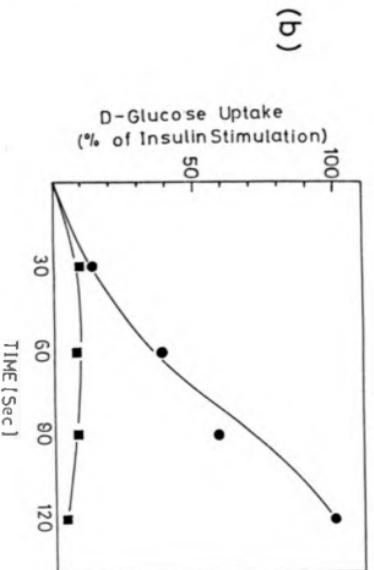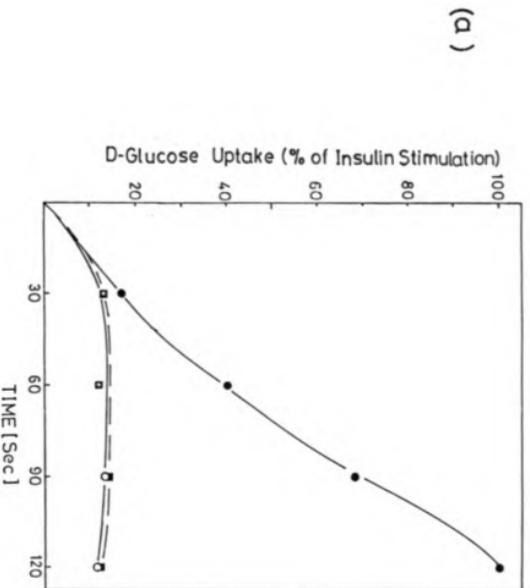
Fig 3.3 The effects of cytochalasin B on stereospecific D-glucose uptake by rat adipocytes at 37°C.

Both basal and insulin-stimulated cells were treated with cytochalasin B (25 μM for 5 min). D-glucose uptake was measure over 120 sec.

⊞ ;Basal level of D-glucose uptake

☐ ;D-glucose uptake in cytochalasin B treated basal cells.

▧ D-glucose uptake in insulin-stimulated cells (100nM for 20 min).

■ D-glucose uptake after insulin-stimulation (100nM for 20 min) followed by incubation with cytochalasin B (25 μM for 5 min)

The results are the mean of quadruplicate determinations.

Fig 3.3
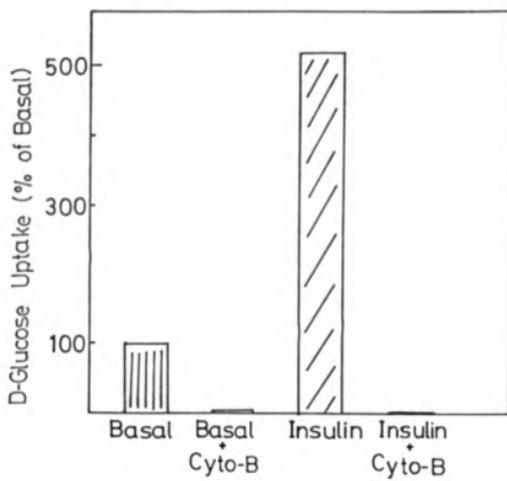
Fig 3.4   The effect of low temperature on stereospecific D-glucose uptake in rat adipocytes.

Adipocytes were incubated at 20°C and a time course for D-glucose uptake was measured after 1hr and 3hr incubations.

□, basal level of D-glucose uptake; ■, D-glucose uptake after insulin stimulation (100nM for 20 min); D-glucose uptake after 1 hr, 0, and 3 hr, ●, incubations at 20°C.

Fig 34
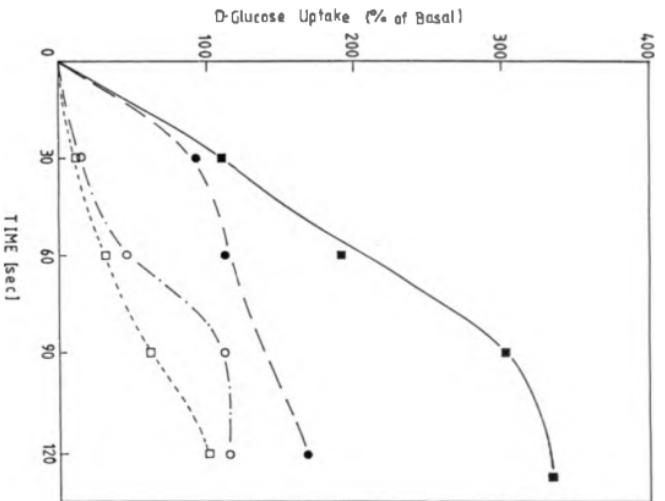
Fig 3.5   Time course for stereospecific D-glucose uptake in rat adipocytes at 37°C. Adipocytes were prepared from collagenase batch no. 10479230-70. ●, basal D-glucose uptake; O, insulin-stimulated (100nM for 20 min) D-glucose uptake.

Fig 3.5
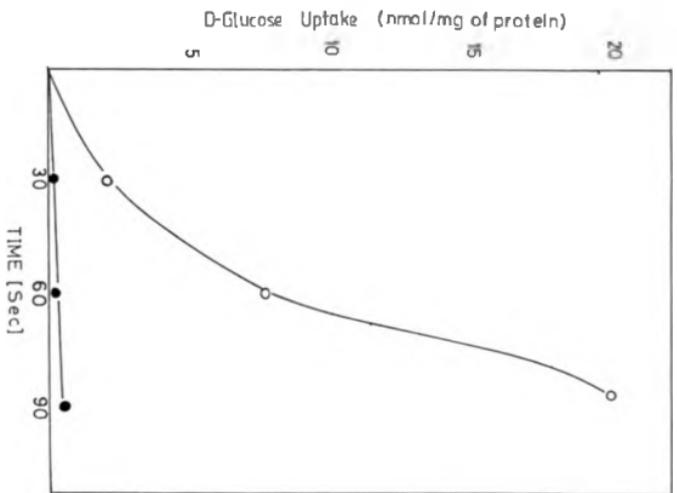
DISCUSSION

Glucose is rapidly metabolised by adipocytes mainly to lipid so that in contrast to a non-metabolised sugar such as 3-0-methylglucose the amount of radioactivity taken up by cells, during uptake experiments, is much higher. Although 3-0-methylglucose has been used to monitor sugar transport in a variety of cell types, including adipocytes (Simpson & Cushman, 1986; Gliemann & Rees, 1983), the aqueous space available to a non-metabolised sugar within the adipocyte is very small, approximately 2% of the cell volume (Gliemann et al., 1972), relative to the extracellular space which gives rise to a high background levels of radioactivity. Also insulin-stimulated transport rate of 3-0-methylglucose is very rapid (Gliemann et al., 1972) which may present additional problems given the small internal water space.

In addition 3-0-methyl-D-glucose has been found to exhibit a greater diffusional permeability across the plasma membrane as compared to L-glucose (Regen & Morgan, 1964). These researchers observed that while D-glucose penetration of rabbit erythrocytes appeared to be entirely by the carrier, the apparent diffusion constant for the L-glucose steroisomer was about one eight that encountered for 3-0-methylglucose. The increased diffusional permeability of this analog is probably related to the presence of a methyl group at C-3 since alkyl substitution at position three of glucose has been shown to accelerate transport (Hillman et al., 1959). Bihler et al. (1985a) have also observed a significant cytochalasin B-insensitive component of 3-0-methyl-D-glucose uptake under both basal and and insulin stimulated conditions, which was clearly in excess of L-glucose uptake. Hence it has been suggested that under some conditions this

monosaccharide may not be the ideal D-glucose analog (Bihler *et al.*, 1985a).

However the use of a metabolised sugar such as 2-deoxyglucose or D-glucose provides a system which allows the initial sugar transported across the membrane to be incorporated into 2-deoxyglucose-6-phosphate or lipids respectively, thus allowing an easier indirect means of measuring transport, provided that the transport step remains rate limiting for metabolism. The glucose concentration (0.5mM) employed in the uptake experiments described herein, is considered to be a concentration at which transport is rate limiting for subsequent metabolism (Cherqui *et al.*, 1983; Horuk *et al.*, 1983). In any event Crofford and Renold (1965) showed that transport of D-glucose into adipose tissue under conditions of adequate diffusion was rate limiting even at glucose concentrations as high as 80mM, which is 160 times higher than the D-glucose concentration used for these transport assays.

Recent studies on the kinetics for transport of D-glucose and 3-O-methylglucose in rat adipocytes by Whitesell & Abumrad, 1985, led to qualitatively similar findings and the transport k_m values measured for glucose with and without insulin were almost identical to those reported earlier by Crofford and Renold (1965). Additionally glucose transport has also been identified as the rate-limiting step for overall *iv vivo* glucose dispersal (Fink *et al.*, 1986). Also the production of volatile (CO_2) or diffusible (lactate) metabolites of glucose is insignificant during the first minutes hence loss of label during a brief assay would be minimal (Whitesell & Abumrad, 1985). Both D-glucose (Halperin and Cheema-Dhadli, 1982; More & Jones, 1983;

Whitesell & Abumrad, 1985) and 2-deoxy-D-glucose (Hyslop *et al.*, 1984, 1985; Green, 1986) uptake have been used as a measure of glucose transport in rat adipocytes.

In Fig. 3.1a, insulin stimulates stereospecific carrier mediated uptake of D-glucose ~ 7-fold. Only a marginal difference between the D_{tot} and the stereospecific ($D_{tot}-L_{tot}$) uptake in both basal and insulin stimulated state was observed. It is notable that the non-specific uptake remains quite small in the stimulated state (Fig. 3.1b) indicating that insulin enhances the stereospecific glucose transport while the passive uptake is not significantly affected. The small non-specific passive diffusion of the L-glucose indicated that the cells were in good condition and were not "leaky". The enhancement of glucose uptake by insulin in these experiments were usually 3- to 7- fold and initially this was thought to be somewhat low since other researchers were apparently obtaining 10- (Gliemann & Rees, 1983), 15- (Horuk *et al.*, 1983) and even 30- fold (Simpson & Cushman, 1986) increases in transport. In view of this a considerable amount of effort was made to improve insulin stimulation of transport.

Confidence in the results is reliant on the preparation of a good sample of adipocytes (cells with low basal uptake of glucose) which depends on a number of factors. Using a favourable batch of collagenase is essential (Gliemann & Rees, 1983). The incubation buffer should contain both D-glucose, which protects against erroneous results due to cell starvation (Whitesell & Abumrad, 1985), and albumin which binds free fatty acids in the medium, reduces degradation of insulin, prevents adsorption to vial walls (Lipkin, 1986a) and hence helps to maintain cell integrity (Gliemann & Rees, 1983). Cell agitation prior to the transport assay is believed to

markedly decrease the basal k_m (Whitesell & Abumrad, 1986), while harsh mechanical treatment of the cells can increase sugar permeability (Vega & Kono, 1979) and hard centrifugation has been shown to increase the number of transporters in the membrane (Kono et al., 1981).

Increasing the pH from 7.0 to 8.0 in an isotonic buffer enhanced basal transport activity approximately 3-fold without having any effect on the plus insulin activity, while increasing buffer hypertonicity at pH 7.4 increased basal activity and also caused a decrease in the plus insulin activity (Toyoda et al., 1986). The size of the rat used is also important; Hansen (1983) showed that cells from 200g rats were metabolically more responsive to insulin than cells from 340g rats. So that any one of the above factors may affect the final outcome of the experiment.

Adipocytes were therefore prepared by a strict method (described in detail in Chapter 2), from rats < 200g, in a Krebs-Henseleit buffer containing fatty acid free bovine serum albumin (BSA), 1%, and 2mM D-glucose. The buffer was filtered twice (minimum foaming), the second time through a $0.22\mu m$ millipore filter to remove any particles that may have been introduced by the BSA. Also after adjustment of the buffer pH by gasing (CO_2/O_2 mixture), some amount of pH shift was observed on standing which was readjusted to maintain a constant pH 7.4 level. Finally care was taken to avoid any centrifugation or harsh mechanical treatment of cells.

Despite these efforts and the use of different batches of collagenase the levels of stimulation rarely got above 10-fold. However although some researchers have claimed enormous levels of increased

transport under insulin stimulation in rat adipocytes others have only reported 3-10 fold increases in transport (Table 3.1) demonstrating that the levels of stimulation obtained in this work was at least in accordance with previously published data.

As a further check of membrane permeability to the passive uptake of glucose, the effect of cytochalasin B on D-glucose uptake was investigated. Cytochalasin B is a potent inhibitor of carrier mediated D-glucose transport, binding to the inner face of the carrier and in so doing altering the carrier conformation such that the transport sites are all recruited to the intracellular surface (Wang et al., 1986). Hence cytochalasin B treatment should result in the complete inhibition of stereospecific D-glucose uptake in both basal and insulin stimulated cells. Figs. 3.2 & 3.3 clearly demonstrates this powerful inhibitory effect of cytochalasin B (25μM). When both insulin stimulated and basal cells were treated with cytochalasin B the level of D_{tot} uptake fell to that of L_{tot} resulting in the complete obliteration of the stereospecific transport (Fig 3.2a). That the uptake of D-glucose in this system is cytochalasin B sensitive, > 96% inhibition in both basal and insulin stimulated cells (Fig. 3.3), is significant and demonstrates that glucose uptake is almost entirely due to carrier-mediated transport and not passive diffusion. Indeed Fig. 3.2 and 3.3 suggests that the cell membranes in these cells were intact and capable of restricting non-specific entry. So that the initial observation of low passive diffusion across the membrane (Fig 3.1), together with the demonstration of near total inhibition of transporters by cytochalasin B with resulting annihilation of stereospecific uptake strongly suggests that the cells were capable of restricting non-specific entry and at the same time could be stimulated by insulin into increasing carrier mediated D-glucose transport.

Table 3.1

Insulin Stimulation of Hexose Uptake in Adipocytes

Hexose	Change V_{max}	Change in K_m	Stimulation -Fold	Reference
MeGlc	3	N	3	May & Mikuleckey 1983
"	5-6	N	5-6	May & Mikuleckey 1982
"	6	N	6	Whitesell & Gliemann, 1979
*	6	N	6	Siegel & Olefsky 1950
*	*	*	7	Kohanski *etal* 1986
2-Deoxyglc	3	N	3	Olefsky, 1978
"	*	*	3	Cherqui, 1986
D-Glc	*	*	3-7	More & Jones 1983
*	*	*	5-6	Douen & Jones 1986
"	2	9.4	19	Whitesell & Abumrad, 1985

* = Not reported   N = Negligible

MeGlc = 3-0-Methylglucose
2-Deoxyglc = 2-Deoxyglucose
D-Glc = D-glucose

As an additional check of the ability of these cells to undergo increased stimulation of transport activity, it was decided to check the proposal that low temperatures were capable of inducing an insulin-like activation of transport activity in fat cells (Vega & Kono, 1979; Ezaki & Kono, 1982). Fig. 3.4 shows the stimulatory effect of low temperatures on the stereospecific D-glucose transport when adipocytes were incubated at $20^{\circ}C$ for 1hr and 3hr periods. This stimulatory effect was reproducible, though the extent of stimulation varied for each batch of cells produced.

Ezaki & Kono (1982) demonstrated that when adipocytes were exposed to low temperatures for prolonged time periods, glucose transport activity in the plasma membrane rich fraction was increased, while that in the Golgi-rich fraction was decreased. They suggested that low temperatures might facilitate the generation of an insulin like signal which induces translocation of glucose transport activity from intracellular storage sites or that changes in incubation temperature might shift the steady state distribution of transport activity between the plasma membrane and the storage sites.

Although Fig. 4 supports the view that low temperatures might induce an "insulin-like" effect one factor that should be considered is the inhibition of endo-and exocytosis at low temperatures ($<20^{\circ}C$) which would result in the inhibition of translocation processes. Nevertheless, Ezaki and Kono (1982) observed a significant increase in transport activity in plasma membrane fractions after incubation at low temperatures and suggested that subcellular membranes do not entirely stop their movements at low temperatures ($20^{\circ}C$ - $10^{\circ}C$). The long incubation periods required to bring about this "insulin-like" effect is probably due to decreased membrane fluidity which might be

hindering the usually rapid response to the generation of the biological signal.

Additionally it has been reported that some factors such as cell agitation and centrifugation, can induce an insulin-like effect. In particular cell agitation is believed to reduce the k_m of the transporter for D-glucose (Whitesell & Abumrad, 1986). It is conceivable that low temperatures might also facilitate a reduction in transporter k_m resulting in enhanced glucose uptake. Again the long time period required could be explained by a slow turn over of the transporter in the membrane caused by a restriction of the transporter conformational changes due to decreased membrane fluidity.

A number of steps have been purported to act in the generation of a biological signal upon insulin binding (Chapter 1, Section 1.6). It might be interesting to determine whether low temperautes might induce a conformational change in the receptor or activate the β subunit tyrosine specific kinase which are thought to be early events in signal generation, or whether low temperature can activate some post-binding event.

Much later on in the research a batch of collagenase (Batch No. 10479230-70) was purchased, which produced cells that exhibited greatly enhanced level of stimulation, approximately 30-fold (Fig 5.). This bears out earlier observations that a "good" batch of collagenase, which presumably yields cells with a low basal uptake, could markedly improve the levels of stimulation (Gliemann & Rees, 1983). Highly purified collagenase is incapable of disintegrating the tissue and the combined action of collagenase and acid protease appear to be necessary (Kono, 1969). However it has been speculated that crude

collagenase may contain proteins with "insulin-like" properties. Collagenase then seems to be an important factor in the cell preparation as Fig. 3.1a (~ 7-fold) and Fig 3.5 (~30-fold) indicate. However, one wonders whether the proposed "insulin-like" properties of the collagenase acts by increasing the levels of uptake in basal or insulin stimulated state or both.

It has been reported that insulin has little or no effect on the transporter k_m but can greatly increase the V_{max} (~ 30 fold) and yet the increase in number of transporters in the membrane upon insulin treatment, as measured by cytochalasin B binding, appears to be only 4- to 5- fold (Table 3.2). So that one might expect the increase in transporter activity to correspond to the magnitude of increase of carriers in the plasma membrane. Indeed some researchers have reported insulin increased transport levels which correlates with this increased number of transporters (Table 3.1).

Based on this it is difficult to conceive a 30-fold stimulation of transport without the involvement of some other factor, such as a decrease in transporter k_m. Such a decrease in the k_m by insulin and other external stimuli (cell agitation and glucose starvation) has been reported by at least one research group (Whitesell & Abumrad, 1985, 1986). These researchers observed a small 2-fold increase in V_{max} for D-glucose uptake which was accompanied by a larger 9-fold decrease in k_m (Table 3.1).

In view of the possible involvement of the transporter k_m in the transport system the question arises as to whether collagenase may in some way serve to modify the transporters with a resulting increase or decrease in k_m. A decrease in the transporter k_m might act to

Table 3.2

Insulin Induced Increase in Plasma Membrane Transporter Number (-fold)	Reference
4	Wardzala *etal*, 1978
5	Cushman & Wardzala, 1980
5.5	Karnieli *etal*, 1981
4-5	Simpson *etal*, 1983
4	Oka & Czech, 1984
5	Ezaki *etal*, 1986

produce cells with high basal permeability while an increase in k_m might yield cells with low basal permeability. It is envisaged that the latter effect might be able to be reversed by insulin hence giving the illusion of enormous insulin stimulation.

In the final analysis what remains is a great discrepancy in the literature as to the levels of stimulation of glucose transport by insulin and the mode by which this event occurs i.e. increasing V_{max} and or decreasing k_m.

Attempts to purchase a large quantity of this apparently "favorable" batch of collagenase proved fruitless. In any event the bulk of the data accumulated herein were obtained prior to receiving collagenase Batch No. 10479230-70. The adipocytes prepared, appeared suitable for measuring D-glucose uptake as evidenced by the small non-specific (passive diffusion) of L-glucose. The cells exhibited stereospecific transport and cytochalasin B directly inhibited the transporter function as shown by the total obliteration of stereospecific transport. Insulin routinely stimulated transport 3- to 7-fold which correlates well with the reported increase in transporter number in the plasma membrane and was also in agreement with levels of stimulation reported by others.

CHAPTER 3

Section b: *The Role of Calcium (Ca^{2+}) in insulin stimulation of glucose transport.*

INTRODUCTION

Calcium (Ca^{2+}) is actively extruded from adipocytes through the action of a high affinity Ca^{2+}- stimulated, Mg^{2+}-dependent adenosine triphosphatase (Pershadsingh & McDonald, 1979). Insulin is capable of inhibiting this plasma membrane ATPase with resulting accumulation of Ca^{2+} within the cells. Hence there is much debate as to whether Ca^{2+} may play some role in the mechanism of insulin action (Reviews: Pershadsingh & McDonald, 1984; Klip; 1984). The following experiments were designed to determine the importance of Ca^{2+} in insulin-stimulation of hexose transport.

RESULTS

To determine if extracellular Ca^{2+} (Ca_e^{2+}) is necessary for insulin stimulation of glucose transport, experiments were performed with adipocytes incubated in a Ca^{2+} free/1mM EGTA buffer. The divalent ion chelator EGTA has a high affinity for Ca^{2+} but a 2×10^4 lower affinity for Mg^{2+} and hence has been used previously as a specific Ca^{2+} chelator (Eckel et al.. 1983), and was used here to remove any excess traces of Ca^{2+} that may contaminate the medium. Fig. 3.6.a., shows the time course of D-glucose uptake for insulin-stimulated cells, in the presence and absence of Ca_e^{2+}. In the absence of Ca_e^{2+} insulin stimulated glucose transport to a significantly lesser extent than in the presence of Ca_e^{2+}. In two independent experiments insulin stimulation of hexose transport in the absence of Ca^{2+} was only $68.8 \pm 0.14\%$ of the control value, Fig. 3.6.b, indicating an inhibitory value of ~30%.

As a further probe into the importance of Ca_e^{2+} the divalent ionophore A23187 was employed. A23187 is a carboxylic ionophore which facilites membrane translocation of Ca^{2+} (Pressman, 1976). Hence if an elevation of cytoplasmic Ca^{2+} is involved in the activation glucose transport activity then treatment of basal adipocytes with A23187 should allow an influx of Ca^{2+} from the external medium into the cytoplasm with a resulting increase in sugar uptake. Fig. 3.7.a, shows the time course for D-glucose uptake in A23187 ($2\mu M$ for 20 min) treated basal adipocytes compared to the control insulin stimulated and basal states. A23187 appeared to partially activate the basal cells. This initial observation was checked in a separate experiment, Fig. 3.7.b, where the ionophore increased basal transport activity ~2.5 fold.

Although A23187 was able to elevate D-glucose uptake to approximately 50% that of the insulin stimulated state (Fig 3.8), it did not appear to act synergistically with insulin since treatment of insulin stimulated cells with ionophore only increased glucose uptake by ~12% (Fig. 3.8) as compared to ~150% increase of basal transport (Fig. 3.7.b). The stimulatory effect of A23187 on basal cells was used to establish a dose response curve for the Ca^{2+} dependence of glucose transport, Fig. 3.9. Maximum stimulation occured between 1-5mM and further demonstrates the importance of Ca^{2+} in D-glucose uptake.

It was decided to adopt two approaches to determine the importance of intracellular Ca^{2+} (Ca_i^{2+}) in hexose uptake: 1) to deplete cells of Ca_i^{2+} through the use of A23187 in conjunction with a Ca^{2+} free/EGTA buffer and 2) to monitor any increases in cytoplasmic Ca^{2+}, that may be induced by insulin, through direct measurements of fluorescence by quin 2. It has previously been

reported that addition o EGTA to cells being loaded with ^{45}Ca in the presence of A23187 caused an almost total loss of label (Bihler *et al.*, 1982 a,b). Adipocytes incubated in a Ca^{2+} free/EGTA buffer containing A23187 ($2\mu M$) failed to undergo insulin stimulation, Fig. 3.10.a. The level of insulin stimulation in these Ca_i^{2+} depleted cells was slightly below that of the basal level. A23187/EGTA treatment also caused a slight depression of the basal state, Fig. 3.10.b.

Quin 2, a fluorescent Ca^{2+} chelator (Tsien *et al.*, 1982), was used to investigate whether insulin is capable of mobilising Ca^{2+} from intracellular stores. However a number of difficulties were encountered (see Discussion) due to the bouyancy of the adipocytes, which hampered any accurate determination of intracellular Ca^{2+} concentrations and also prohibited a proper analysis of possible insulin effects on Ca^{2+} release. Indeed simple agitation of the cells, either by the use of a plastic stirrer or by removing and gently tapping the vial, was enough to produce a sharp transient rise in fluorescence (Fig. 3.11.a.). Given the shortcomings of the experiment, insulin did not appear to induce any rapid increase in fluorescence to coincide with the time span (seconds) needed to activate the glucose transport system (Fig. 3.11.b.). Increased and decreased fluorescence upon lysis of adipocytes with triton X-100 (1%) and addition of EGTA respectively, Fig. 3.11.c. indicates the existence of a Ca^{2+} pool within these cells.

Fig 3.6.a. The effects of depletion of Ca_e^{2+} on insulin stimulation of stereospecific D-glucose uptake in rat adipocytes at 37°C. Adipocytes were transfered to a modified Krebs-Henseleit buffer ($CaCl_2$ omitted) after preparation. To one portion of these cell Ca^{2+} (2.5mM) was reintroduced into the medium and a time course for basal, ●, and insulin-stimulated (100nM for 20 min), 0, D-glucose uptake was obtained. The second portion of cells were treated with 1mM EGTA for 5 min, followed by insulin (100nM for 20 min) and a time course for D-glucose uptake was performed, □

Fig 3.6.b. The effect of depletion of Ca_e^{2+} on insulin stimulation of stereospecific D-glucose uptake. Adipocytes were prepared as in the legend to Fig 3.6.a. D-glucose uptake were measured for 120 sec by insulin-stimulated (100nM for 20 min) cells in the presence, open bar , and absence (1mM EGTA - Ca^{2+} free buffer), hatched bar , of Ca_e^{2+}. Results are the mean ± SD of quandruplicate determinations.

Fig 3.6
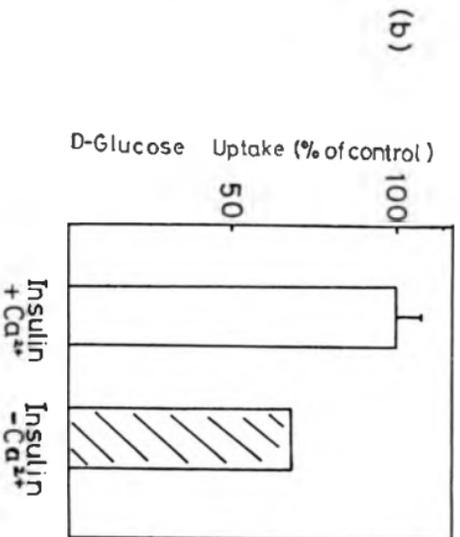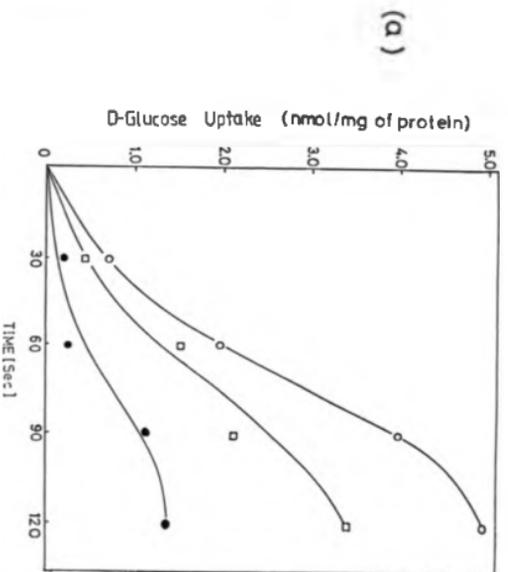
Fig 3.7.a. Time course for the effect of A23187 on stereospecific
D-glucose uptake in adipocytes at 37°C.
0, D-glucose uptake after insulin stimulation (100nM for 20 min);
●, basal level of D—glucose uptake; ■; D-glucose uptake after
incubation of basal cell with A23187 ($2\mu M$ for 20 min).

Fig 3.7.b. Effect of A23187 on stereospecific D-glucose uptake measued
for 120 sec. Hatched bar represents the basal level of uptake while
the open bar indicates D-glucose uptake in adipocytes treated with
A23187 ($2\mu M$ for 20 min). Results are the mean ± SD.

Fig 3·7

(a)

D-Glucose Uptake   (nmol/10⁶ Cells/60 Sec)

(b)

D-Glucose Uptake (% of Basal)
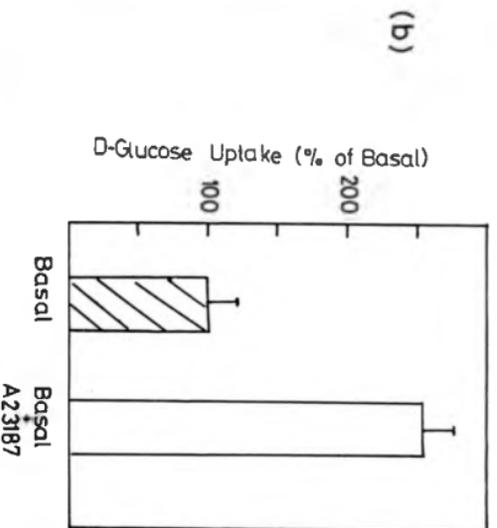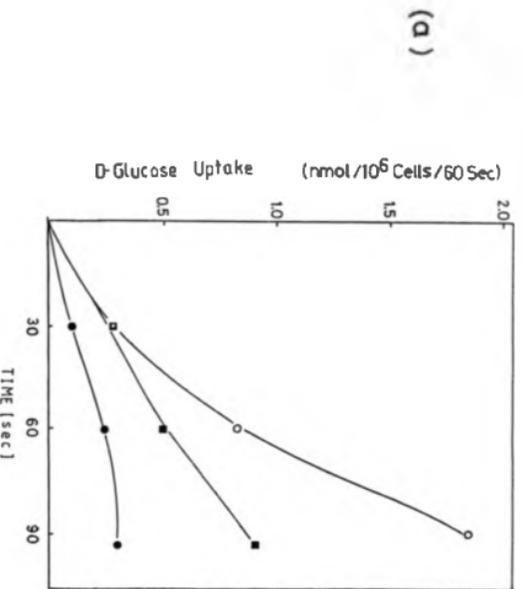
Fig. 3.8

The effect of A23187 on the stereospecifc D-glucose uptake by basal and insulin stimulated adipocytes at 37°C. D-glucose uptake was measured for 120 sec.

D-glucose uptake after insulin stimulation (100nM for 20 min).

D-glcuose uptake in basal adipocytes treated with A23187 (2μM for 20 min).

D-glucose uptake in insulin-stimulated cells (100nM for 20 min) treated with A23187 (2μM for 20 min)

Results are the mean ± SD of quadruplicate determinations.

Fig 3.8

D-Glucose Uptake (% of control) vs Insulin (control), Basal + A23187, Insulin + A23187
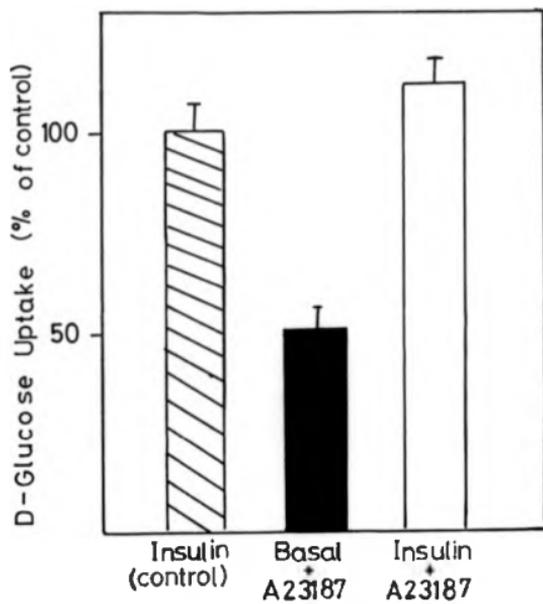
Fig. 3.9

Dose response for the effect of A23187 on the enhancement of stereospecific D-glucose uptake by rat adipocytes at 37°C.

Adipocytes were transferred to a modified Krebs-Henseleit buffer ($CaCl_2$ omitted) after preparation. Cells were then incubated with increasing concentrations Ca_e^{2+}. The suspensions were treated with A23187 ($2\mu M$ for 20 min) and D-glucose uptake was measured for 60 sec.

Fig 3.9

D-Glucose Uptake (nmol/mg prot/ 60 Sec)

[Ca²⁺] mM
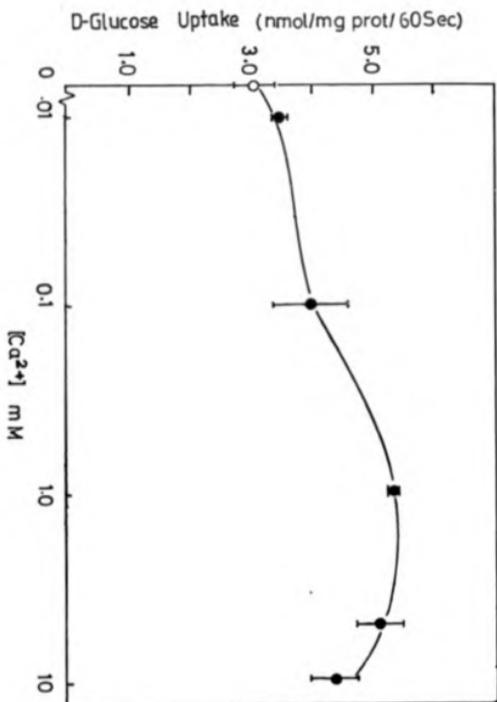
Fig 3.10

The effect of EGTA and A23187 on the stereospecific uptake of D-glucose in rat adipocytes at 37°C.

a)   After cell preparation adipocytes were transfered to a modified Krebs-Henseleit buffer ($CaCl_2$ omitted) and the resulting suspension divide into three portions. $CaCl_2$ (2.5mM) was introduced into two of these portions which were used to measure, ●, basal level of D-glucose uptake and ; ○, D-glucose uptake after insulin stimulation (100nM for 20 min).   The third portion of cells, □, was treated with EGTA (1mM for 5 min) followed by A23187 (2μM for 20 min) then insulin stimulation (100nM for 20 min).

b)   ○, basal level of D-glucose uptake in the presence of 2.5mM Ca^{2+}; □, D-glucose uptake after treatment with A23187 (2μM for 20 min) in the absence of Ca_e^{2+} (Ca^{2+} free buffer containing 1mM EGTA).

Fig 3·10
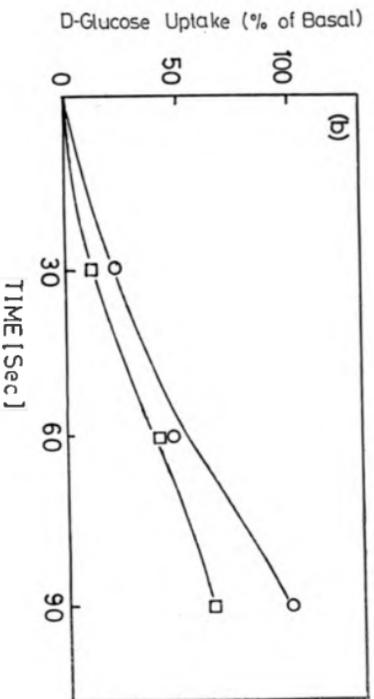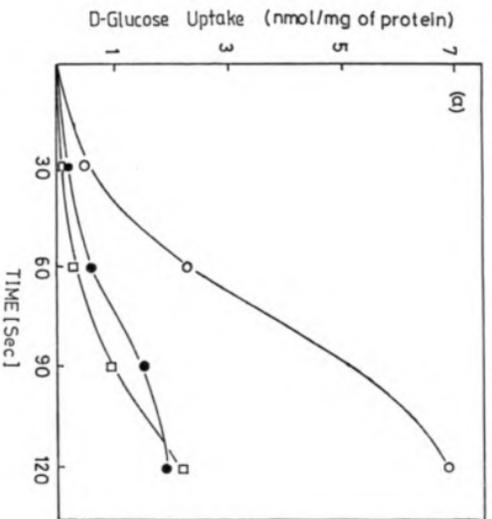
Fig. 3.11

Fluoresence measurements of $[Ca^{2+}]_i$ in rat adipocytes at $37^{\circ}C$ using quin 2.

Adipocytes were loaded with quin 2/AM ($100\mu M$) for 40 min. Cells were then washed twice with Krebs-Henseleit buffer (1% BSA, 2mM D-glucose) and 2 mls of this suspension transfered to the cuvette.

a)   Fluoresence measured after the heterogenous cell suspension was disturbued through gentle agitation of the cuvette.

b)   Fluoresence measured after the addition of insulin (100nM)

c)   Fluoresence measued after the addition of Triton X-100 (1%) and EGTA (5-15mM)

Fig 3·11

Arbitrary Fluoresence
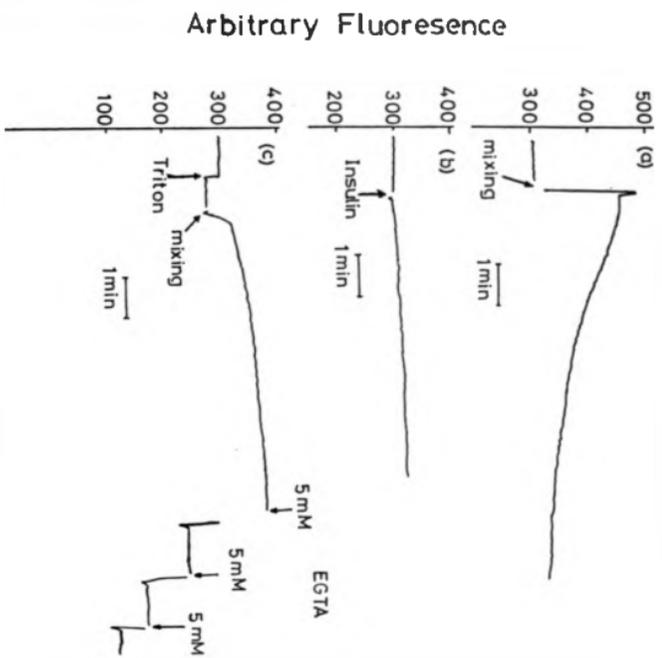
DISCUSSION

Although the direct role of Ca^{2+} in the mechanism of insulin action is controversial, substantial indirect evidence implies its essential importance. This includes: a requirement for Ca_c^{2+} for some of insulin's effects on cellular metabolism, several insulin-sensitive enzymes are Ca^{2+} dependent, some Ca^{2+} - altering compounds (e.g. local anesthetics and heavy metals) mimic the effects of insulin, insulin alters Ca^{2+} fluxes in intact cells and insulin alters Ca^{2+} homeostasis in plasma membranes (Pershadsingh & McDonald, 1984). However no substance has been unambiguously identified to participate in the enhancement of glucose transport.

Insulin is known to hyperpolarize adipocytes (Beigelman & Hollander, 1962) but the ionic basis of the hyperpolarised state remains unclear (Moore, 1983; Pershadsingh et al., 1986b). Insulin affects a number of plasma membrane associated ion-transport systems including stimulation of active $Na^+ + K^+$ transport (i.e. the $Na^+ - K^+$ pump) and inhibition of the high affinity Ca^{2+} pump, both of which are primary active ATP dependent transport systems. In addition insulin also increases the activity of Na:H exchange systems and stimulates Na^+ coupled amino acid co-transport (Moore, 1983). It is conceivable then, that ionic changes in the cytoplasmic plasma membrane region might facilitate activation of hexose transport and indeed both hyperpolarization (Marunaka, 1986; Zierler & Rogus, 1980) and Ca^{2+} (Bihler et al., 1985a, Taylor et al., 1979), have been purported to be essential for enhancement of sugar transport.

The physiological stimulation of exocytosis has been shown to be accompanied by elevation in cytosolic Ca^{2+} (Case, 1984). Electrical

excitation of neuronal plasma membranes reaching the nerve endings causes an increase in the cytosolic Ca^{2+} activity which triggers the fusion of the synaptic vesicles with the plasma membrane (Akerman & Nicholls, 1983). Moreover, the secretion of both neurotransmitters and a variety of hormones can be induced by Ca^{2+} ionophores (Akerman & Nicholls, 1983; Case, 1984) which further implicates Ca^{2+} in the exocytosis process. It has been speculated that within 1 sec of inhibition of the $(Ca^{2+} + Mg^{2+})$ - ATPase intracellular Ca^{2+} content increases approximately 10-fold with subsequent increases in the amount of Ca^{2+} bound to the plasma membrane (Pershadsingh & McDonald, 1984). Hence given the resemblence of recruitment of transporters to the fusion of intracellular secretary granules with the plasma membrane occuring during exocytosis it is possible that the build up of cytoplasmic Ca^{2+} may play an important role in the fusion of the transporter rich cytoplasmic vesicles, which contain a high concentration of phosphatidylserine (a relatively fusogenic lipid in the presence of Ca^{2+}) on their external bilayer, to the plasma membrane.

However several research groups have failed to observe any dependence for Ca^{2+} in sugar transport in rat adipocytes (Williams *et al.*, 1986, Shechter, 1984) and in other cell types (Hall *et al.*, 1982, Klip, *et al.*, 1984, Eckel, 1983). A comprehensive report by Klip *et al.*, (1984), on hexose transport in L6 muscle cells, indicated that the use of a Ca^{2+} free buffer and depletion or modification of Ca_i^{2+} levels did not inhibit transport while A23187 failed to enhance transport. Further the direct determination of fluorescence in these cells using quin 2 did not show differences between basal and insulin stimulated cells.

Nonetheless, it has previously been proposed that the activation of glucose transport by insulin in adipocytes may be associated with an increased cytoplasmic Ca^{2+} concentration (Clausen, 1970) and Ca^{2+} increased both basal and insulin-stimulated apparent rates of glucose transport (~ 40%) by increasing the V_{max} of the glucose transport system without altering the K_m (Taylor et al., 1979; Bihler et al., 1985b). This observation is interesting since an increase in V_{max} fits well with the concept of transport recruitment and might imply a direct role for Ca^{2+} in membrane fusion. Also, certain heavy metals Ni^{2+}, Zn^{2+} and La^{3+} which antagonise Ca^{2+} entry (Bihler et al., 1980) and the Ca^{2+} channel blocker D600 (Bihler et al., 1985b) significantly inhibited insulin stimulation of sugar transport. In addition removal of Ca^{2+} dramatically diminished insulin stimulated hexose transport in rat adipocytes (Taylor, 1979) and myocytes (Bihler, 1985b). In the latter case the 70% decrease in transport observed on omission of Ca^{2+} from the incubation medium was partially reversed by subsequent return to a Ca^{2+} containing medium.

The data presented here supports the view that Ca^{2+} is essential for insulin stimulation of glucose transport. Adipocytes prepared in a normal Krebs-Henseleit buffer containing Ca^{2+} showed a significant decrease in the insulin stimulation of glucose transport when transfered to a Ca^{2+} - free buffer containing 1mM EGTA (Fig. 3.6.) This removal of Ca_c^{2+} from the medium resulted in a reduction, (~ 30%) but not complete iradication of stimulation indicating that Ca_c^{2+} might be essential but not critcial for glucose transport. The observation is in agreement with Bihler et al. (1985b), though these researchers demonstrated a much more significant reduction (~ 70%) of the insulin stimulated rise in V_{max} and proposed that the effect of insulin was strongly dependent on Ca_c^{2+}.

The importance of Ca_e^{2+} in the enhancement of sugar transport is again demonstrated in Fig. 3.7 where treatment of basal cells with A23187 in a medium containing ample Ca^{2+} caused a notable rise in D-glucose uptake. The fact that A23187, which facilitates membrane translocation of Ca^{2+}, could elevate transport activity indicates that a build up in intracellular Ca^{2+}, from extracellular sources might be important for hexose transport. A23187 appears to be capable of boosting glucose uptake to approximately $1/2$ that of insulin stimulated state (Fig. 3.8) which again suggests that although Ca_e^{2+} is essential for stimulation, other factors which cannot be determined here, are also essential and may act in unison with Ca^{2+} to induce maximum stimulation. Although A23187 has been found to stimulate sugar transport in thymocytes (Reeves, 1975), cultured chick embryo breast muscle (Schudt et al., 1976), rat heart and diaphragm (Bihler, 1980) and avian erythrocytes (Carruthers & Simons, 1978), it did not enhance transport in adipocytes (Taylor 1979; Bonne, 1977), in contrast to the observations made here (Fig. 3.7, 3.8, 3.9). Oddly enough Taylor et al., 1979 determined that omission of Ca_e^{2+} from the medium inhibited transport but failed to observe any stimulation of sugar uptake by A23187. The reasons for these discrepancies are unclear.

Treatment of insulin-stimulated cells with A23187 resulted in a small and relatively insignificant increase in glucose uptake (Fig. 3.8) suggesting that little or no synergism is occuring between insulin and this ionophore. It is possible that insulin-induced changes in Ca^{2+} flux across the plasma membrane is sufficient for maximal response and further increasing the Ca^{2+} intake, with A23187, does not have any significant impact on transport activity.

The ability of A23187 to enhance hexose transport (Fig. 3.7) indicates the involvement of Ca_c^{2+} in the activation of this process, however that a concentration dependence for this stimulatory effect can be obtained (Fig. 3.9) whereby increasing Ca_c^{2+} concentration increases the amount of sugar taken up must strongly implicate Ca^{2+} as having an important role in sugar transport. At concentrations greater than 5mM some amount of inhibitory effect on transport was observed which might be due to interference a) with cell integrity or b) with changes in plasma membrane viscosity. It has been reported that noradrenalin, via α adrenergic receptors and other Ca^{2+}-mobilizing hormones, increases lipid fluidity by displacing a small pool of Ca^{2+} bound to phospholipids thus removing the mechanical constraints brought about by this ion (Burgess et al., 1983). Hence it might be envisaged that a large continuous influx of Ca_c^{2+} might generate an antagonistic effect whereby a decrease in membrane fluidity occurs thus impairing fusion of transport vesicles with the plasma membrane.

Although Ca_c^{2+} appears to be necessary for maximum activation of glucose transport it probably serves to inflate the cytoplasmic Ca^{2+} levels or replenish Ca_i^{2+} stores (Putney, 1986). Hence depletion of Ca_i^{2+} by use of Ca^{2+}-free/1mM EGTA buffer and A23187 should present a situation whereby Ca_i^{2+} should move freely outward, along a concentration gradient, and be mopped up by EGTA. This scheme has been previously employed to determine the importance of Ca_i^{2+} in insulin action and have yielded conflicting results. A23187/EGTA combination failed to inhibit insulin stimulation of glucose transport in rat soleus muscle and isolated cardiocytes whereas as A23187/EDTA (chelates both Ca^{2+} and Mg^{2+}) caused significant inhibition of hexose transport thus suggesting a role for Mg^{2+} in sugar transport (Hall et

al., 1982; Eckel *et al.*, 1983). On the other hand Bihler *et al.* (1985a) found that stimulation of hexose transport was abolished in cells depleted of Ca_i^{2+} by A23187/EGTA treatment. A similar occurance was observed here (Fig. *3.10*). Removal of Ca_i^{2+} depressed the basal activity and completely abolished the insulin stimulated D-glucose uptake strongly suggesting an intimate involvement of Ca_i^{2+} in hexose transport.

Studies with the ionophore A23187 are open to criticism however, since one major assumption is that A23187 has no other effect upon the membrane other than increasing permeability to Ca^{2+}. So that an attempt was made to monitor insulin induced release of Ca^{2+} from cytoplasmic stores using quin 2. This agent could be easily loaded into the adipocytes via its acetoxymethyl ester, qunin 2-AM (see Methods, Chapter 2), however one of the major problems encountered thereafter was the inability to obtain a completely homogenous solution. The very nature of adipocytes, high triglyceride content, meant that the cells floated easily. Introduction of a magnetic stirrer only marginally improved the situation since vigourous spinning had to be avoided so as to prevent cell breakage and also to avoid activation of the cells (Vega & Kono, 1979; Whitesell & Abumrad 1985, 1986) which, if it entails increases in cytoplasmic Ca^{2+}, would defeat the entire experiment. Diluting the adipocyte suspesion only managed to present these problems on a lesser scale.

An additional problem was realised when it was observed that agitation of the basal cells to improve mixing caused a transient increase in fluorescence, (Fig. 3.11,a) which probably relates to increased distribution of cells in the excitation path. So that this method of stirring could not be used for ensuring proper mixing of

insulin with the cells. Further, the time taken for such a procedure would not detect any sudden increased fluoresence that might be due to the presence of insulin. In any event when insulin (100nM) was injected below the top layer of cells into the lower layer, which was being gently stirred, no increased fluorescence was observed (Fig. 3.11.b), tentatively suggesting that Ca_i^{2+} stores were not released upon insulin treatment of adipocytes. However given the short comings of this system, this suggestion can only be made with some reservation.

Lysis of the cells with triton X-100 caused an initial decrease in fluorescence, which was probably related to the inability of the suspension to form a uniform dispersion, since upon gentle agitation sustained increased fluorescence was observed (Fig. 3.11.c). The Ca_i^{2+} released into the medium could be mopped up by excess EGTA (15mM) resulting in decreased fluorescence and indicating the existence of Ca^{2+} within the cells. However the complications confronted with the inadequacy of adipocytes to yield an unvarying suspension prohibited proper analysis of the proposed ability of insulin to mobilize Ca^{2+} from intracellular stores (Delfert et al., 1986).

Although it has been reported that quin 2 does not inhibit glucose transport in L6 muscle cells (Klip et al., 1984) recently Pershadsingh et al., 1986a observed that quin 2 inhibited the stimulation of glucose transport and oxidation in adipocytes, further supporting the hypothesis that Ca_i^{2+} may be essential for hexose transport (Czech, 1977; Pershadsingh & McDonald, 1984). The dramatic decrease in glucose uptake observed on depletion of Ca_i^{2+} (Fig. 3.10) as compared to only a partial reduction of transport activity on exclusion of Ca_e^{2+} (Fig. 3.6) might provide some indication as to the relative importance of these two Ca^{2+} pools in the activation of

hexose transport. It might be speculated that Ca_i^{2+} may be critical for hexose transport and may serve to initiate intracellular events associated with activation of transport activity and is subsequently assisted by an influx of Ca_e^{2+}. One might envisage a scenario as that depicated in Fig. 1.6 (Chapter 1) whereby insulin induced increase in cytoplasmic IP_3 releases Ca^{2+} from the edoplasmic reticulum. The subsequent conversion of $IP_3 \rightarrow IP_4$, in a Ca^{2+} dependent reaction, might serve to open plasma membrane Ca^{2+} gates allowing an influx of Ca_e^{2+} and hence maximization of certain Ca^{2+} dependent processes.

Apart from the possible direct involvement of Ca^{2+} in the fusion of transporter vesicles with the plasma membrane, it is conceivable that Ca^{2+} may act indirectly to enhance insulin action and consequently hexose transport. The insulin receptor contains a Ca^{2+} binding site (Williams & Turtle, 1984) and Ca^{2+} binding to the receptor serves to increase its affinity (Williams et al., 1986). Insulin receptor phosphorylation, which is thought to be a prerequisite for insulin induced activation of sugar transport (Kohanski et al., 1986, Ellis et al., 1986), has been shown to be a calmodulin sensitive, Ca^{2+} dependent process (Graves, et al., 1986; Plehwe et al., 1983). In addition Ca^{2+} is believed to be intimately associated with changes in membrane potential (Pershadsingh et al., 1986b) which has recently been proposed as a possible mediator on insulin stimulated sugar transport (Marunaka, 1986). Indeed it has been suggested that a regulatory role of Ca^{2+} on insulin sensitive plasma membrane events, either directly or via changes in the membrane potential, seems irrefutable (Pershadsingh & McDonald, 1984).

Although the data presented here was in agreement with some

reports it was also in disagreement with others. This situation is not easily explained but may possibly be due to different experimental procedures or in some cases tissue specificity. These initial Ca^{2+} transport experiments were performed concurrently with other studies (Chapters 4 & 5) and eventually gave way to the latter. The experiments performed here gave an indication that Ca^{2+} may be an essential requirement for glucose transport in rat adipocytes. However, much more work would be required to fully establish Ca^{2+} as an indisputable intermediary in insulin stimulation of D-glucose transport.

C H A P T E R   4

INTRODUCTION

Phenylarsine oxide (PhAsO) is a trivalent arsenical which reacts with sulphydryl groups and has previously been reported to inhibit internalization of protein into oocytes (Wallace & Ho, 1972) and receptor mediated endocytosis of protein nexin (low et al., 1981), EGF (Willey & Cunningham, 1982) and insulin (Knutson et al., 1983; Draznin et al., 1984). However the mechanism of action of this arsenical is largely unexplored. PhAsO reacts with sulphydryl groups and since other sulphydryl reactive compounds inhibit mitochondrial function it is possible that the inhibitory effects of PhAsO on endocytosis might be related to lowering of cellular ATP. Irreversible inhibition of ATP production could lead to general debilitation of cell integrity and a loss in the capacity to mount a complex event like endocytosis (Hertel et al., 1985). Indeed agents that lower ATP levels have been found to inhibit endoytosis (Ciechanover et al., 1983, Clark & Weigel, 1985). If the observed effects of PhAsO results from non-specific interactions (i.e. lowering of cellular ATP) then this agent would be a less useful tool in the elucidation of the molecular mechanism of endocytosis (Hertel et al., 1985).

However despite the fact that PhAsO is capable of reacting with sulphydryl groups, 100μM PhAsO, a concentration 10 times higher that thought to effectively inhibit all protein encorporation (Wallace & Ho, 1972), did not affect cellular ATP content in alveolar macrophages (Kaplan et al., 1985) and human astrocytoma cells (Hertel et al., 1985), while Frost & Lane (1985) observed only a small 10% decrease in cellular ATP in 3T3-L1 adipocytes using 18μM PhAsO. Hence the effect of PhAsO cannot be explained on the basis of inhibition of

energy metabolsim and it has been suggested that the ability of PhAsO to inhibit endocytosis under conditions where cellular ATP levels remain unchanged is due to selective interaction with biological molecules containing vicinal sulphydrl groups (Hertel *et al.* 1985).

Trivalent arsenicals form stable ring structures with molecules having appropriately spaced vicinal sulphydryl groups; five membered rings appear to be most stable (Thompson, 1948; Webb, 1966). It is this ability of PhAsO to react with vicinal or paired thiol groups to form stable cyclic thoarsenite complexes that is thought to potentiate PhAsO's ability to inhibit protein internalization rather than any effect on cellular ATP (Wallace & Ho, 1972; Walker-Smith & Payne, 1983). 1,2 and 1,3 - dithiols react with PhAsO to yield cyclic phenyldithioarsenites (Hannestad & Sorbo; 1980):

1,2 dithiol   Dithiarsane

1,3 Dithiol   Dithiarsolane
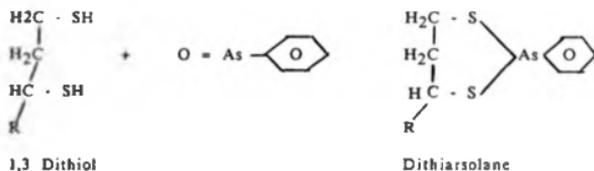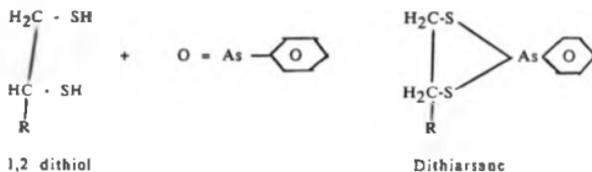
Insulin receptors, D-glucose transporters and mediators of insulin action, are all thought to be associated with the plasma membrane. The question arose then as to what effect might PhAsO have on

insulin stimulation of hexose transport/uptake? Could PhAsO:a) trap receptors and/or transporters in the membrane thereby causing greatly enhanced gluocse uptake, upon insulin stimulation, through prolonged signal generation and glucose influx, or b) does PhAsO interact with some other component(s) associated with the system such as hormone binding or mediator production? The following experiments report on the effect of PhAsO on insulin stimulation of D-glucose uptake.

RESULTS

Fig 4.1 shows a time course for the effect of PhAsO on stereospecific D-glucose uptake. PhAsO (10μM) inhibited both basal and insulin-stimulated adipocytes. Simultaneous treatment of adipocytes with insulin (50nM) and PhAsO (10μM), for 20 min, showed significant inhibition; the level of uptake falling below that of the basal level. Although PhAsO (10μM) caused substantial inhibition of glucose uptake, transport remained stereospecific (Fig. 4.1b) and the total L-glucose was ~30% that of total D-glucose uptake. No stimulation of uptake occured if cells treated with PhAsO (10μM) were subsequently treated with insulin (100nM for 20 min) (Fig. 4.2). In fact whether PhAsO was added prior to or after insulin stimulation the extent of inhibition appeared to be about the same (Fig. 4.2).

The inhibitory effect of PhAsO (10μM) on both basal and insulin stimulated cells, monitored over an 8 min time period, is demonstrated in Fig. 4.3.; D-glucose uptake being measured for 60 sec after specific time intervals. PhAsO acts rapidly dramatically inhibiting insulin-stimulated uptake within 1 min (Fig. 4.3a). Basal cells were also rapidly inhibited after 1 min. (Fig. 4.3b) and in a dose dependent manner since 10μM PhAsO brought about more severe inhibition than 5μM PhAsO. The rapidity with which phAsO acts is borne out in Fig. 4.4. When adipocytes were treated simultaneously with insulin (50nM) and PhAsO (10μM), inhibition was apparent within 30 sec (Fig. 4.4). In addition this inhibitory effect of PhAsO appears to be of long duration since adipocytes incubated with PhAsO (10μM) and insulin (50μM) showed no increase in sugar uptake over a 60 min time period (Fig 4.5).

The ability of PhAsO (10μM) to inhibit stereospecific D-glucose uptake as compared to cytochalasin B is shown in Table 4.1. Although PhAsO (10μM) caused -70% and -87% inhibition of glucose uptake in basal and insulin stimulated cells respectively, this was less than the near total inhibition of transport exhibited by cytochalasin B (25μM).

Time courses for D-glucose uptake in the presence of insulin and PhAsO are shown in Fig. 4.6. The degree of uptake inhibition is dependent on the PhAsO concentration, more inhibition occuring at higher concentrations. The reducing agent dithiothreitol (200μM) partially reversed the PhAsO inhibition of D-glucose uptake, Fig. 4.7a; here adipocytes were incubated with 10μM PhAsO for 5 min, followed by the addition of 200μM dithiothreitol and after 1 hr insulin (100nM) was added and D-glucose uptake assayed. Dithiothreitol alone had no significant effect on the basal level of D-glucose uptake (Fig. 4.7b).

Fig. 4.8 shows the effect of different PhAsO concentrations on D-glucose uptake in insulin-stimulated cells which clearly indicates that the initial inhibition by PhAsO is transient and that recovery of D-glucose uptake occurs in a dose-dependent manner since lower PhAsO concentrations allowed recovery of approximately 90% of the original activity. In 5 independent experiments using 1μM PhAsO the percentage recovery was found to be $86.0 \pm 8.5\%$ (Table 4.2).

Fig. 4.9 shows the dose-response curves for D-glucose uptake for cells treated simultaneously with insulin and PhAsO and for cells treated first with insulin and then with PhAsO. Since PhAsO acts

rapidly (Figs. 4.3; 4.4) the simultaneous treatment with insulin and PhAsO gives a dose-response curve for cells with receptor-rich plasma membranes whereas the curve for insulin-stimulated cells is a dose-response curve for transporter-rich plasma membranes. It is clear that at the lower PhAsO concentrations ($< 1\mu M$) these dose-response curves differ significantly.

The recovery of transport activity observed after initial inhibition of uptake at low PhAsO concentrations (Fig. 4.8) also persists in the presence of monensin (Fig. 4.10). PhAsO ($1\mu M$) added to cells treated first with insulin (100nM for 20 min) then monensin ($25\mu M$ for 20 min) caused a significant inhibition of D-glucose transport within 2 min and here again the transport activity begins to recover and returns to approximately 90% or its original activity after 20 min.

Monensin, a carboxylic ionophore that preferentially exchanges Na^+ for H^+ ions (Pressman, 1976), is thought to inhibit dissociation of internalized hormone-receptor complexes and prevent recycling of occupied or unoccupied receptors (Carpentier *et al.*, 1986b; Wileman *et al.*, 1984). Treatment of basal or insulin stimulated cells with monensin ($25-100\mu M$) for 20 min. did not inhibit D-glucose uptake (Fig. 4.11) and interestingly higher monensin concentrations ($100\mu M$ for 20 min) appeared to exert a small (~20%) but notable enhancement of the insulin stimulated state (Fig. 4.12).

Fig. 4.1.a.

Time course for the effect of PhAsO on stereospecific D-glucose uptake by rat adipocytes at 37°C.

●, D-glucose uptake after insulin stimulation (50nM for 20 min); ■ basal level of D-glucose uptake; □, basal cells treated with PhAsO (10μM for 20 min); 0, D-glucose uptake after incubation with insulin (50nM) and PhAsO (10μM) added simultaneously for 20 min. Adipocytes were incubated for the required time period and D-glucose uptake was quenched by addition of ice-cold stopping buffer.

Fig 4.1.b.

D- and L- glucose uptake after incubation with insulin (50nM) and PhAsO (10μM) added simultaneously for 20 min.

0, Total D-glucose uptake; △ Total L-glucose uptake; ●, stereospecific D-glucose uptake ($D_{tot} - L_{tot}$)

Fig 4.1
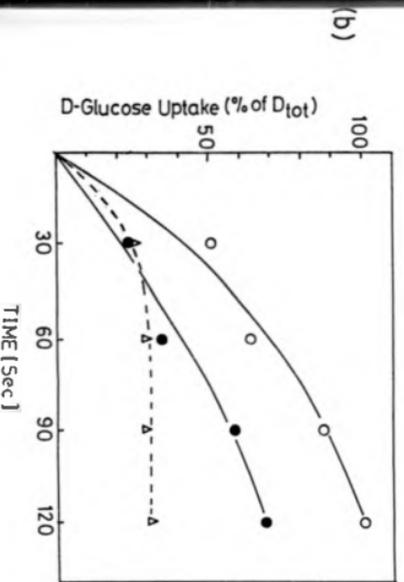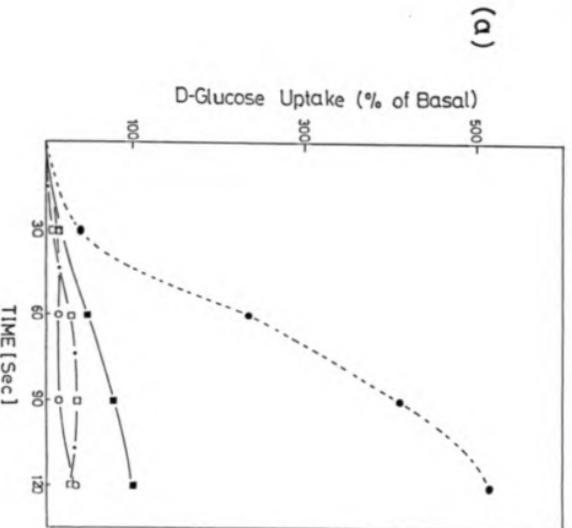
Fig 4.2

Time course of the effect of phenylarsine oxide on stereospecific D-glcuose uptake by basal and insulin stimulated rat adipocytes at 37°C.

0, D-glucose uptake after insulin stimulation (100nM; 20 min); ■, D-glucose uptake after insulin stimulation (100nM; 20 min) followed by PhAsO treatment (10μM; 5 min); □, D-glucose uptake after PhAsO treatment (10μM; 5 min) followed by insulin treatment (100nM, 20 min).

Fig 4.2
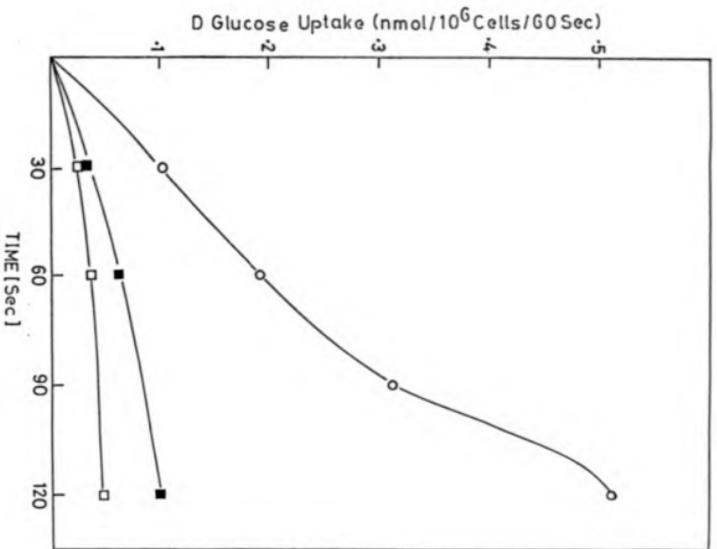
Fig. 4.3

The effect of PhAsO on stereospecific D-glucose uptake in rat adipocytes at $37^{\circ}C$.

a) Adipocytes were incubated with insulin and D-glucose uptake (in 60 sec.) was measured. These insulin stimulated cells were treated with PhASO ($10\mu M$) and D-glucose uptake (in 60 sec) was measured after 1,3,5 and 8 min.

b) Basal cells were treated with $5\mu M$ and $10\mu M$ PhAsO and D-glucose uptake (in 60 sec) was measured after 1,3,5 and 8 min. □, basal level of glucose uptake; 0, D-glucose uptake in basal cells treated with $5\mu M$ PhAsO; ●, D-glucose uptake in basal cells treated with $10\mu M$ PhAsO.

Fig 4·3
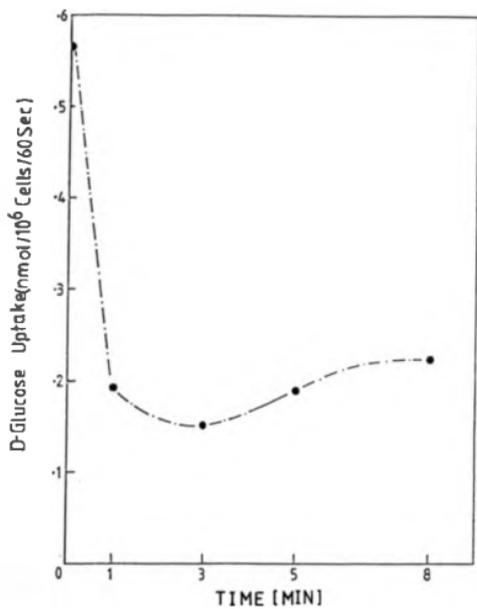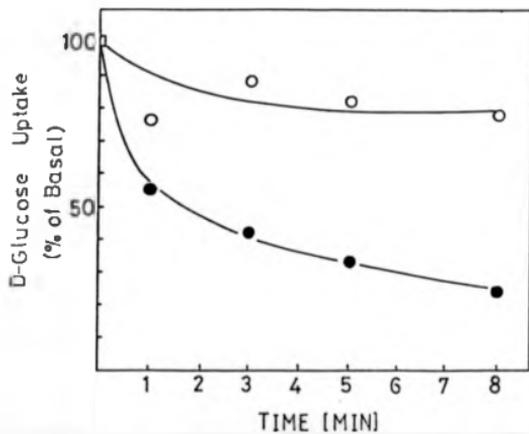
Fig 4.4

The effect of phenylarsine oxide on the stereospecific uptake of D-glucose by rat adipocytes over a short time period at 37°C.

0, Cells treated with insulin (50nM); ●, cells treated simultaneously with insulin (50nM) and PhAsO (10μM). D-glucose uptake (in 60 sec) was measured after specific time intervals.

Fig 4.4
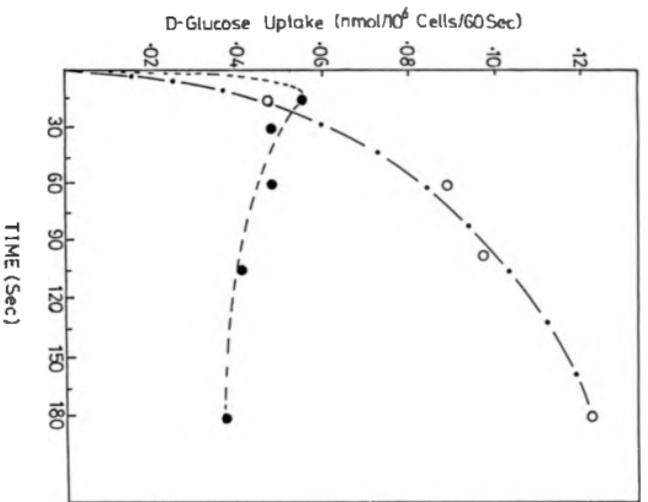
Fig 4.5

The effect of phenylarsine oxide on the stereospecific uptake of D-glucose by rat adipocytes over an extended time period at 37°C.

0, cells treated with insulin, 50nM; ●, cells treated simultaneously with insulin (50nM) and PhAsO (10μM). D-glucose uptake (in 60 sec) was measured after specific time intervals.

Fig 4.5
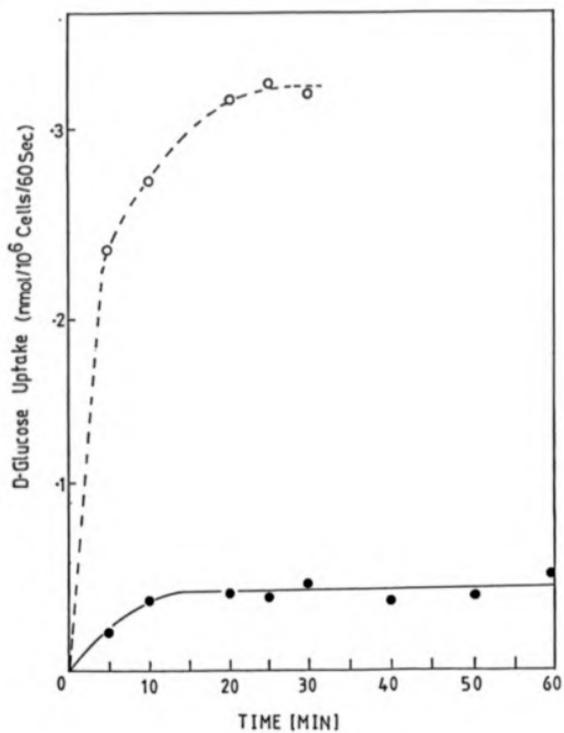
Table 4.1

Comparison of PhAsO and cytochalasin B on the stereospecific inhibition of carrier mediated D-glucose uptake in rat adipocytes at 37°C.

Inhibtor	% Inhibition of stereospecific D-glucose uptake (in 120 sec)	
	Basal	Insulin
Cytochalasin B (25 μM)	96.3 ± .6	99 ± .25
PhAsO (10 μM)	70.5 ± 3.5	87.33 ± 6.5

Results are the mean ± SD of quadruplicate determination.

Fig. 4.6.

The effect of different concentrations of phenylarsine oxide on stereospecfic D-glucose uptake by rat adipocytes at 37°C.

•, Adipocytes treated with insulin (100nM); □, Adipocytes treated simultaneously with insulin (100nM) and PhAsO (5µM); 0, Adipocytes treated simultaneously with insulin (100nM) and PhAsO (10µM). D-glucose uptake (in 60 sec) was measured after specific time intervals.

Fig 4.6
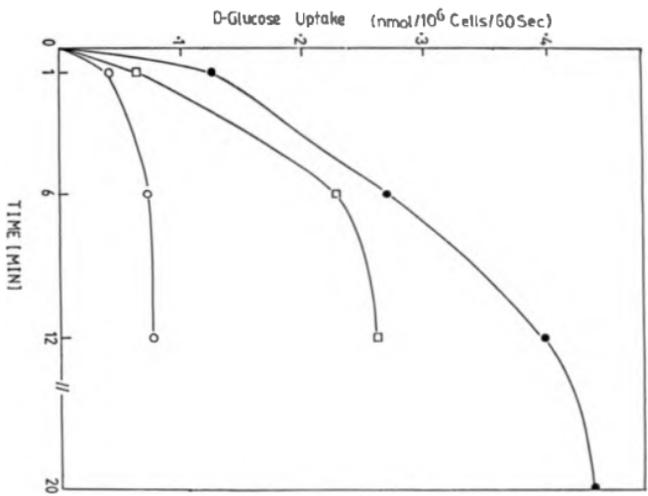
Fig 4.7.a.

The effect of dithiothreitol (DTT) on phenylarsine oxide inhibition of D-glucose uptake by rat adipocytes at 37°C.

O, cells treated with insulin (100nM); ●, cells treated with PhAsO (10μM; 5 min) then insulin (100nM); □, cells treated with PhAsO (10μM; 5 min) then DTT (200μM; 60 min) then insulin (100nM). D-glucose uptake (in 60 sec) was measured after incubation for 1,3,5 and 8 min.

Fig 4.7.b.

The effect of Dithiotheitol (DTT) on stereospecific D-glucose uptake in rat adipocytes at 37°C.

□, Basal level of D-glucose uptake; 0, D-glucose uptake after insulin stimulation (100nM for 20 min); ▲, D-glucose uptake in basal cells treated with DTT (200μM for 60 min).

Fig 4 7
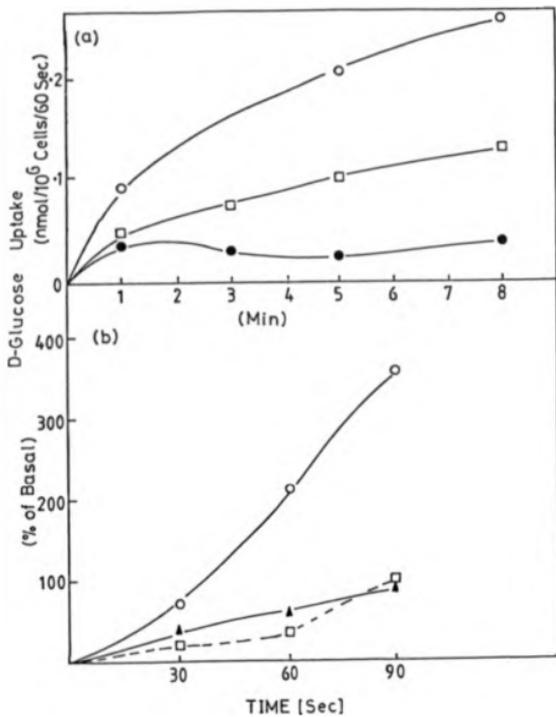
Fig. 4.8.

The effect of different phenylarsine oxide concentrations on the stereospecific D-glucose uptake by insulin stimulated rat adipocytes at 37°C.

Adipocytes were treated with insulin (100nM for 20 min) and D-glucose uptake (in 60 sec was measured). Insulin stimulated cells were then treated with PhAsO (at different concentrations) and D-glucose uptake (in 60 sec) was measured after specific time intervals.

●, Insulin stimulated cells treated with PhAsO (1μM); 0, Insulin stimulated cells treated with PhAsO (5μM); □, Insulin-stimulated cells treated with PhAsO (10μM).

Fig 4.8
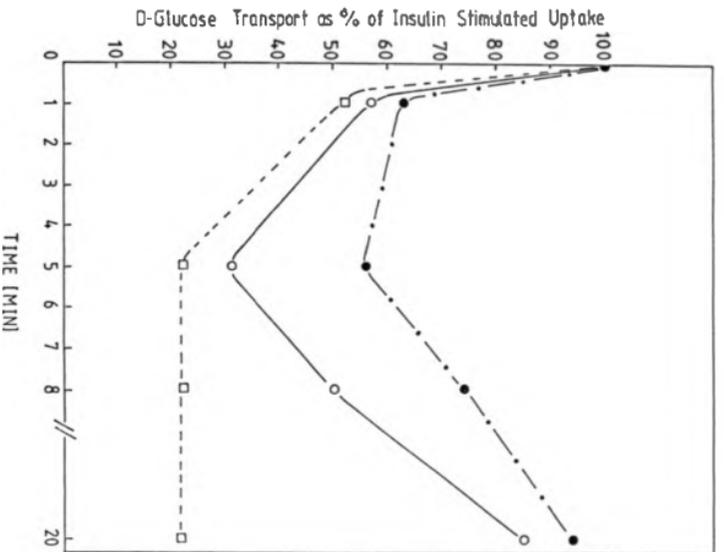
Table 4.2

Recovery of transport activity for PhAsO (1μM) inhibition of stereospecific D-glucose uptake in insulin stimulated cells

nmoles D-glucose uptake (10^6 cells/60 sec)		% Recovery After 20 min
Original activity	Recovered state	
0.47	0.42	89
0.56	0.52	93
0.56	0.41	73
0.35	0.33	94
0.38	0.31	81

Fig. 4.9

Dose response for phenylarsine oxide inhibition of stereospecific D-glucose uptake by adipocytes exhibiting different membrane states at 37°C.

"Receptor-rich" membrane state:

0, D-glucose uptake (in 60 sec) by rat adipocytes treated simultaneously with PhAsO (varying concentrations) and insulin (100nM) for 10 minutes.

"Transporter-rich" membrane state:

●, D-glucose uptake (in 60 sec) by rat adipocytes treated first with insulin (100nM for 10 min) then PhAsO (varying concentrations) for 1 min.

Fig 4.9
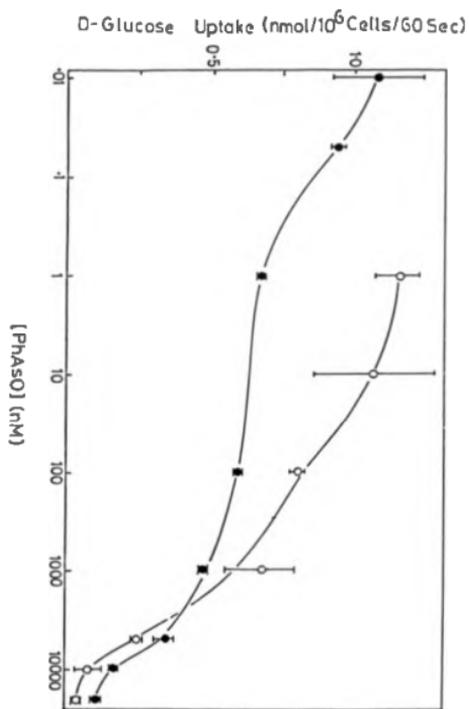
Effect of phenylarsine oxide and monensin on the stereospecific take of D-glucose in insulin stimulated rat adipcocytes at 37°C.

Cells were treated with insulin (100nM) for 20 min, then onensin (25µM) for 20 min, followed by PhAsO (1µM). D-glucose take was measured for 60 sec at specific time intervals after ldition of PhAsO.

Fig 4.10

Effect of phenylarsine oxide and monensin on the stereospecific uptake of D-glucose in insulin stimulated rat adipocytes at 37°C.

Cells were treated with insulin (100nM) for 20 min, then monensin (25μM) for 20 min, followed by PhAsO (1μM). D-glucose uptake was measured for 60 sec at specific time intervals after addition of PhAsO.

Fig 4·10
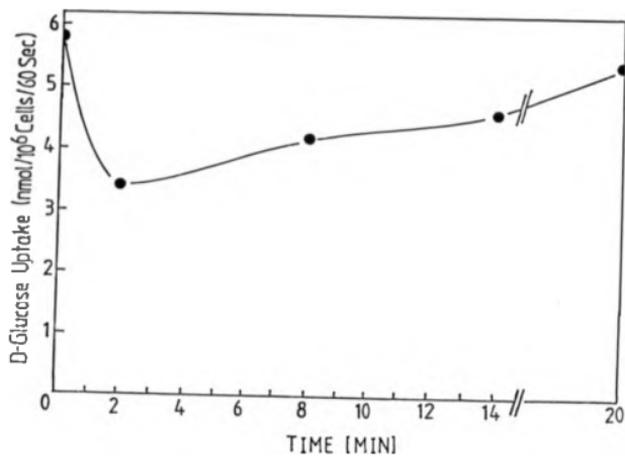
Fig 4.11

Effect of monensin on stereospecific D-glucose uptake by rat adipocytes at 37°C.

a)   Time course for the effect of monensin on stereospecific uptake of D-glucose by rat adipocytes.

0, Basal level of glucose uptake; ■, basal level in the presence of monensin ($25\mu M$ for 20 min); ●, D-glucose uptake after insulin stimulation (100nM for 20 min); □, cells treated with insulin (100nM) for 20 min, followed by monensin ($25\mu M$) for 20 min.

b)   Effect of monensin on stereospecific basal uptake.

Adipocytes were treated with $25\mu M$ or $100\mu M$ monensin for 20 min and D-glucose uptake was measured for 120 sec.

Basal level of D-glucose uptake.

D-glucose uptake after incubation with $25\mu M$ monensin for 20 min.

D-glucose uptake after incubation with $100\mu M$ monensin for 20 min.

Result are then mean ± SD for triplicate determinations.

Fig 4.11
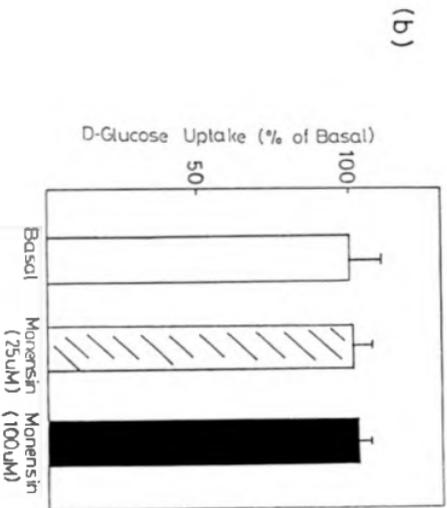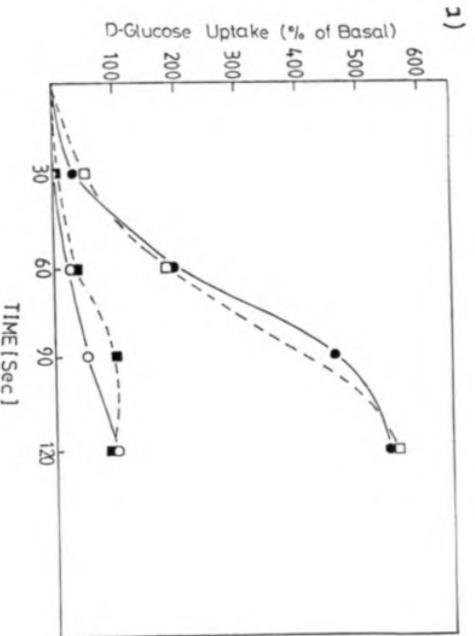
Fig 4.12

Time course for the effect of monensin on insulin stimulation of D-glucose uptake by rat adipocytes at 37°C.

0, D-glucose uptake after insulin stimulation (100nM for 20 min); •, D-glucose uptake in cells treated first with insulin (100nM for 20 min) then with monensin (100 μM for 20 min).

Results are the mean of quadruplicate determinations.

DISCUSSION

PhAsO inhibited stereospecific D-glucose uptake in both insulin stimulated cells and basal state cells (Figs 4.1; 4.3). The rate of uptake inhibition is very rapid being apparent within 30 seconds (Fig 4.4) and the extent of inhibition is dependent on PhAsO concentration (Fig 4.3b; 4.6). The inhibitory effect of PhAsO appears to be permanent since addition of insulin to PhAsO-treated cells did not alter the inhibitory effect of the arsenical (Fig. 4.2) and no recovery of transport activity was observed over extended time periods in insulin-treated PhAsO-inhibited cells (Fig. 4.5). This is consistent with the covalent interaction of PhAsO with sulphydryl groups to form stable dithioarsenite complexes (Wallace & Ho, 1972) and it has been reported that extensive washing of PhAsO treated cells does not reverse its effects (Hertel, et al., 1985).

That PhAsO could inhibit stereospecific D-glucose uptake in both insulin-stimulated and basal cells was in partial agreeement with Frost & Lane (1985) who observed that pretreatment of 3T3-L1 adipocytes in culture with PhAsO markedly inhibited insulin's ability to enhance hexose uptake but failed to observe any inhibitory effect of PhAsO on the basal state, in contrast to observations made here. These investigators suggested that PhAsO inhibition of hexose transport might be due to uncoupling of the transporter activation process rather than any direct inhibition by PhAsO on the hexose transporter or insulin binding to its receptor. However, although Frost & Lane (1985) found no inhibition of basal 2-deoxyglucose uptake in 3T3-L1 adipocytes in culture, other workers in our laboratory have also observed inhibition of 2-deoxyglucose uptake in basal state rat adipocytes suggesting possible differences between isolated and cultured cells.

Nevertheless, the very rapid and dramatic inhibition of insulin stimulated cells by PhAsO (Fig 4.3; 4.8) suggests that this agent might be acting directly on the transporter to inhibit its function. However, PhAsO's ability to depress the basal states (Fig's 4.1; 4.3) might also imply that arsenical is also exerting some inhibitory effect on insulin receptors. Fig. 4.13 illustrates some possible sites of action of PhAsO.

Effects on Metabolism

PhAsO is a hydrophobic molecule, has limited solubility in pure water and it is possible that it may partition into the hydrophobic regions of the adipocytes, such as the cell membranes and fat, and possibly interfere with cell metabolism. Such an occur nce would be relevant to the observations made here. However several lines of evidence suggest that PhAsO does not significantly alter intracellular metabolism at concentrations where it effectively inhibited endocytosis and sugar transport. Firstly high concentrations of PhAsO (18-$100\mu M$) did not alter cellular ATP content in a variety of cell types (Discussed above). In addition, $100\mu M$ PhAsO did not affect the activties of alkaline phophodiesterase, latate dehydrogenase or β-galactosidase when alveolar macrophages were treated with the arsenical for 10 min (Kaplan *et al.*, 1985). Furthermore, Frost & Lane (1985) found that hexokinase activity in digitonin extracts of adipocytes treated with $20\mu M$ PhAsO did not differ from the activity of control cells and work done by other researchers in our laboratory have also indicated that PhAsO (10-$20\mu M$) did not affect hexokinase action.

Rat feeding studies have shown that dietary PhAsO did not affect the levels of serum glutamic-oxalacetic transamine activty, serum

Fig. 4.13

Possible sites of action of PhAsO
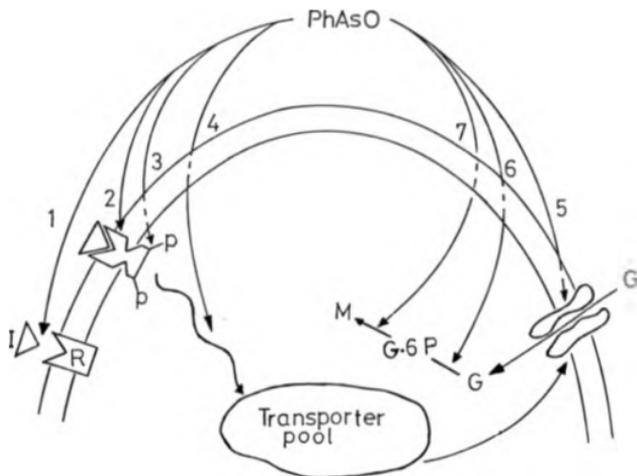
PhAsO might act to inhibit

1. Insulin (I) binding to its receptor (R).
2. Insulin-induced receptor conformational changes.
3. β-subunit autophosphorylation (P).
4. Signal transduction.
5. Transporter (T) function.
6. Hexokinase activity.
7. General cell metabolism (M).

lactic dehydrogenase activity, serum inorganic phosphate, hematocrits, whole blood pyruvate, urinary nitrogen excretion, or gross serum protein profiles examined by electrophoresis (Siewicki & Leffel, 1980). It is not entirely surprising then that Devaskar & Karycki (1985) found that PhAsO did not affect the metabolic activity and cell viability in brain cells while Trowbridge & Draznin (1986) reported that PhAsO *per se* did not affect either the viability of or intracellular proteolysis in hepatocytes. Knutson *et al.*, (1983) showed that although PhAsO ($10\mu M$) totally blocked insulin-induced translocation of receptors into the intracellular compartment in fibroblasts it did not inhibit recyling of already internalized receptors; an energy dependent process (Huecksteadt *et al.*, 1986). Moreover, PhAsO could be used reliably for up to three hours without any cytotoxic effects that might result in the loss of cell viability (Knutson *et al.*, 1983).

Interestingly, Wallace & Ho (1972) observed that internalization of extracellular proteins in amphibian oocytes was markedly inhibited by PhAsO ($10\mu M$) without any apparent cytotoxic manifestations and suggested that the plasma membrane, which is exposed first and to the highest concentrations of the arsenical, is probably the main site of action. With regard to this, electron microscopic examination of PhAsO-treated marocphages ($100\mu M$ for 10 min at 37^oC) revealed no striking abnormalities in membrane integrity or any evidence of cell dissolution (Kaplan, 1985). Fig 4.1b, shows that although PhAsO inhibited sugar uptake into rat adipocytes, uptake re ined stereospecific indicating that membrane integrity was not affected.

It is apparent therefore, that PhAsO does not obstruct intracellular metabolic events. However this arsenical has been shown

to inhibit insulin stimulation of sugar (3-0-methyl-D-glucose and 2-deoxyglucose) transport in 3T3-L1 adipocytes (Frost & Lane, 1985) and since the major rate-limiting step in D-glucose uptake is transport it is assumed that the effects of phenylarsine oxide described here are associated with the mechanism of D-glucose transport and its simulation by insulin as concluded by Frost & Lane (1985).

Effects on Receptors and Transporters

Since it does not seem likely that PhAsO affects intracellular metabolism in such a way as to be responsible for the observed inhibition of hexose uptake in might be reasonable to assume that sulphydryl groups involved in maintaining the structure and function of both the receptors and transporters might be a possible target for modification by PhAsO. The subunits of the insulin receptor complex, $\alpha2\beta2$, are held together by interchain disulphide bridges and strong non-covalent interactions (Massague et al., 1980). In addition two functionally distinct sets of disulphide bonds may be involved in insulin receptor microclustering in the adipocyte plasma membrane (Schweitzer et al., 1980; Jarett & Smith, 1983).

The insulin receptor α subunit contains a large number of cysteine residues (Ullrich et al., 1985) and similar high cysteine regions have also been observed in the primary structures of the human and Drosophila epidermal growth factor receptors. The fact that these regions of high cysteine content are conserved among hormone receptors as well as across diverse species suggests that they play an important role in the function of these receptors (Pike et al., 1986). It has been suggested that in a compact structure a region of high cysteine residues might embody the ligand binding region (Gammeltoft

& van Obberghen, 1986).

Insulin binding to its receptor triggers a conformational change within the α subunit (Czech *et al.*, 1981) and subsequently autophosphorylation of the β-subunit (Gammeltoft & van Obberghen, 1986). The latter has been shown to be requisite for insulin stimulation of glucose transport (Kohanski *et al.*, 1986; Ellis *et al.*, 1986). Recently it has been shown that, insulin activation of the receptor/kinase involves alterations of intramolecular disulphides (Wilden *et al.*, 1986). Also class I disulphide bonds are required for both insulin binding and autophosphorylation (Pike *et al.*, 1986). Further $\alpha\beta$, dimer-dimer interactions appear to be critical to the receptor's intrinsic kinase activity and it has been speculated that the insulin induced receptor conformational changes might facilitate this $\alpha\beta$-$\alpha\beta$ interaction (Boni-Schnetzler *et al.*, 1986). Furthermore, maximal activity of the receptor seems to require a reduced sulphydryl group at or near the active site (Shia *et al.*, 1983).

It has been proposed that the D-glucose transporter contains 12 membrane spanning segments (Mueckler *et al.*, 1985) which forms a pore through which sugars are transported. Transport is facilitated by a conformational change within the transporter (Lowe & Walmsley, 1986; Holman & Rees, 1982) and May (1985) has shown that exofacial sulphydryl groups on the D-glucose transporter are important for the proper functioning of the carrier. The exofacial groups may be required for maximal activity of the transporter and possibly be involved in the regulation of transport rates be insulin.

It is clear therefore, that sulphydryl groups have important structural and functional roles in both receptors and transporter.

Hence the ability of PhAsO to react with vicinal or paired thiol groups suggests that it could react with essential sulphydryl groups in the insulin receptor and the sugar transporter. It does not of course follow that such a reaction would necessarily inhibit receptor or transporter function or exclude the possibility that the inhibitory effect of PhAsO on stereospecific D-glucose uptake results from reaction of PhAsO with some other essential component involved in the mechanism of insulin stimulated sugar transport.

Knutson *et al.*, (1983), Draznin & Trowbridge (1984), Jones *et al.*, (1986) have all indicated that PhAsO does not prevent insulin binding to its receptor and data presented in Chapter 5 concur with this observation in the range upto $10\mu M$ PhAsO but above this level significant inhibition of binding occurs. However, although PhAsO does not inhibit insulin binding it inhibits internalization (Chapter 5) and may inhibit the biochemical functioning of the insulin-receptor complex. Should direct reaction occur between PhAsO and receptors the resulting inhibition is not primarily a consequence of the effect on the extent of insulin binding but on the subsequent functioning of the insulin-receptor complex. The importance of sulphydryl groups in receptor structure and function is evident.

The reducing agent dithiothreitol seems to potentiate the effects of phosphorylation of the insulin receptor/kinase (Pike *et al.*, 1986; Sweet *et al.*, 1986), possibly by releasing structural constraints of the insulin receptor, allowing greater expression of the intrinsic tyrosine kinase activity (Pike *et al.*, 1986). Hence it is posible that PhAsO interaction with sulphydryl groups present within the receptor may present steric constraints which might render the tetramer more rigid thus hindering its ability to undergo or maintain a desirable

conformational change and also inhibit β-subunit phosphorylation. However this remains to be determined.

The inhibitory effect of PhAsO can be partially reversed by reduction with dithiothreitol (DTT) which presumably cleaves the dithioarsinite complex (Fig. 4.7a). The reduction process is primarily associated with effects resulting from prior reaction with PhAsO since 0.2mM DTT has no significant effect on cells not previously treated with PhAsO. Whereas the effects of PhAsO can be reversed by bifunctional sulphydryl agents (DTT; 2,3 dimercaptopropanol), monofunctional sulphydryl drugs (2 mercaptoethanol; glutathione) are incapable of reversing the effects of the arsenical (Bernier et al., 1987; Walker-Smith & Payne, 1983 , 1984; Sanadi et al., 1981). The reversibility of PhAsO action with dithiols is consistent with the interaction of PhAsO with vicinal sulphydryl groups to forms stable ring structures.

Interestingly, Frost & Lane (1985) also observed only partial reactivation of sugar transport upon addition of 2,3 mercaptopropanol (0.2mM) to cells previously treated with PhAsO (20μM) in agreement with Fig. 4.7a. Hence the inability of the reducing agents to effect total reversibility in this system suggests that these dithiols may themselves be acting to disrupt insulin stimulation of sugar transport since DTT did not appear to have any effect on the basal state (Fig. 4.7b).

However, class I disulphide bridges linking the two $\alpha\beta$ halves of the receptor can also be reduced by low concentration of DTT (0.5mM-1mM) (Massague & Czech, 1982; Czech et al., 1981). Hence although the $\alpha\beta$ units remain associated by non-colvent interactions

(Sweet *et al.*, 1986) should the class I disulphide bridges be essential for complete functioning of the insulin receptor as indicated by Pike *et al.*, (1986) then at the concentration of DTT used (0.2mM) only partial recovery of insulin-stimulated D-glucose transport would be expected as is observed.

In addition although DTT has been found to enhance β-subunit phosphorylation in the already "activated" receptor/kinase, both the binding and tyrosine kinase activities of the affinity purified unphosphorylated receptor/kinase were abolished by this reducing agent; DTT inhibited insulin binding 40-50% in human placenta (Pike *et al.*, 1986). Furthermore, data presented in Chapter 5 demonstrates that insulin binding to adipocytes as 15°C is inhibited in a dose-dependent fashion by DTT in the range 0.1 to 1.0mM (inhibition at 0.2mM is approximately 20%), and supports the observations made by Pike *et al.*, (1986). Hence although DTT appears capable of reducing the dithioarsenite complexes formed between PhAsO and sulphydryl groups, its own ability to reduce insulin binding and inhibit receptor phosphorylation (shown to play an important role in hexose transport) might impair complete reversal of the insulin stimulated state.

In addition any PhAsO which has been freed from cleavage of dithioarsenite will still be in the medium and will presumably continue to react with sulphydryl groups within proteins (receptor/transporter etc) and cause some amount of inhibition. A dynamic state might be envisaged whereby an equilibrium is established between the free and complexed sulphydryl groups:

$$
\begin{array}{c}
R \\
HC - S - H \\
| \\
HC - S - H \\
R
\end{array}
\quad
\underset{DTT}{\overset{PhAsO}{\rightleftarrows}}
\quad
\begin{array}{c}
R \\
HC - S \\
| \diagdown \\
 \diagup AsPh \\
HC - S \\
R
\end{array}
$$

Such a situation then would result in only a partial reversal of the inhibited state as seen in Fig. 4.7a. However, because of the presence of a much higher DTT concentration (20-fold) than PhAsO, it is likely that the equilibrium position of such a reaction (above) would favour a shift to the left, whereby considerable amounts of free sulphydryl groups would exist. Hence it might be suggested that it is the disruptive effect of DTT on both insulin binding and receptor tyrosine phosphorylation that might be largely responsible for the partial reversibility observed.

The plasma membrane of adipocytes in the basal state is receptor-rich but contains relatively few transporters. Approximately 90% of the cellular insulin receptors are in the plasma membrane in basal cells (Arsenis *et al.*, 1985; Marshall, 1985a) while only 10% of the cell's transporter are on the surface of unstimulated cells (Simpson *et al.*, 1983). Treatment of basal cells with insulin results in a rapid redistribution of the receptors and transporters between the plasma membrane and the golgi rich fraction. Surface derived receptors are seen within cells as early as 30 seconds and accumulates intracellularly at the rate of ~ 20,000/min (see Fig. 1.3, Chapter 1), resulting in approximately 40% of the receptors being internalised in 6 min. and a new steady state is maintained for up to 2 hours (Marshall, 1985a). In a similar fashion, signal transduction from insulin receptor complexes causes recruitment of hexose carriers such that transporters in the membrane increase 4-5 fold with a corresponding decrease in the intracellular pool (Simpson *et al.*, 1983). Hence D-glucose transport after treatment of basal and insulin-stimulated cells with PhAsO will reflect the effects of PhAsO on the receptor-rich and the transporter-rich plasma membrane respectively.

The dose-response curves (Fig 4.9) in which stereospecific D-glucose uptake in basal and insulin-stimulated cells is shown as a function of PhAsO concentration demonstrate that transport inhibition in cells with receptor-rich (i.e. in cells treated simultaneoulsy with PhAsO and insulin) and transporter-rich (i.e. in cells stimulated by insulin and then inhibited with PhAsO) plasma membranes are not the same at low PhAsO concentrations. That such a distinction can be made suggests that PhAsO may inhibit the functions of both the receptor system and the transporter system directly.

Low doses of PhAsO, 0.01-100nM, caused greater inhibition of uptake in cells with membranes rich in transporters, as compared to cells with receptor rich membranes. If, as suggested by Wallace & Ho (1972), the plasma membrane is the main site of action of the arsenical, then in a transporter-rich membrane a large number of carriers would be available for direct, immediate reaction and possible inhibition by small quantities of PhAsO. However in a receptor-rich plasma membrane the bulk of the transporters are contained in the cytoplasm and might be protected from small insignificant doses of PhAsO. In the latter situation the majority of the cells receptors would be exposed to the inhibitor, so that if the insulin-receptor complex or the biological signal generated from it were the main site of action of the arsenical then one would expect a result in reverse to that of Fig. 4.9, i.e. low doses of PhAsO should be expected to cause a greater inhibition of cells containing receptor-rich plasma membranes.

Alternatively it might be suggested that the inhibition of a limited number of receptors by a small quantity of PhAsO might not be sufficient to cause transport inhibition since it is thought that

only a small percentage of receptors are required for generating maximal effects of insulin, spare receptor hypothesis (Gammeltoft & Gliemann, 1973; Kono & Barham, 1971). Additionally it might be considered that if both receptors and transporters were equally inhibited then one might anticipate the identical dose response curves regardless of the membrane state (receptor-rich or transporter-rich) or PhAsO concentration.

Such is the case at high PhAsO concentration, 5 to $20\mu M$, where saturation of the glucose transport system seems to be occuring. Though an entirely accurate assessment of a possible inhibitory effect of PhAsO on receptors or transporters cannot be ascertained from Fig. 4.9, there is an indication that this agent might be impeding transporter function rather than receptor activity but does not exclude the possibility that PhAsO also reacts with a component of the coupling system between receptor and transporter.

PhAsO does not inhibit insulin binding (Chapter 5) and it has been reported that neither basal nor insulin activated autophosphorylation of partially purified triton X-100 solubilized receptors was affected by prior incubation with PhAsO ($20\mu M$) (Frost & Lane, 1985). In addition PhAsO does not impair the function, i.e. ligand induced desensitization, of the β-adrenergic receptors even though receptor internalization was completely blocked (Kassis *et al.*, 1986; Hertel *et al.*, 1985; Feldman *et al.*, 1986). It is likely therefore, that PhAsO exerts its inhibitory effect on hexose uptake at a site distal to the insulin receptor.

Mediators which have been proposed for insulin action include Ca^{2+}, through inhibition of membrane bound ($Ca^{2+} + Mg^{2+}$)- ATPase

(McDonald *et al.*, 1982; Pershadsingh & McDonald, 1984), G-proteins (Rapiejko *et al.*, 1986; Houslay *et al.*, 1986; Rodbell, 1985), degradation products of inositol phospholipid hydrolysis through the activation of phospholipase C (Goldman *et al.*, 1986; Saltiel *et al.*, 1986) an uncharacterised plasma membrane glycoprotein (Hayes & Lockwood, 1986) and cell surface thiol/disulphide groups (Morgan *et al.*, 1985). It is interesting that these proposed intermediaries are all associated with the plasma membrane and hence may be susceptible to rapid interaction with PhAsO. Although it is conceivable that the arsenical might interact with and inhibit one of these systems, the simplest interpretation of the data would be to assume that inhibition by PhAsO results from direct reaction of PhAsO with the transporters.

Direct action with transporters would result in inhibition of basal transport as well as insulin-stimulated transport. Table 4.1 compares the inhibitory effect of PhAsO on the uptake of D-glucose with cytochalasin-B, an acknowledged inhibitor of the glucose carrier. Although PhAsO caused significant inhibition of sugar uptake in both basal and insulin stimulated states, the extent of inhibition was noticeably less than that induced by cytochalasin-B which caused almost total inhibition of transport.

If one assumes that like cytochalasin B, PhAsO exerts its inhibitory effect through the direct interaction with the transporter, then the inability of the arsenical to cause maximal inhibition of the transporter may be a reflection of the broad specificity of this agent. That is, PhAsO would react with vicinal sulphydryl groups within any protein present in the medium, whereas cytochalasin B reacts specifically with the transporter resulting in greater inhibition of transport activity. Additionally, cytochalasin B rapidly penetrates

the plasma membrane and will combine with gluclose transporters regardless of their cellular location. However, if as suggested by Wallace & Ho (1972), the primary site of action of the arsenical is the plasma membrane then it is likely that essentially membrane bound transporters might be affected leaving some carriers within the cell unaltered.

Even if PhAsO does penetrate the cell its broad specificity will probably render some percentage of transporters impervious to its action. Further, PhAsO inhibits protein internalization but has no effect on exocytosis (Knutson *et al.*, 1983) and since many integral membrane proteins have been found to recycle at a finite basal rate, 50-200% of the plasma membrane surface area internalized per hour, even in the absence of a stimulus (Mellman, *et al.*, 1986) then some amount of cycling of uninhibited intracellular transporters from the Golgi region to the plasma membrane might still occur giving rise to a small but persisitent amount of stereospecific D-glucose uptake in PhAsO treated basal or insulin stimulated cells (Table 4.1; Figs 4.1 - 4.5). However it is not clear why basal transport is inhibited in rat adipocytes (Table 4.1) but not in 3T3-L1 adipocytes (Frost & Lane, 1985). It is possible that cultured adipocytes may possess a lower efficiency for membrane exhange than rat adipocytes, however this remains to be determined.

The effect of PhAsO on the transport system is more clearly demonstrated, when insulin-stimulated adipocytes are treated with 1,5 and $10\mu M$ PhAsO (Fig. 4.8) significant inhibition of glucose tranpoprt occurs after 1 min. If PhAsO was reacting with and inhibiting the insulin receptor or a component of the coupling system between the receptors and transporters then a slow, time-dependent decrease of

transport activity should occur upon treatment of insulin-stimulated cells with PhAsO. This however does not occur and addition of PhAsO to insulin-stimulated adipocytes results in a large rapid fall in transport activity suggesting direct transporter inhibition.

Interestingly, at low PhAsO concentrations, transport recovers after inhibition. The extent of inhibition and subsequent recovery is dependent on the PhAsO concentration. The inhibition observed after 1 min for all three PhAsO concentrations (1,5 and $10\mu M$) is very similar (Fig. 4.8) and probably indicates that a fixed amount of transporter in the membrane is inhibited. With increasing time the higher PhAsO concentration, $10\mu M$, appears to be capable of saturating the transport system resulting in increased inhibition and little or no recovery in observed (Fig. 4.8, 4.3b). At low PhAsO concentrations, 1 and $5\mu M$, recovery of transport activity is of the order of 90% after 20 min (Fig 4.8; Table 4.2).

Transport recovery could be due to internalized receptors, protected from PhAsO, recycling to the plasma membrane, binding insulin, and causing recruitment of latent carriers. This proposal is not unreasonable since PhAsO had been found to effectively inhibit internalization of insulin-receptor complexes in fibroblasts, without preventing recycling of previously internalized receptors (Knutson et al., 1983). Further, although mechanisms for membrane retrieval between various intracellular compartments and the plasma membrane are in place to ensure the balance in membrane flow, acidtropic agents, which inhibit exocytosis of internalized membrane receptors, have been shown to cause a massive redistribution of membrane into the cells; more than half of the cell surface is internalized after addition of acidtropic bases (Mellman et al., 1986).

So that given PhAsO's ability to inhibit internalization of extracellular proteins (Wallace & Ho, 1972; Walker-Smith & Payne, 1983) without affecting exocytosis (Knutson, 1983) it is probable that PhAsO might impose a situation opposite to that created by acidtropic agents whereby inhibition of endocytosis may result in a massive redistribution of membrane to the cell surface. Such an occurence has been described in rabbit alveolar macrophages whereby PhAsO treatment resulted in a two to three fold increase in surface receptor activity for macroglobulin-protease complexes, diferric transferrin, and mannose terminal glycoproteins (kaplan et al.. 1985).

A similar event in rat adipocytes would allow recovery of transport activity at low PhAsO concentraion and the presence of extracellular insulin may expedite the translocation of carriers. In addition since PhAsO inhibits protein internalization and it is also capable of directly impeding transporter function, as suggested by its ability to cause a marked rapid decline in insulin-stimulated transport activity (Fig. 4.3, 4.8), then the initial group of inhibited carriers would remain membrane bound and hence the new carriers must arise by the recruitment mechanism (Karnieli et al.. 1981; Kono et al.. 1981). One possible model for the sequence of events from basal cells through insulin-stimulated to PhAsO inhibited cells and finally to the transport recovered state is schematically shown in Fig. 4.14. Since low PhAsO concentrations permit recovery whereas high concentrations do not, it is possible that high concentrations may saturate the total cell transporters possibly by penetrating the membrane and acting intracellularly or by inhibiting "new" carriers as they are recruited to the membrane, while low concentrations of PhAsO may possess limited activity, inhibiting only the initial plasma membrane bound

Fig 4.14.

Schematic model for the recovery of insulin-stimulated stereospecific D-glucose transport in rat adipocytes following phenylarsine oxide inhibition.

◊ = Receptors;　　○ = Transporters;　　─□ = PhAsO; ◄ = Insulin

1)　Basal cell; the plasma membrane contains numerous receptors but relatively few transporters.

1)→2)　Redistribution of receptors and transporters between the plasma membrane and the intracellular storage sites under the influence of insulin.

2)→3)　Inhibition of the surface bound transporters and receptors.

Internalized receptors and transporters protected from PhAsO (at low concentrations).

3)→4)　Recovery process - internalized receptors recycling to the membrane binding insulin and transmiting signals to latent transporters

Fig 4.14

(1) Basal

(2) Stimulated

Insulin(▼)

PhAsO(▷-) (1 uM)

(4) Recovered

(3) Inhibited

D·G
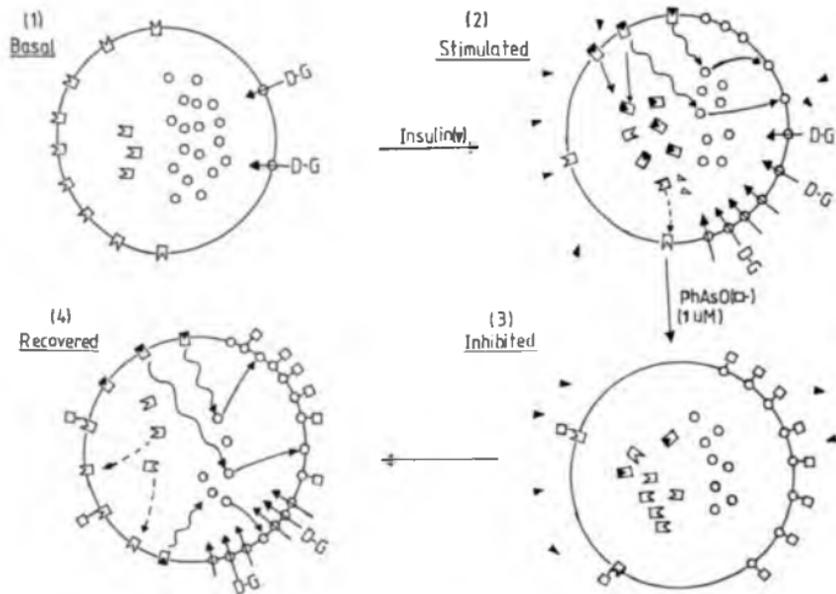
transporters. In any event the internalized transporters (and receptors) certainly seem to be protected against PhAsO at low concentrations.

Recently Whitesell & Abumrad (1985, 1986) proposed that apart from causing recruitment of intracellular transporters insulin also significantly lowers the K_m of the carrier for D-glucose (from 75mM to 8mM). It is probable then that PhAsO might transiently inhibit modulation of the transporter affinity by insulin which might result in temporary inhibition of transport activity. However since PhAsO acts covalently this concept seems unlikely. Additionally should the focus of PhAsO be the as yet undefined mechanism of coupling between insulin-receptor complexes and transporters the effect of PhAsO might be reversed as PhAsO diffuses into other hydrophobic domains such as the fat. However, since low PhAsO concentrations permit recovery whereas high concentrations do not it is apparent that the site or sites of action of PhAsO are saturable.

Interestingly it has been proposed that thiol/disulphide groups on the cell surface and those immediately inside the cell membrane may be critical in the mediation of insulin action and additionally intracellular thiol (glutathione) concentrations may be important for the maintenance of full expression of some of insulin's biological activities (glycogen and lipid synthesis) (Morgan *et al.*, 1985). Although these researchers did not investigate the importance of these thiol/disulphide groups in hexose transport, should they prove to be essential, then it is possible that PhAsO may interfere with these thiol/disulphide groups. It might also be suggested, that transient inhibition of transport activity observed at low PhAsO concentration might be due to reversal of the PhAsO effect with an intracellular dithiol since glutathione (a monothiol) would be incapable of reversing

PhAsO action.

Although there are many similarities between the experiments shown here and that described by Frost & Lane (1985) for the effect of PhAsO on sugar transport, such as inhibition of insulin-stimulated hexose uptake, the rapidity with which the arsenical works and the partial reversibility with dithiols, two fundamental differences were observed: the inhibition of basal uptake by PhAsO observed here and not found by Frost & Lane (1985) and more importantly the observed effects of PhAsO on insulin stimulated cells. Whereas Fig. 4.8 shows a rapid marked decrease in glucose uptake that is apparent within 1 min of addition of PhAsO to insulin-stimulated adipocytes, Frost & Lane (1985) observed a slow time dependent decrease in transport activity for a similar experiment using 3T3-L1 adipocytes in culture. The latter found that the transport activity gradually approached the basal state but even after one hour the level of sugar uptake in PhAsO treated insulin-stimulated cells was still notably higher than basal cells. This effect seemed to parallel the withdrawal of insulin from the medium and hence these researchers concluded that PhAsO does not cause any direct inhibition of the glucose carrier but instead inhibits some component of the coupling system between the receptors and transporters. In a subsequent publication from the same laboratory (Bernier et al., 1987) these investigators observed that insulin stimulates phosphorylation of a tyrosine residue(s) on a 15kDa cytosolic protein only when 3T3-L1 adipocytes were treated with PhAsO. Hence they proposed an intermediary signalling role for the phosphorylated 15kDa protein in insulin-stimulated glucose uptake.

On examination of the system used here and that used by Frost & Lane (1985) and Bernier et al., (1987) two differences are evident:

The hexose used in the experiments (D-glucose as opposed to 2-deoxyglucose) and the cells used (isolated rat adipocytes as opposed to 3T3-L1 adipocytes in culture). Could the differences observed in the data be due to one or both or these factors? D-glucose is rapidly transported across the plasma membrane where it is converted first to glucose 6 phosphate and then undergoes substantial intracellular metabolism. Although transport remains rate limiting for metabolism (Crofford & Renold, 1965; Whitesell & Abumrad, 1985) and D-glucose has been used as a measure of transport (More & Jones, 1983; Whitesell & Abumrad, 1985) it was considered whether PhAsO might in some way affect metabolism thereby yielding peculiar results. However several lines of evidence (Discussed above) strongly indicated that PhAsO does not obstruct intracellular ATP production, general cell metabolism and cell integrity. Additionally it was decided to repeat some of these D-glucose uptake experiments using 2-deoxyglucose. Indeed work done by other researchers in our laboratory using 2-deoxyglucose (used by Frost & Lane, 1985) in isolated rat adipocytes yielded almost identical results to that shown here. In particular, PhAsO treatment to insulin-stimulated cells resulted in a rapid decrease in 2-deoxyglucose transport that was apparent within 1 min of treatment with the arsenical in contrast to that obtained by Frost & Lane (1985). Hence it appears that although cultured adipocytes and isolated rat adipocytes share common similarities there may also be some differences.

It might be speculated that the transport system of adipocytes in culture may not be as well defined as that of freshly isolated rat adipocytes. It is interesting that whereas Frost & Lane (1985) reported that insulin acutely activates hexose transport 15- to 20-fold, Kohanski, Frost & Lane (1986) reported only 7 fold increase in

transport activity in insulin stimulated 3T3-L1 adipocytes. Such a discrepancy may reflect the inconsistency of the transport system in this cell type.

The data presented here suggests that PhAsO most probably combines with and directly inhibits the D-glucose transporter possibly by reacting with sulphydryl groups at or near the transport site. It is also apparent that a significant fraction of transporters remain unexposed to the extracellular medium after insulin treatment and the sulphydryl groups on the transporter are critical for glucose uptake as found by May (1985). Recent work done by others in our laboratory have shown that PhAsO inhibits cytochalasin B binding in rat adipocyte plasma membrane preparations, further indicating that this arsenical interacts directly with the hexose carriers and hence could possibly prevent sugar transport. Of interest also, is the observation that PhAsO can inhibit both the carrier mediated transport systems for peptide transport in the scutellum of germinating barley embryos (Walker-Smith & Payne, 1984) and pyrimidine nucleoside transport in germinating pollen (Kamboj & Jackson, 1985) suggesting a possible general involvement of vicinal sulphydryl groups in transporter function.

The effect of PhAsO on receptors is less well established and it would be interesting to thoroughly investigate PhAsO affects on receptor phosphorylation. While the results can be explained in terms of the recruitment mechanism the action of PhAsO on the coupling mechanism between receptors and transporters cannot be understood until the transduction process itself and any "second messengers" involved are more clearly defined.

Effects of Monensin/PhAsO

Following internalization, insulin-receptor (I-R) complexes are found localised within endosomes/CURL (Carpentier *et al.*, 1986 a,b) and acidification to pH 5.0 occurs almost immediately after endocytosis in these organelles (Tycko & Maxfield, 1982). The acidic environment of endosomes facilitates receptor ligand dissociation and subsequently recycling of receptors occurs while the ligands undergo lysosomal degradation. (See Fig. 1.2, Chapter 1) (Geuze *et al.*, 1983, 1984; Willingham & Pastan, 1985). Alkalinization of the endosomal compartment with acidtropic agents (chloroquine, dibuciane Tris, NH_4Cl) and carboxylic ionophores (monensin, nigercin, X537A) prohibits dissociation of hormone-receptor (H-R) complexes with a resulting accumulation of these complexes within the cells. Monensin ($<10\mu M$) is effective in raising the pH of endocytotic vesicles above 6.0 within 1-2 min (Maxfield, 1982). Furthermore, it has been shown that addition of monensin to hepatocytes after receptor ligand dissociation has occured results in rebinding of ligand molecules (Wolkoff *et al.*, 1984).

Recently Huecksteadt *et al.*, (1986) observed that although monensin markedly inhibited recycling of I-R complexes in rat adipocytes, covalently coupled complexes were capable of recyclying in the absence of monensin indicating that dissociation of internalized I-R molecules is not a requisite for receptor recycling and that agents that collapse intracellular pH gradients inhibit recycling of H-R complexes by a mechanism other than prevention of ligand dissociation. In any event, whatever the mode of action, monensin inhibits recycling of not only H-R complexes, but also free receptors within intracellular vesicles (Wileman *et al.*, 1984, Mellman *et al.*, 1986).

The combined action of insulin, monensin and PhAsO was employed to further investigate the mechanism of transporter recruitment.

I-R complexes are rapidly internalized and a new steady state of receptor distribution is attained within 6 min such that approximately 40% of total cell receptors is localized within the cell (Marshall 1985a). Hence treatment of insulin stimulated cells with monensin should lead to intracellular entrapment of : I-R complexes already within the cell, new I-R molecules being continually internalized through insulin-induced receptor mediated endocytosis, and free insulin receptors awaiting transition to the plasma membrane. Since signal transduction is thought to originate from plasma membrane bound I-R complexes (Cheng & Larner, 1985) then one might expect that reduction in the number of cell surface receptors might impair or reduce the magnitude of the signal and consequently the response.

However treatment of insulin stimulated cells with monensin (25-100 μM) for 20 min did not inhibit D-glucose uptake (Figs. 4.11a, 4.12). This suggests that, in the case of insulin stimulated cells either: (a) the hexose carriers initially sequested to the surface are still present and functional in the membrane 20 min after the addition of monensin, (b) the internalized complexes are still biologically active, (c) the initial signal generated by the I-R complex is independent of internalization of the complex and is still stimulating the system or (d) changes in the cytoplasmic ionic environment caused by the ionophore may affect other ion concentrations; both hyperpolarization (Marunaka, 1986; Zierler & Rogus, 1980) and Ca^{2+} (Bihler *et al.*, 1985b; Tyalor *et al.*, 1979) have been suggested to be involved in the activation of glucose transport.

Although elevation of intracellular Ca^{2+} has been found to enhance sugar uptake (Chapter 3, Section b), monensin (25-100μM) did not have any effect on the basal state Fig. 4.11b. A small increase in transport activity at 100μM monensin was observed in insulin stimulated adipocytes (Fig 4.12), however whether this is due to changes in the ionic environment is uncertain. Nevertheless, because monensin does not potentiate transport activity in the basal state (Fig 4.11) it seems unlikely that any monensin-induced ionic fluxes participate in the observed transport enhancement (Fig 4.12).

Addition of PhAsO (1μM) to monensin treated insulin-stimulated adipocytes resulted in a marked inhibition of D-glucose transport within 2 min in accordance with Fig. 4.8. Intriguingly the transport activity of monensin treated cells recovers to approximately 90% of its original activity in 20 min. This sequence of events is extremely similar to that obtained for adipocytes treated with low doses of PhAsO in the absence of monensin and indicates that the presence of monensin does not weaken the biological signal. Additionally, although monensin impairs receptor recycling (Huecksteadt *et al.*, 1985 Carpentier *et al.*, 1986,b) it does not prevent transporter translocation (Ezaki *et al.*, 1986). So that since PhAsO apparently acts rapidly to inhibit the plasma membrane bound transporters (Fig. 4.3; 4.8) the recovery of transport activity in the presence of monensin probably arises from recruitment of intracellular carriers. But since recycling of insulin receptors is impaired by monensin then it seems feasible that the internalized I-R complexes might still be biologically active.

Interestingly Jeffrey *et al.*, (1985) observed that intracellular entrapment of insulin using acidtropic agents did not inhibit activations of glucose transport, acetyl-CoA carboxylase or pyruvate

dehydrogenase and suggested that insulin induced signals stimulating these processes are produced at an early stage of the interaction of insulin with the adipocytes, prior to the formation of the endosome. In addition it has been found that regulation of surface receptors continued after the removal of insulin, indicating that insulin generates a signal which can sustain receptor regulation in the absence of the ligand (Marshall *et al.*, 1984). Taken together, it might be suggested that upon inhibition of surface transporters by PhAsO, the continued presence of the initial signal might facilitate recovery of transport.

Of interest also is the observation that although monensin substantially inhibits receptor recycling its action is not absolute and a small amount of recycling does occur (Huecksteadt *et al.*, 1986, Mellman *et al.*, 1986). Whether this is linked to the ability of some I-R complexes to undergo retroendocytosis through an acidtropic insensitive pathway (Marshall, 1985b) is not clear. So that since some receptors do cycle in the presence of monensin it is possible that on returning to the surface membrane they may rebind insulin, still present in the medium, and effect a biological signal(s).

However because of the restriction imposed by monensin on receptor recycling coupled with the inhibitory effect of PhAsO on the glucose carriers one might expect any recovery of transport activity to be somewhat slowed. This does not appear to be the case; compare Fig. 4.8 and 4.10.

Ueda *et al.*, (1985) determined that monensin induced intracellular accumulation of I-R complexes in rat adipocytes by blocking acidification of endocytotic vesicles and that the accumulated

complexes retain a weak, but significant, capacity to stimulate both glucose transport and phosphodiesterase activity. This proposal is in some agreement with the observations made here although the rapidity with which recovery occurs after PhAsO treatment in the presence of monensin suggests that a relatively strong signal is still operative in the system. Hence in view of the current recruitment hypothesis (Kono *et al.*, 1981, Karnieli *et al.*, 1981) and the reported biological effects of monensin, it might be suggested that the internalized I-R complex may continue signal transduction.

CHAPTER 5

INTRODUCTION

The following experiments were designed to further investigate the observations made earlier for the effects of PhAsO, dithiothritol (DTT) and monensin on D-glucose uptake. The approach used here was to explore the effects of these agents on insulin binding to rat adipocytes and adipocyte plasma membranes, under various conditons in order to gain a closer insight into the mechanism of action of the insulin receptor and the activation of hexose transport.

RESULTS

Fig. 5.1 shows a time course for the binding of insulin to adipocytes at 15°C. Non-specific binding was determined in the presence of high excess could insulin ($10 \mu M$). Within the first half hour binding increased rapidly. In the next 30 min a state of equilibrium binding was approached and binding appeared to be fully equilibrated after one hour in accordance with Jones et al., 1986. On this basis binding was routinely measured after a 60 min incubation period, unless otherwise stated.

The temperature dependence of insulin binding to rat adipocytes is shown in Fig 5.2a. Adipocytes were preincubated at the specified temperature (15°C, 22°C, 30°C, 37°C) for one hour prior to the addition of "hot" iodoinsulin, after which insulin binding was monitored over a two hour time period. After 10 min there was no significant difference in the quantity of radiosotope associated with the cells at different temperatures, however with increasing time a dramatic reduction in insulin binding was observed at high temperatures, 30°C and 37°C, while a marked increase in binding was obtained at lower temperatures, 22°C

and 15°C. Such a display of temperature sensitivity of insulin binding to rat adipocytes probably reflects the extent of membrane activity at high and low temperatures i.e. continuous endocytosis and hence ligand destruction occuring at high temperatures as opposed to limited internalization/ligand-destruction at low temperatures. Fig 5.2b shows a thorough time course of insulin binding at 20°C, a temperature shown to mimic insulin stimulation of hexose transport (Fig 3.4, Chapter 3). Insulin associates rapidly with adipocytes at this temperature reaching a maximum at 40 min, after which loss of cell associated radioactivity occurs, thus suggesting that at 20°C some amount of endocytosis is still occuring.

Fig. 5.3 shows the time course for the binding of insulin to adipocytes in the absence and presence of PhAsO (10μM) at 15°C and 37°C. It is clear that PhAsO does not have a marked effect on insulin binding at concentrations upto 10μM at 15°C where the rate of internalization is slow, whereas at 37°C the inhibition of internalization by PhAsO is clearly seen. Insulin binding as a function of PhAsO concentration at 15°C is illustrated in Fig 5.4. PhAsO within the concentration range 0.01 to 10μM, did not alter insulin binding to adipocytes, however at concentrations exceeding 10μM a significant decrease in binding occurs. Of interest is the ability of PhAsO (>10μM) to inhibit the "apparent" insulin binding, while at the same time increasing the non-specific binding (Fig 5.4a). Hence the actual inhibition (Fig 5.4.b) is much greater than it initially appears. The maximal inhibitory effect of the arsenical on insulin binding was attained at a concentration of 100μM.

Since the data shown in Fig. 5.4 was obtained in the presence of a low insulin concentration (39pM) it was considered whether higher insulin

concentrations might induce some change within the receptor (conformational or otherwise) and possibly override PhAsO inhibition on binding. Fig. 5.5 shows insulin binding to adipocytes as a function of PhAsO concentration at 15°C in the presence of 25nM insulin containing tracer amounts of hot ^{125}I-iodoinsulin. Again low concentrations of PhAsO did not alter insulin binding, while high PhAsO concentrations (50 to 100μM) reduced binding and also increased the non-specific attachment of the radioisotope resulting in marked inhibition of insulin binding.

The binding of insulin at 15°C to isolated rat adipocytes plasma membranes from basal and insulin-stimulated cells, in the presence or absence of PhAsO (10μM) is shown in Fig. 5.6. This concentration of PhAsO did not inhibit binding in isolated adipose cells (Fig. 5.3, 5.4) and although substantial amounts of ^{125}I-iodoinsulin binds to PhAsO-treated plasma membranes a small but notable decrease in binding was observed in the presence of the arsenical.

Fig. 5.7, 5.8., 5.9 and 5.10 show the binding isotherms for insulin binding to adipocytes in the absence or presence of PhAsO (10μM, 25μM and 40μM) respectively. In these experiments the curves obtained by Klotz analysis (Klotz, 1982) did not turn over about an expected inflection point thus indicating that saturation binding was not attained. In any event the corresponding Scatchard plots appeared linear and were used to obtain the binding constants and extrapolated to give the total number of binding sites. The binding constants and the receptor number per cell for the Scatchard analysis is summarized in table 5.1. The number of binding sites per cell was ~1.5 x 10^5 and was comparable to the 1.68 x 10^5 sites obtained for swine adipocytes (Etherton and Walker, 1982). Quite a large variation exists in the literature for the binding

constant of the insulin receptor (see Jones *et al.*, 1986 for references). The value obtained here was 0.102 x 10^9 M^{-1} and compares with 0.33 x 10^9 M^{-1} obtained for insulin binding at 15°C to rat adipocytes (Jones *et al.*, 1986).

Dithiothreitol (DTT) has been found to partially reverse PhAsO inhibition of D-glucose uptake in rat adipocytes (Fig 4.7 Chapter 4). Hence it was considered whether DTT might be capable of reversing, either partially or completely, PhAsO's inhibitory effect on insulin binding. Fig 5.11 shows the effects of both PhAsO and DTT on insulin binding to rat adipocytes at 15°C. High PhAsO concentrations inhibited insulin binding as expected and in accordance with Figs 5.4 and 5.5, however quite unexpectedly DTT also inhibited insulin binding in a dose dependent fashion between 0.1 and 1.0mM in contrast to previously reported effects of DTT on insulin binding in isolated rat adipocytes plasma membranes (Schweitzer *et al.*, 1980). A more thorough dose response for the action of DTT on insulin binding, Fig 5.12, clearly demonstrates this inhibitory effect of DTT on insulin binding. Almost complete inhibition of binding, ~96% is attained at 2mM DTT concentration. It is notable that the combination of both DTT and PhAsO caused some reversal of the inhibitory effect of both these agents on insulin binding (Fig 5.11)

Dose response curves for the effects of PhAsO on D-glucose uptake in basal (receptor-rich) and insulin-stimulated (transporter-rich) adipocytes have been shown to be dissimilar at low PhAsO concentrations (< 1µM) (Fig. 4.9, Chapter 4). The basis for such an experiment relies on published data that insulin induces a cellular redistribution of both receptors and glucose transporters such that a new steady state of distribution of these proteins is attained. A number of researchers have

demonstrated insulin induced transporter translocation (Table 3.2, Chapter 3) while Marshall (1985a), and Arsenis *et al.*, (1985) have reported quntitatively on insulin induced receptor internalization in rat adipocytes.

Nevertheless, it was decided to conduct experiments to clarify whether insulin treatment of adipocytes does induce and maintain a new steady state of receptor distribution. Such a demonstration would lend further support to the proposals made for Fig 4.9. An acetic acid wash technique similar to that used by Draznin *et al.*, (1984) and Trowbridge and Draznin (1986) for removing cell-surface ^{125}I-iodoinsulin in rat hepatocytes was initially employed here.

Fig 5.13 shows the effects of acetic acid (0.2M) in 0.5M NaCl, on the removal of cell associated iodoinsulin. Adipocytes were allowed to bind ^{125}I-insulin for 30 min. The Krebs-Henseleit buffer, in which the cells were incubated, was removed and replaced with an acetic acid (0.2M)/NaCl (0.5M) solution, pH2.5. After specificed times either: a) adipocytes were retrieved directly from the acid medium and counted in order to determine the efficiency of hormone stripping or b) cells were washed free of the acid medium, with two consecutive washings with Krebs-Henseleit buffer (pH7.4), and adipocytes were further incubated with ^{125}I-iodoinsulin and the amount of insulin rebound after 30 min was determined.

Although it appeared that acetic acid was quite capable of removing insulin from the cell surface rebinding of iodoinsulin to acid washed cells appeared very erratic. Indeed a batch of acid washed control cells showed a much greater increase in binding than normal unwashed cells. This indicated that the acid solution was affecting the integrity of the cell membrane. Closer invesigation of this technique, Fig. 5.14, revealed

that acetic acid treatment of adipocytes caused a substantial increase in non-specific binding. A 10 min exposure of adipocytes to acetic acid (0.2M) pH2.5, resulted in an enormous 27-fold increase in binding as compared to control non-specific binding levels, further demonstrating the inadequacies of this technique for stripping bound insulin from rat adipocytes.

By trial and error a reliable acid wash procedure was developed for removing plasma-membrane bound insulin using less severe conditions. A low pH glycine HCl buffer (50mM glycine/0.15M NaCl, pH 3.5 for 5 min) was found to be quite effective for stripping insulin from the cells (Fig. 5.15). As shown in Fig. 5.15a, there was no significant difference between insulin binding at 15°C in control (unwashed) or acid-washed adipocytes, while the glycine/HCl solution was completely effective in stripping bound insulin from the cells. It is evident that this more moderate low pH medium did not increase non-specific binding. Furthermore, incubation of acid washed cells with ^{125}I-iodoinsulin at 15°C resulted in complete rebinding of insulin (Fig. 5.15.b).

This improved method was used to ascertain the extent of insulin-induced receptor redistribution in adipocytes. Transference of insulin-stimulated adipocytes (100nM for 20 min at 37°C) to a low temperature bath (15°C for one hour) followed by acid washing and binding of iodoinsulin to resuspended cells resulted in a marked decrease in the amount of cell associated radioisotope (Fig 5.16). The binding capacity of insulin-stimulated adipocytes was ~63% that of the basal cells suggesting that ~37% of the surface receptors were internalized upon insulin treatment.

To further investigate the state of receptor distribution in basal

and insulin stimulated rat adipocytes binding experiments were conducted on plasma membranes prepared from these cells. Fig. 5.17 show binding isotherms for insulin binding to plasma membranes. Scatchard analysis revealed a substantial degree of concavity in both basal and insulin-stimulated membranes in contrast to those obtained for whole cells (Fig. 5.7) while a plot of the insulin bound versus the log of the free insulin, did not yield a complete sigmodal curve for the free insulin concentrations covered.

A time course for the effect of monensin ($25 \mu M$) on insulin binding to rat adipocytes at $37^\circ C$ is shown in Fig 5.18. This carboxylic monovalent ionophore is thought to deacidify endosomal compartments, thereby preventing hormone-receptor (H-R) dissociation with resulting accumulation of internalized complexes (Ueda *et al.*, 1985; Carpentier *et al.*, 1986b). Indeed compared to untreated cells, this concentration of monensin did significantly increase the cell associated radioactivity over a two hour period. However, it would appear that $25 \mu M$ monensin was not completely effective in restricting dissociation of internalized complexes since a considerable decrease from the initial ^{125}I-iodoinsulin bound after 10 min occured with time. This loss in cell-associated radioisotope could clearly be seen when compared to PhAsO ($10 \mu M$) which retains almost all of its initial activity.

Table 5.2 compares the powerful inhibitory effect of PhAsO ($10 \mu M$) on receptor mediated endocytosis at $37^\circ C$ with control and monensin ($25 \mu M$)-treated cells. The data is expressed as a percentage of the initial iodoinsulin bound in 10 min. In the absence of PhAsO or monensin the rapidly internalized insulin receptor (I-R) complexes are soon degraded, after 1 hour >50% of the initial bound insulin is loss and after 2 hours only minute (~13%) amounts remain associated with

the cells. Both PhAsO (10μM) and monensin (25μM) restrict loss of radioisotope from the cells, though to different extents, and by two different methods. PhAsO (10μM) inhibits internalization, and hence degradation, of the surface bound insulin. This agent effectively inhibited any loss in activity over 60 min and after 2 hours only a small (~14%) loss in activity was observed. Monensin on the other hand, allows internalization but prevents dissociation of H-R complexes. This ionophore, at the concentration studied appeared to be quite effective in restricting hormonal degradation over a short, 30 min, period. However with increasing time the amount of ligand degraded also increased although the rate of hormone destruction was significantly slower than in the absence of the ionophore.

A time course for the effect of different monensin concentrations on insulin binding to adipocytes is shown in Fig 5.19. 100μM monensin restricted intracellular ligand degradation to a considerably greater extent than 25μM monensin. However the effect of 100μM monensin was not absolute, and ligand degradation still occured though at a much reduced rate. A dose response curve for the effect of monensin on insulin binding at 37°C is shown in Fig. 5.20. Adipocytes were treated with varying monensin concentration (5 to 200μM) for 30 min at 37°C. " Hot" insulin was then added and the quantity of radioisotope still associated with the cells after 2 hours was determined. 100μM monensin was the most effective concentration for limiting insulin degradation. Binding was somewhat reduced at monensin concentrations >100μM suggesting that the cell membrane integrity was being affected.

Fig 5.1

Time course for uptake of insulin by adipocytes at 15°C.

0.2ml aliquots of an adipocyte suspension, containing ~0.9 x 10^6 cells per ml, were incubated for the indicated times at 15°C with 67 pM ^{125}I-iodoinsulin. The cells were separated from the free insulin by oil flotation as described in Methods (Chapter 2). ●, total bound insulin; ■, non-specifically bound insulin; 0, specifically bound insulin.

Fig 5.1
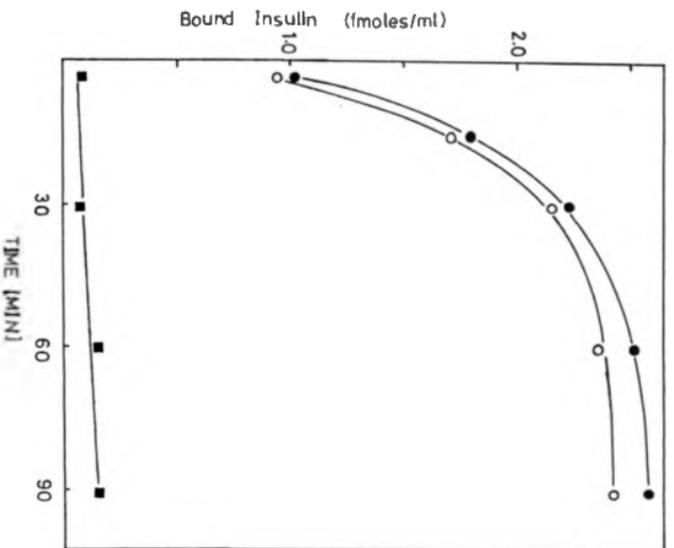
Fig 5.2

Time course for the temperature dependence of ^{125}I-iodoinsulin binding by rat adipocytes.

a)   0.2ml aliquots of adipocyte suspension, containing ~3 x 10^6 cells per ml, were incubated for the indicated times with 33 PM ^{125}I-iodoinsulin.   Insulin binding at 15°C (●), 22°C (△),30°C (■) and 37°C (▽).

b)   0.2ml aliquots of an adipocyte suspension, containing ~0.7 x 10^6 cells per ml, were incubated with 72 pM ^{125}I-iodoinsulin at 20°C for the specific times.

Fig 5.2
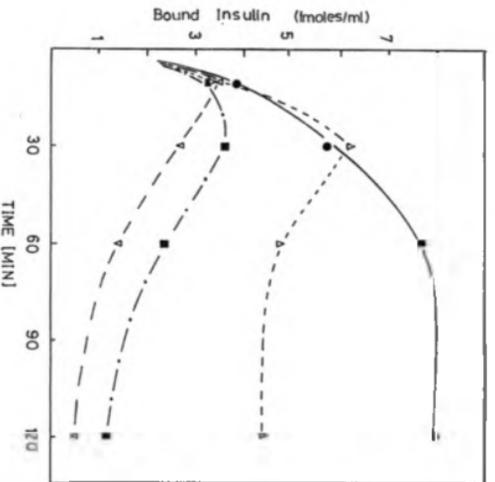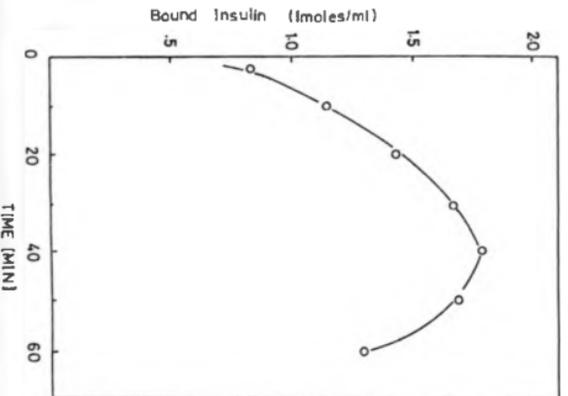
Fig 5.3

Time course for the effect of phenylarsine oxide on insulin
binding by rat adipocytes at 15°C and 37°C.

0.2ml aliquots of an adipocyte suspension, containing
~2.7 x 10^6 cells per ml, were preincubated at 15°C or 37°C
in the absence or presence of 10μM PhAsO for 1hr. The
reaction was started by addition ^{125}I-iodoinsulin (26 pM)
and binding was monitored over 2 hrs. □, insulin binding at
15°C; ■, insulin binding at 15°C in the presence of PhAsO
(10μM); 0, insulin binding at 37°C; ●, insuling binding at
37°C in the presence of PhAsO (10μM).

Fig 5.3
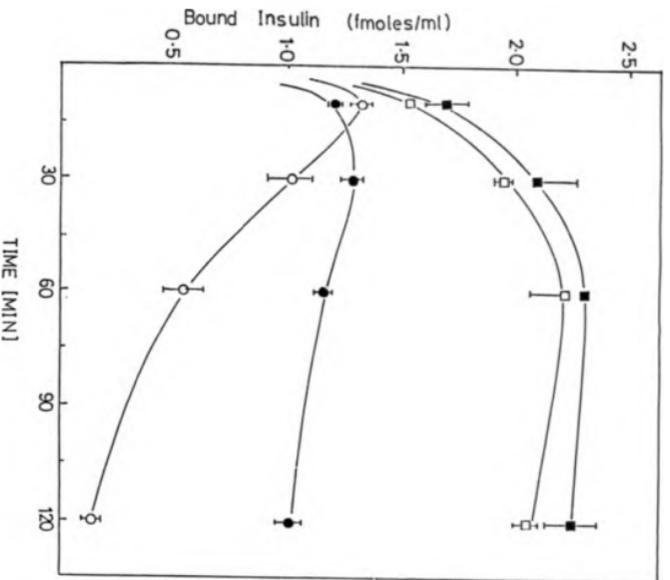
Fig 5.4

Effect of phenylarsine oxide on ^{125}I-iodoinsulin binding to rat adipocytes at 15°C.

0.2ml aliquots of an adipocyte cell suspension, containing ~3.4 x 10^6 cells per ml, were preincubated with varying PhAsO concentrations at 15°C for 1 hr. The reaction was initiated by the addition of 39 pM ^{125}I-iodoinsulin and binding allowed to proceed for 1 hr. Non-specific binding was determined in the presence of 10μM cold insulin.

a)   0, total bound insulin; □ non-specifically bound insulin.

b)   ●, specifically bound insulin.

Fig 5.4
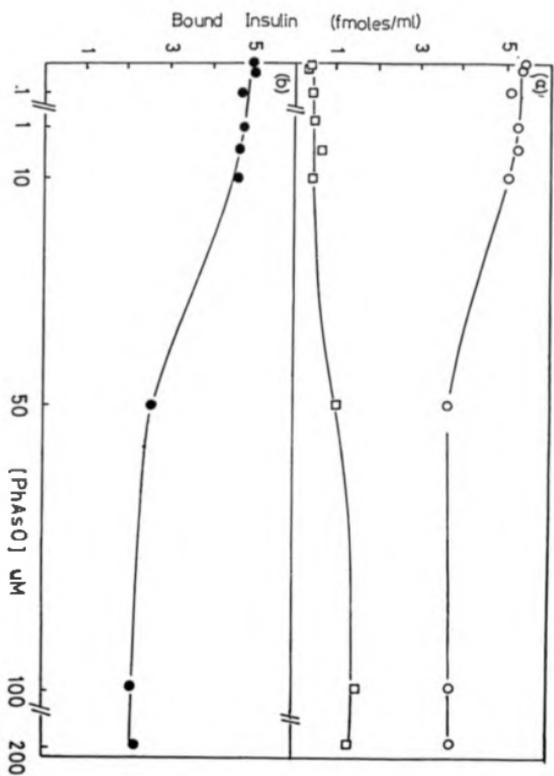
Fig 5.5

Effect of PhAsO on ^{125}I-iodoinsulin binding to rat adipocytes at 15°C in the presence of 25nM cold insulin.

0.2ml aliquots of an adipocyte suspension containing ~5 x 10⁶ cells per ml were preincubated with varying PhAsO concentrations at 15°C for 1 hr. The reaction was initiated by the addition of 0.17ml of stock insulin solution containing both cold and "hot" insulin (0.7 μCi/ml). Final concentration of insulin in the medium was 25nM cold and 31 pM "hot". Non-specific binding was determined in the presence of 10μM cold insulin. ●, total bound insulin; 0, non-specifically bound insulin. The specifically bound insulin was taken to be the difference in the smooth curves.

Fig 5.5
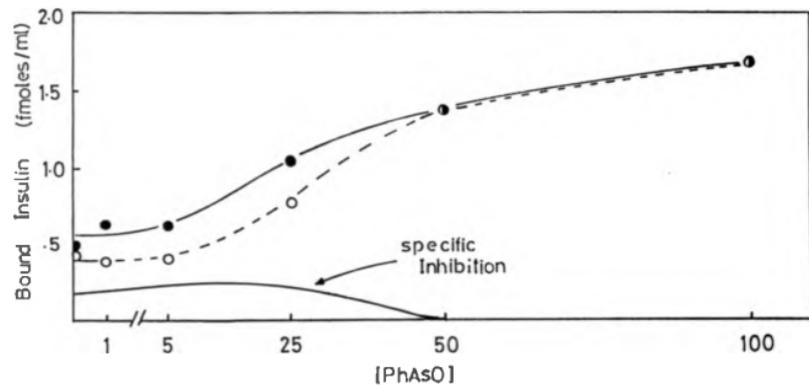
Fig 5.6

Effect of PhAsO on ^{125}I-iodoinsulin binding to rat adipocyte plasma membranes at 15°C.

Plasma membranes isolated from basal and insulin stimulated rat adipocytes were preincubated at 15°C in the presence and absence of PhAsO for 1 hr. ^{125}I-iodoinsulin was added and binding was carried out for 1 hr. Each vial contained 60 μg of membrane suspension and 15pM ^{125}I-iodoinsulin.

Insulin binding in basal plasma membranes.

Insulin binding in PhAsO treated basal plasma membranes.

Insulin binding to insulin-stimulated plasma membranes.

Insulin binding to PhAsO treated insulin-stimulated plasma membranes.

Fig 5.6
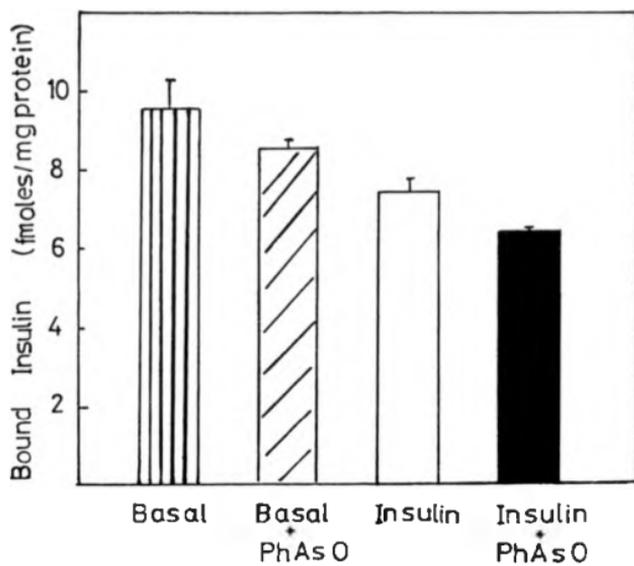
Fig 5.7.a

Scatchard plot for insulin binding to rat adipocytes at 15ºC. The bound/free ratio plotted against bound insulin per 10^9 cells. The slope determined by a straight line least squares fit.

Fig 5.7.b.

Binding isotherm for insulin to adipocytes at 15ºC. Bound insulin per 10^9 cells plotted against the log of the free insulin concentrations.

Fig 5.7
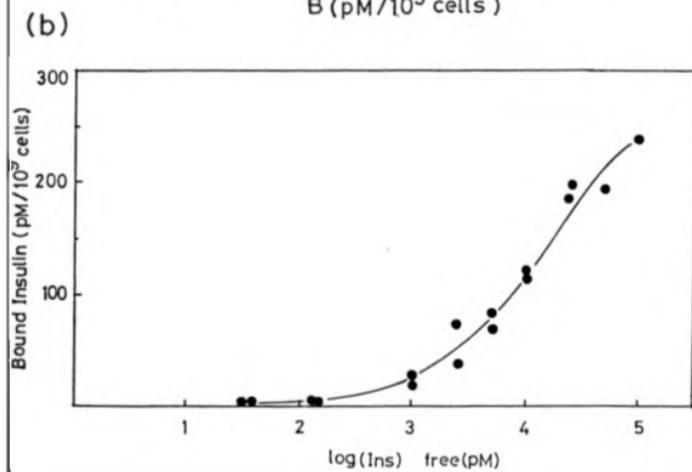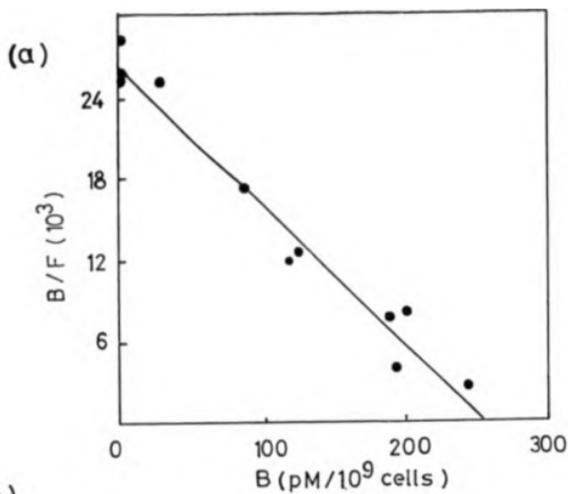
Fig 5.8.a.

Scatchard plot for insulin binding to rat adipocytes at 15°C in the presence of $10\mu M$ PhAsO. Bound/free ratio plotted against bound insulin per 10^9 cells. The slope determined by a straight line least squares fit.

Fig 5.8.b.

Binding isotherm for insulin to adipocytes at 15°C in the presence of $10\mu M$ PhAsO. Bound insulin per 10^9 cells versus log of the free insulin concentration.

Fig 5.8

(a)
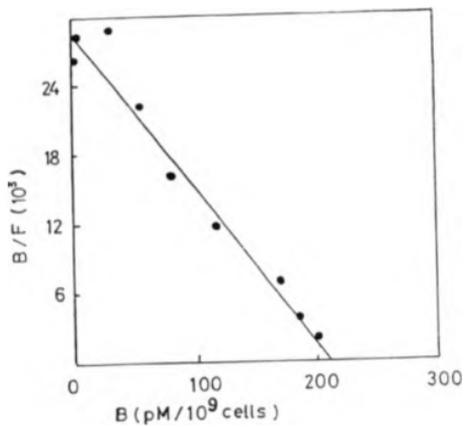
(b)
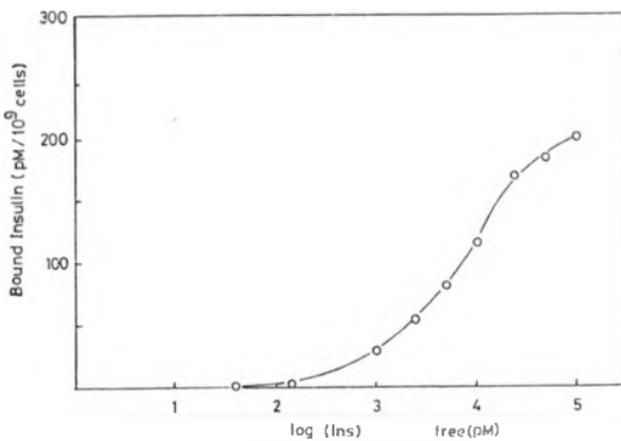
Fig 5.9.a.

Scathard plot for insulin binding to rat adipocytes at 15°C in the presence of 25 μM PhAsO. Bound/free ratio versus bound insulin per 10^9 cells. Slope determined by a straight line least squares fit.

Fig 5.9.b.

Binding isotherm for insulin to adipocytes at 15°C in the presence of 25 μM PhAsO. Bound insulin per 10^9 cells versus log of the free insulin concentration.

Fig 5.9
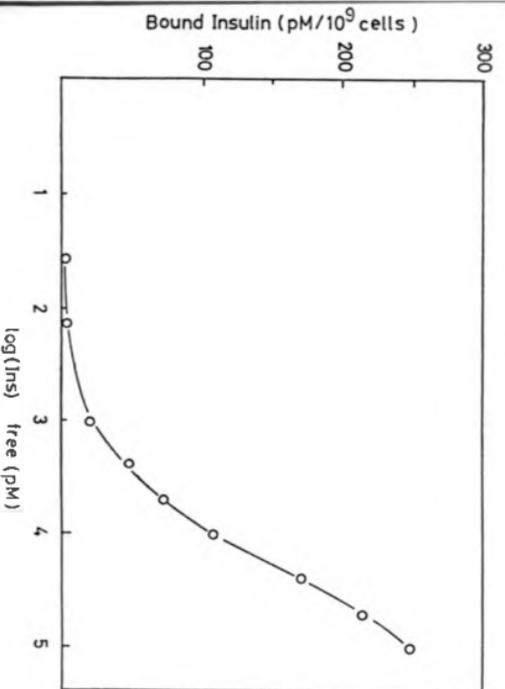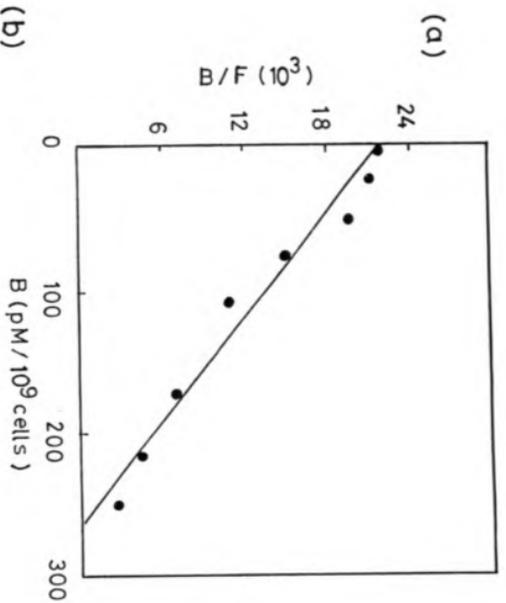
Fig 5.10.a.

Scatchard plot for insulin binding to rat adipocytes at 15°C in the presence of 40µM PhAsO. Bound/free ratio versus bound insulin per 10^9 cells. Slope determined by a straight line least squares fit.

Fig 5.10.b.

Binding isotherm for insulin to adipocytes at 15°C in the presence of 40µM PhAsO. Bound insulin per 10^9 cells versus log free insulin concentration.

Fig 5.10
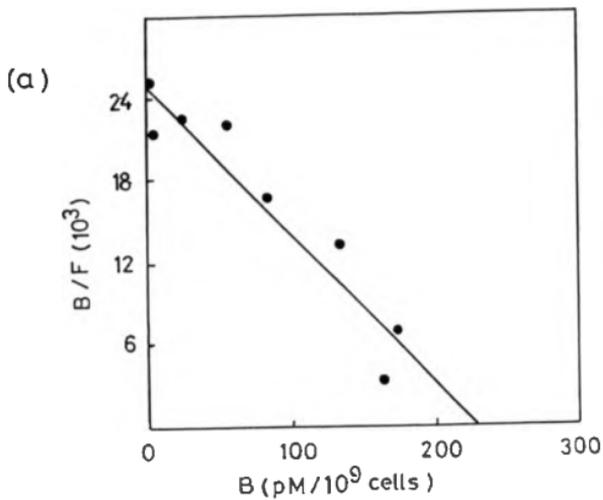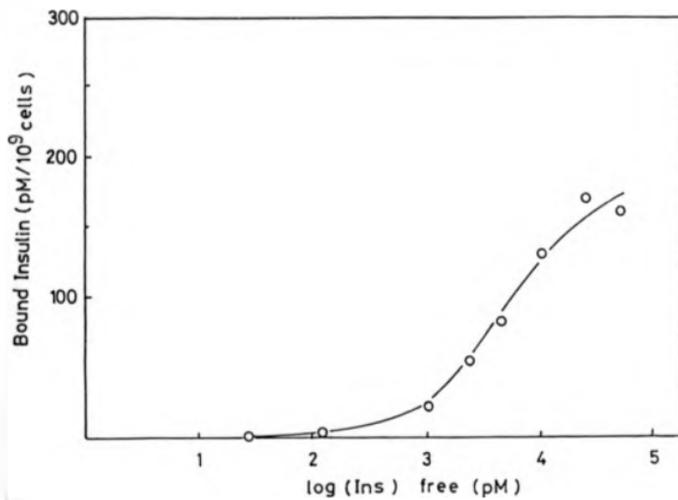
Table 5.1.

The effect of PhAsO on insulin binding as determined by Scatchard Anaylsis.

[PhAsO] μM	No. of Binding Sites per cell (10^5)	Binding Constant $10^9 M^{-1}$
Control	1.5 ± .06	.102 ± .006
10	1.26 ± .057	.134 ± .009
25	1.57 ± .072	.082 ± .006
40	1.29 ± .124	.108 ± .015

Fig 5.11

Effect of phenylarsine oxide and dithiothreitol (DTT) on insulin binding to rat adipocytes at 15°C.

1ml aliquots of adipocyte suspension containing ~3.4 x 10⁶ cells per ml, were incubated at 37°C with PhAsO (10 - 100μM for 5 min), DTT (100 - 1000μM for 30 min) and PhAsO (10 - 100μM) for 5 min followed by DTT (100 - 1000μM) for 30 min. The ratio of PhAsO to DTT in suspensions treated with both these agents was 1:10. Following incubations at 37°C, 0.2mls of the treated cells were pipetted into 1.16 mls Krebs-Henseleit buffer (1% BSA, 2mM glucose) and the suspensions were equilibrated for 1 hr at 15°C before incubating with 35pM ¹²⁵I-iodoinsulin for 1 hr. ●, insulin binding to PhAsO treated adipocytes; □, insulin binding to DTT treated cells; 0, insulin binding to adipocytes treated PhAsO followed by DTT.

Fig 5.11
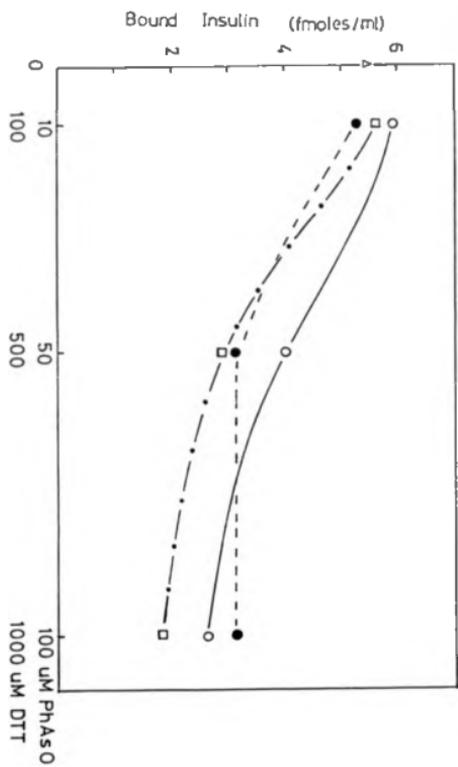
Fig 5.12

Effect of dithiothreitol (DTT) on insulin binding to rat adipocytes at 15°C.

0.8mls of adipocyte suspension, containing ~3.48 x 10^6 cells per ml, were incubated with DTT (100 - 1000 μM for 30 min) at 37°C. Next 0.2ml of DTT treated cells were transfered to 1.16 mls of Krebs-Henseleit buffer (1% BSA; 2mM glucose) and the suspensions were equilibrated for 1 hr at 15°C. ^{125}I-iodoinsulin (39pM) was the added and insulin uptake after 1 hr was determined. Non-specific binding was determined in the presence of 10μM cold insulin. □, total bound insulin; △, non-specifically bound insulin; 0, specifically bound insulin.

Fig 5.12
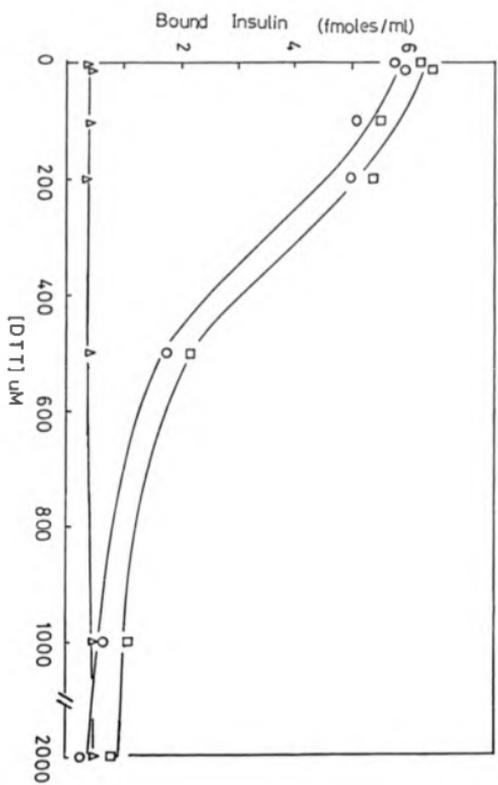
Fig 5.13

Low pH (acetic acid) wash of surface bound insulin at 15°C.

0.4mls of adipocyte suspension, containing ~2 x 10^6 cells per ml, were incubated with ^{125}I-iodoinsulin (35pM) for 30 min at 15°C. The normal Krebs-Henseleit incubation buffer was then removed from the suspension and replaced by the acid solution (pH 2.5) for the times specified. Adipocytes were then washed free of the acid solution and used for direct rebinding of ^{125}I-iodoinsulin (33 pM) for 30 min. The total aqueous volume in which cells were suspended (3.06 mls) was kept constant after each wash.

□, specific insulin binding to control unwashed cells; ο, non-specifically bound insulin to control (unwashed) cells; Δ, specifically bound insulin to adipocytes that were acid washed (for 3 min) prior to resuspension in Krebs-Henseleit buffer (pH 7.4); ●, cell associated ^{125}I-iodoinsulin after insulin binding at 15°C for 30 min, followed by acid washing for the specificed times; ■, cell associated ^{125}I-iodoinsulin after insulin binding at 15°C for 30 min, followed by acid washing for the specified times and rebinding of ^{125}I-iodoinsulin (15°C for 30 min).

Fig 5.13
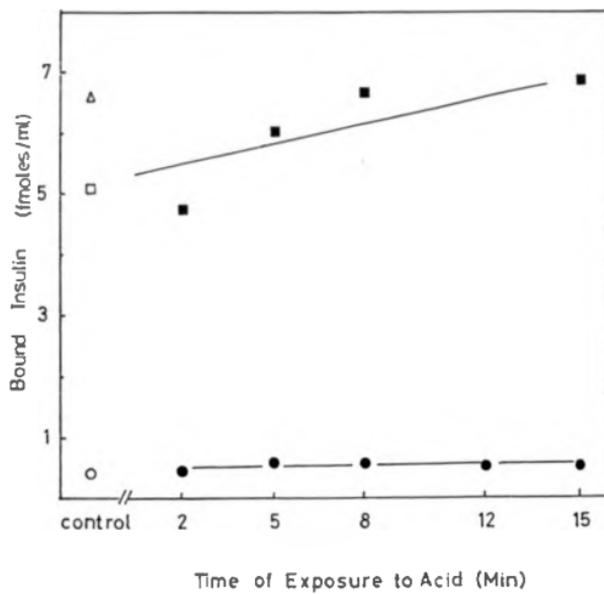
Fig 5.14.

Further investigation of the low pH (acetic acid) wash of surface bound insulin at 15°C.

0.4mls of adipocyte suspension, containing ~2.7 x 10^6 cells per ml, were incubated with ^{125}I-iodoinsulin (31 pM) for 30 min at 15°C. The acid wash procedure was carried out as in the legend to Fig 5.13. Δ ; specifically bound insulin to control (unwashed) cells; 0, non-specifically bound insulin to control (unwashed) cells; ●, cell associated ^{125}I-iodoinsulin after insulin binding at 15°C for 30 min followed by acid washing for the specified times; □, cell associated ^{125}I-iodoinsulin after insulin binding at 15°C for 30 min, followed by acid washing for the specified times and rebinding of insulin (15°C for 30 min); ◉, non-specifically bound insulin to adipocytes that were acid washed for the times indicated; ▲, specifically bound insulin after acid washing and rebinding.

Fig 5.14

Time of Exposure to Acid (Min)
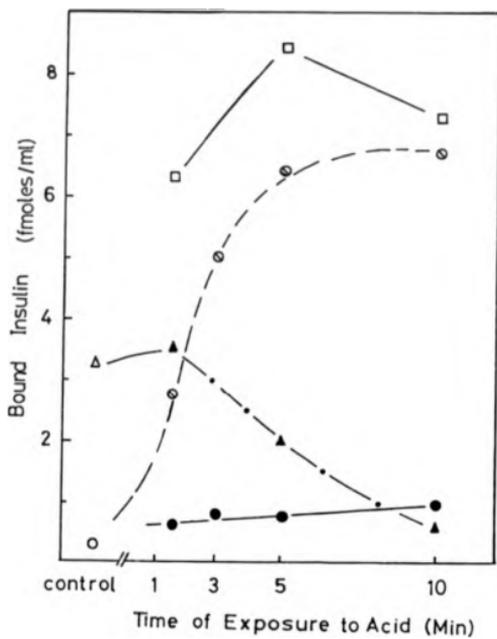
Fig 5.15.a.

Low pH (glycine/HCl buffer) wash to surface bound insulin at 15°C.

0.4mls of adipocytes suspension, containing ~1 x 10^6 cells per ml, were incubated with ^{125}I-iodoinsulin (31pM) for 60 min at 15°C. Krebs-Henseleit incubation buffer was then removed from the suspension and replaced by the glycine/HCl buffer (pH 3.5) for 5 min. The amount of cell associated activity was determined at various stages.

☐ Insulin binding to normal control (unwashed) cells.

■ Non-specifically bound insulin to control cells.

▨ Total insulin binding to adipocytes that were acid washed.

▥ Non-specifically bound insulin to adipocytes that were acid washed.

◩ Cell associated ^{125}I-iodoinsulin after insulin binding at 15°C for 1 hr, followed by acid washing for 5 min.
Results are the mean ± S.D.

Fig 5.15.b.

Rebinding of ^{125}I-iodoinsulin to acid washed adipocytes.

Open box, insulin binding to adipocytes at 15°C for 60 min; hatched box, Insulin binding to adipocytes after binding at 15°C for 60 min, followed by acid washing for 5 min and rebinding of ^{125}I-iodoinsulin at 15°C for 60 min.
Results are the mean ± SD of quadruplicate determinations.

Fig 5.15
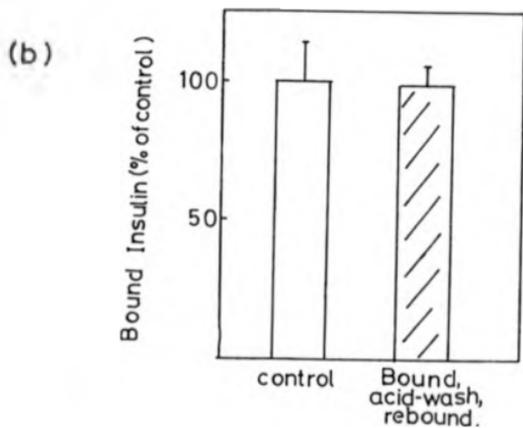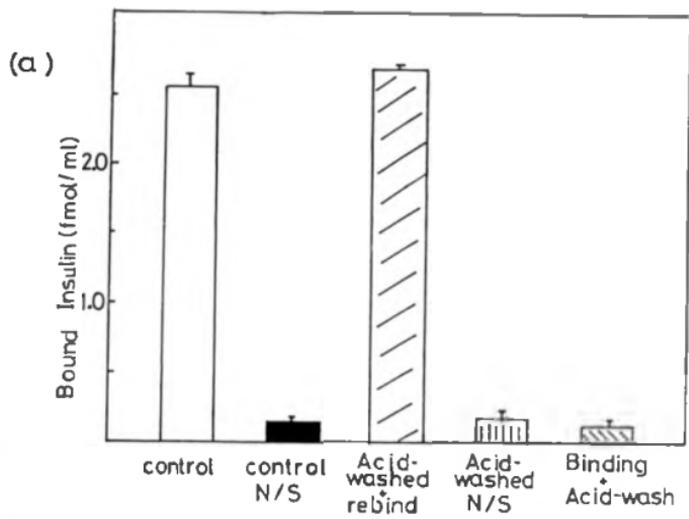
Fig 5.16

Insulin induced receptor redistribution in isolated rat adipocytes.

^{125}I-iodoinsulin binding was carried out at 15°C. 0.4ml aliquots of adipocyte suspension (~3 x 10^{6} cells per ml) were equilibrated at this temperature for 1 hr prior to incubation with ^{125}I-iodoinsulin (39pM) for 1 hr.

Open box, insulin binding to basal cells.; hatched box, insulin binding to adipocytes treated with cold insulin (100nM) for 20 min at 37°C, followed by equilibration at 15°C for 60 min, acid washing (glycine/HCl buffer pH 3.5) for 5 min and incubation with ^{125}I-iodoinsulin for 60 min.

Fig 5.16
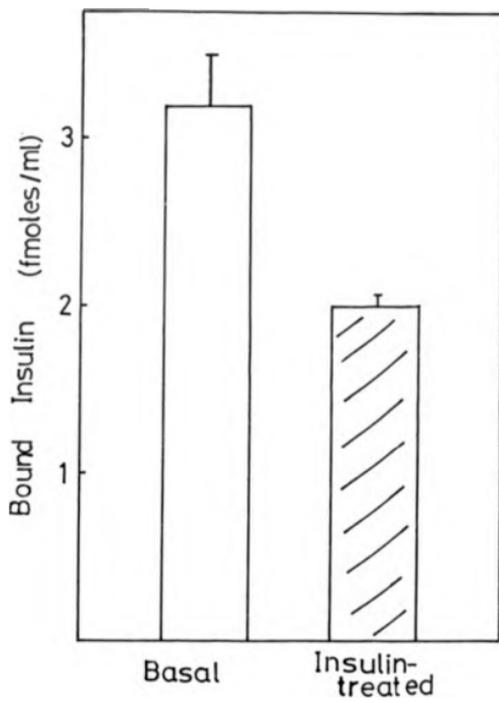
Fig 5.17.a.

Scatchard plot for insulin binding to basal and insulin stimulated rat adipocytes plasma membranes at 15°C. Bound/free ratio plotted against bound insulin pM per mg protein.

Fig 5.1.7.b.

Binding isotherm for insulin to basal and insulin stimulated rat adipocyte plasma membranes at 15°C. Bound insulin per mg protein versus log of the free insulin concentration.

Fig 5.17
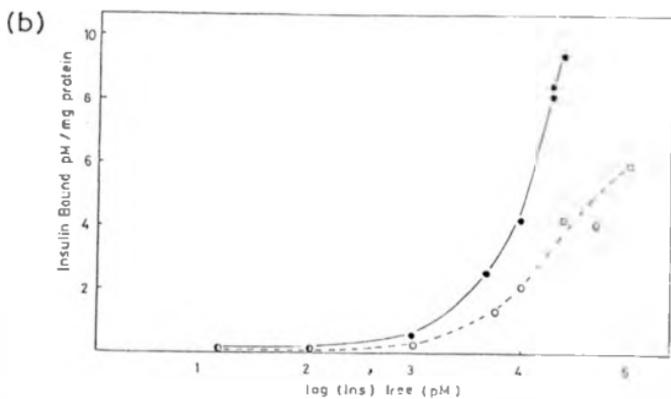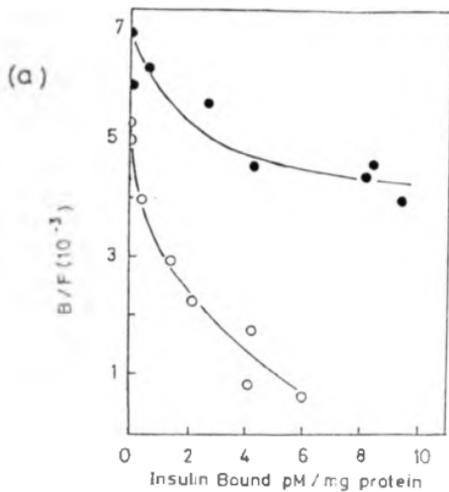
Fig 5.18

The effect of phenylarsine oxide and monensin on insulin binding to rat adipocytes at 37°C.

0.2ml aliquots of an adipocyte suspension, containing ~1.5 x 10^6 cells per ml, were equilibrated with either PhAsO (10μM) or monensin (25μM) for 20 min prior to the incubation with ^{125}I-iodoinsulin (39pM). Cell associated activity was monitored over 2 hrs.

0, insulin binding to control cells; $\square$, insulin binding to PhAsO - treated cells; $\triangle$, insulin binding to monensin -treated adipocytes

Fig 5.18
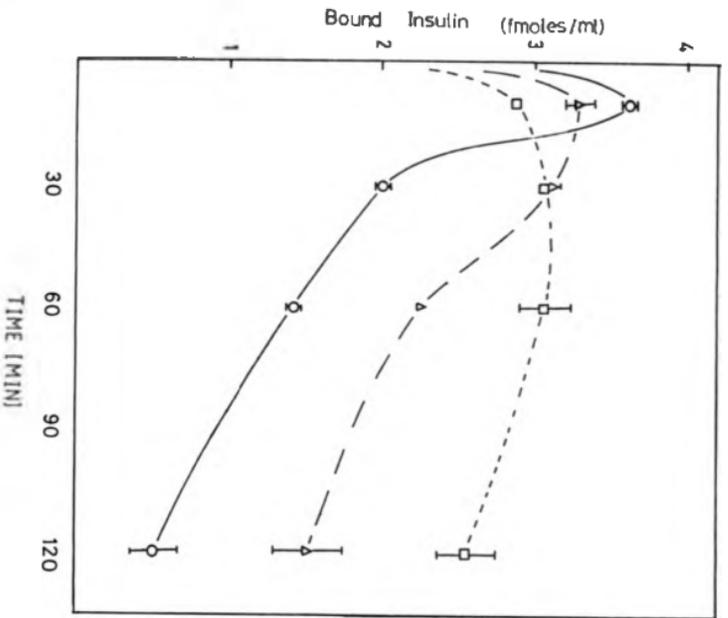
Table 5.2.

The effect of phenylarsine oxide and monensin on insulin binding to rat adipocytes at 37°C.

% of initial iodoinsulin bound in 10 min at 37°C

Time (hr)	Control	PhAsO	Monensin (25μM)
0.5	72 ± 9	106 ± 0%	91 ± 8
1	40 ± 2	101 ± 7	61 ± 11
2	13 ± 2	86 ± 4	40 ± 11

Adipocytes were equilibrated with PhAsO (10μM) or monensin (25μM) for 20 min prior to incubation with ^{125}I-iodoinsulin. Results are mean ± SD of 4-6 determinations.

Fig 5.19

The effect of different monensin concentrations on insulin binding to rat adipocytes at 37°C.

0.2ml aliquots of an adipocyte suspension, containing ~3.88 x 10^6 cells per ml, were equilibrated with either 25μM or 100μM monensin for 20 min prior to the incubation with ^{35}pM ^{125}I-iodoinsulin. Cell associated activity was monitored over 2 hrs. 0, insulin binding to cells incubated with 100μM monensin; ● insulin binding to cells incubated with 25μM monensin.

Fig 5.19
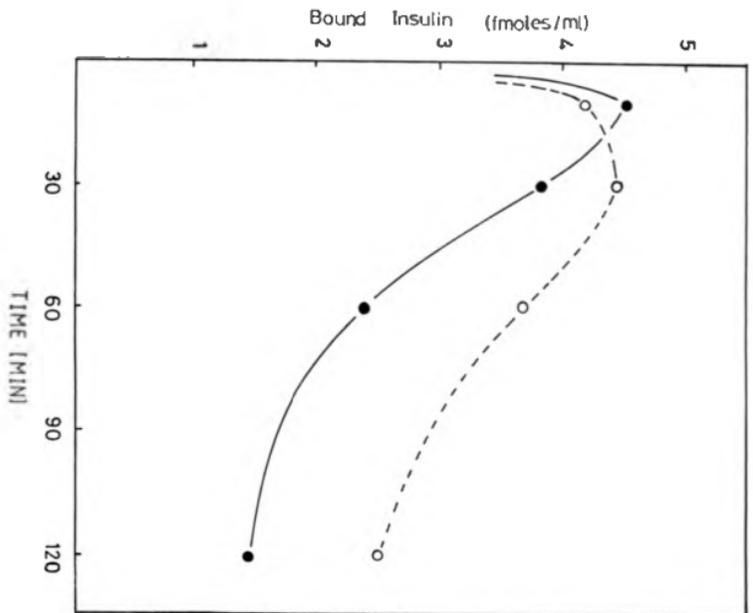
Fig 5.20

Dose response curve for the effect of monensin on insulin binding to rat adipocytes at 37°C.

0.2ml aliquots of an adipocyte suspension, containing ~2.5 x 10^6 cells per ml, were treated with a range of monensin concentrations (5 to 200 μM) for ~30 min. The suspensions were then incubated with 31pM ^{125}I-iodoinsulin for 2 hrs after which the amount of cell associated activity was determined.

Fig 5.20
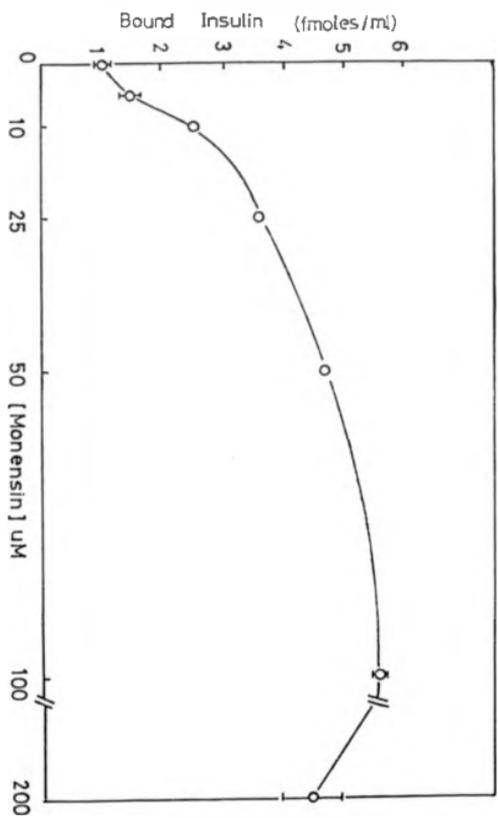
DISCUSSION

Temperature Dependence of Insulin Binding

It has previously been reported that insulin mediated receptor endocytosis is both temperature and energy sensitive (Suzuki & Kono, 1981; Huecksteadt *et al.*, 1986). The rate of insulin-induced receptor internalization is progressively reduced as the temperature decreases with minimal internalization occuring at 16^oC (Marshall 1985a). At relatively higher temperatures (37^oC) where the plasma membrane is more fluid internalization of the insulin receptor complexes occurs rapidly, $t_{\frac{1}{2}} = 2.7$ min, and subsequent ligand destruction also occurs quickly, $t_{\frac{1}{2}} = 8$ min (Marshall, 1985a).

The temperature sensitivity of insulin binding is depicted in Fig 5.2. Insulin binding to adipocytes at 15^oC increases steadily and equilibrates within 60 min. The integrity of endocytosis within these cells is clearly shown at 37^oC where cell associated radioactivity decreased rapidly after an initial maxima at 10 min and within two hours only a small quantity of radioisotope was associated with the cells; approximately 13% of the initial maximal amount bound after 10 min (Table 5.1). This temperature dependence for insulin binding to adipocytes is in agreement with previous reports (Huecksteadt *et al.*, 1986; Marshall, 1985a). However the apparent inability of low temperatures (20^oC) to completely inhibit internalization of insulin-receptor complexes (Fig 5.1, 5.2) suggests that some amount membrane activity is occuring and indicates that the insulin-like effects of low temperatures on D-glucose uptake (Fig 3.4, Chapter 3) occurs via translocation of intracellular carriers.

Although the low temperature-induced enhancement of sugar transport observed in Chapter 3 (Fig 3.4) occured at 20°C where some amount of membrane movement is still possible (Fig 5.2.b), and hence would allow recruitment of intracellular transporters, Ezaki & Kono (1982) observed insulin-like effects at 10°C where virtually no internalization should occur. However, it has recently been reported that although receptor internalization was prevented at 16°C recycling of insulin receptors still occured (Hueckesteadt *et al.*, 1986). Interestingly PhAsO, an inhibitor of protein internalization, does not affect exocytosis (Knutson *et al.*, 1983). Therefore it might be suggested that exocytosis still occurs under conditions where endocytosis is prevented (low-temperatures; PhAsO-treatment). Hence it might be feasible that transporter recruitment could explain the ability of low temperatures to mimic insulin action as suggested by Ezaki & Kono (1982).

Effects of PhAsO on Insulin Binding

Since receptor internalization is inhibited at 15°C then the extent of insulin binding to PhAsO-treated and untreated cells at this temperature should reveal any inhibitory effect of this agent on insulin binding. Fig 5.3 showed that the magnitude of insulin binding at 15°C was unaffected by PhAsO (10μM) in agreement with previous reports (Jones *et al.* 1986, Knutson *et al.*, 1983, Draznin *et al.*, 1983). At 37°C ^{125}I-iodoinsulin rapidly associates with both PhAsO (10μM)-treated and untreated adipocytes and after 10 min no significant difference in the extent of binding occured. However with increasing time the amount of radioisotope associated with control cells decreased rapidly presumably through internalization and degradation of the ligand. PhAsO inhibited these events due to its ability to

prevent receptor-mediated endocytosis resulting in no substantial loss in activity within the first hour and only a minor loss (~14%) in cell associated activity after the second hour (Table 5.1, Fig. 5.3). This effect then clearly emphasizes PhAsO's capability to restrict protein internalization.

The inhibitory effect of PhAsO (20μM) on hexose transport, without inhibiting binding of insulin to its receptor, has led to the speculation that this agent does not affect the functioning of the insulin receptor (Frost & Lane, 1985). Fig. 5.4 however, illustrates that while relatively low concentrations of the arsenical (0.01-10μM) did not inhibit insulin binding higher concentrations did reduce binding to the receptor, suggesting that PhAsO interacts directly with the insulin receptor and at sufficiently high concentrations inhibits hormone binding most probably through inducing a conformational change(s) within the $\alpha_2\beta_2$ tetramer.

It is curious that non-specific binding increases as a function of PhAsO concentration and might be attributed to: a) PhAsO's adeptness to inhibit protein internalization without inhibiting exocytosis (Knuston et al., 1983) such that sequestration of intracellular protein might present an increased protein surface area in plasma membrane for non-specific attachment ; b) distortion of exisiting plasma membrane proteins, through interaction with vicinal sulphydryl groups, to such an extent as to cause severe conformational deformities of the tertiary structure of intergral membrane proteins resulting in unnatural sites for non-specific insulin binding. The marked inhibitory effect of high PhAsO concentrations on insulin binding, 55% inhibition at 100μM PhAsO (Fig. 5.4), strongly suggests that the arsenical was imposing structural constraints on the insulin receptor. Hence it is very

164.

likely that the large increases in non-specific binding might also be due to structural distortion of cell surface molecules.

The presence of a large amount of cold insulin (25μM) in the medium did not prevent or reverse PhAsO inhibition of binding or the significant increase in non-specific binding (Fig 5.5) suggesting that PhAsO action is indendent o the presence of insulin. The data in Chapter 4 indicates that PhAsO (10μM) interacts with and directly inhibits the D-glucose carriers. Hence, although PhAsO does not appear to inhibit insulin binding at concentrations where it effectively inhibited D-glucose uptake, its ability to inhibit binding at high concentrations may reflect the arsenical's property of imposing structural contraints on proteins with resulting conformational modifications. It is possible then that even at lower concentrations (1-10μM) PhAsO may directly impose conformational restrictions on the insulin receptor and/or D-glucose transporter to such an extent as to cause improper functioning of these molecules with resulting inhibition of transport activty.

Scatchard analysis of the effect of PhAsO (10-40μM) on insulin binding did not reveal any dramatic alteration in receptor affinity or in the total number of receptors (Table 5.1). The total number of binding sites in the absence of PhAsO was found to be 1.5×10^5 per cell and though in the presence of 10μM and 40μM PhAsO the number of binding sites was somewhat smaller, 1.26×10^5 and 1.29×10^5 respectively, at 25μM PhAsO the receptor number was 1.57×10^5. Hence within the limits of experimental error it appears that PhAsO does not affect insulin binding in rat adipocytes. This observation is surprising since Fig. 5.4 and Fig 5.5 indicate that binding was being affected.

Since the half maximal inhibition of binding appears to be $-28\mu M$ (Fig 5.4) then one might expect Scatchard analysis to substantiate this observation This however was not the case and in addition PhAsO did not appear to alter the binding constant (Table 5.1). Although it seems likely that some direct interaction between PhAsO and insulin receptors is occuring, and at sufficiently high concentrations binding inhibition occurs regardless of the insulin levels (Fig 5.4, 5.5), it is not clear why this agent did not have any significant impact on receptor affinity or receptor number at the PhAsO concentrations studies, as determined by Scatchard analysis.

Effects of PhAsO/DTT on Insulin Binding

The reducing agent DTT facilitated a small amount of reversal of PhAsO inhibition of insulin binding (Fig 5.11).   However, oddly enough , adipocyte treatment with DTT alone resulted in severe inhibition of binding (Figs 5.11, 5.12). It has been reported that 1mM DTT increases insulin binding 3-fold in isolated rat adipocytes plasma membranes at 37°C, while higher DTT concentrations apparently inhibited binding (Schweitzer et al., 1980). Jarett & Smith (1983) also observed increased binding to DTT-treated adipocyte plasma membrane, and it was proposed that low DTT concentraions (<1mM) disrupts the microaggragation of the receptors on isolated plasma membranes (Schweitzer et al., 1980, Jarett & Smith, 1983).   In addition 0.5mM DTT is capable of cleaving class 1 disulphide bridges within the receptor and twice as much ^{125}I-insulin binds to receptors dissociated into $\alpha\beta$ halves (Czech et al., 1981).

It is quite clear however that DTT (<1mM) in some way interrupts insulin binding in whole cells at 15°C in contrast to Schweitzer *et al.*, (1980) Jarett & Smith (1983) and Czech *et al.*, (1981). The reason(s) for this difference is unclear but it may be related to the fact that these researchers used isolated plasma membranes while whole cells were used here. Since the two α subunits of the insulin receptor exist almost exclusively on the outer surface of the plasma membranes (Ullrich *et al.*, 1985) then one might expect that class I disulphide bridges linking these subunits to react similarly to DTT whether membrane preparations or whole cells are used. However the disparity in the observations for the effects of DTT on insulin binding to intact cells (Fig 5.11; 5.12) and membrane preparations indicate some differences exists between the receptor state in intact cells compared to isolated membranes. It might be speculated that inhibition of insulin binding by DTT in whole cells may not be entirely due to reduction of class I S-S bonds but some additional factors(s), related to hormone binding, is associated with receptors only in intact cells and may be inhibited by DTT.

A substantial amount of evidence has suggested that the insulin receptor may exist as a high molecular weight complex (Helmerhorst *et al.*, 1986; Fehlmann *et al.*, 1985). A serine kinase has been found to copurify with the receptor after wheat germ lectin chromatography but is lost after further purification by anti-receptor-antibodies (Ballotti *et al.*, 1986) suggesting that the $\alpha_2\beta_2$ tetramer may represent only the "core" of the complete receptor. Recently, Helmerhorst *et al.*, (1986) have shown that although the α-subunit is the major insulin binding subunit a number of other proteins may be participating in insulin binding. A considerable amount of processing is involved in membrane preparations and it is possible that the final receptor state (structural

or otherwise) may not be identical in plasma membrane preparations and intact cells and hence may be responsible for the differences in the data shown here and that reported in the literature.

Additionally, data obtained from membrane preparations must be viewed with caution since membranes lack "sidedness". It is possible that in whole cells DTT might react somewhat selectively with the receptors while in isolated plasma membranes DTT might be free to interact with portions of the receptor which might ordinarily be protected in whole cells. It is curious that $10\mu M$ PhAsO which does not alter insulin binding in rat adipocytes (Figs 5.3 & 5.4) causes some inhibition of binding in isolated adipocyte basal and insulin stimulated plasma membrane (fig 5.6), suggesting a greater freedom of interaction between PhAsO and the receptors. Hence DTT may also possess increased freedom of action in membrane preparations.

Another factor which might account for the different effects of DTT on insulin binding (Figs 5.11, 5.12) to that reported (Schweitzer *et al.*, 1980; Jarett and Smith 1983; Czech *et al.*, 1981) might be the temperature at which binding was performed. In the experiments performed here adipocytes were treated with DTT at 37°C for 30 min prior to conducting binding at 15°C, whereas the above researchers performed both DTT treatment and insulin binding at 37°C. Should the different temperatures affect the binding constant then some variance in binding might be expected.

Whether temperature or other factors are responsible for the observations made in Fig 5.11 and 5.12 remains to be determined. The results obtained for the effect of DTT on insulin binding clearly shows that this agent incites considerable reduction in binding to

adipocytes. Because PhAsO and DTT both interact with sulphydryl groups and both inhibit binding it is apparent that sulphydryl groups within the intact receptor are critical for binding. Recently Pike *et al.*, (1986) observed ~40-50% decrease in binding upon DTT (2mM) treatment of purified placental insulin receptors and reasoned that the integrity of class I disulphide bridges were critical for insulin receptor binding and function. This finding is similar to the observation made here although rat adipocytes exhibited greater than 95% binding inhibition at 2mM DTT (Fig 5.12) and based on the dissimilarities with plasma membranes, it might be speculated that factors in addition to class I bonds, which are sensitive to reducing agents, also operate to ensure maximal hormone receptor association in this cell type.

In contrast to PhAsO, DTT did not increase non-specific binding (Fig 5.12). This is quite reasonable since, if as proposed above, PhAsO may increase non-specific binding through imposing constraints on the tertiary structure of cell surface proteins then DTT may act to destroy protein teritary structures by reducing sulphydryl bridges. So that DTT and PhAsO may act in an antagonistic manner. Hence although individually these agents may inhibit binding, when combined together they may act to relieve the inhibitory effect of each other as is observed in Fig 5.11. The binding inhibition for PhAsO (10μM to 50μM) and DTT (100μM-500μM) is quite similar. The individual inhibition of PhAsO (50μM) and DTT (500μM) was ~45% each whereas the combined action of these agents (50μM PhAsO plus 500μM DTT) caused less inhibition (~30%) representing an overall binding increase of 15%. As the DTT concentration increases (500μM - 1000μM) inhibition increases and surpasses maximal PhAsO (100μM) inhibition. However PhAsO (100μM) is still able to antagonise DTT (1000μM)

action and again cause ~15% reversal of binding inhibition. Since the ratio of [PhAsO] to [DTT] throughout the system was consistently 1:10 and the reversal of inhibition throughout appeared to be of the order of 15%, then this might indicate the existence of an equilibrium between the antagonising action of these two agents to interact with sulphydryl groups.

Insulin Induced Receptor Redistribution

The basis of this work was to test the proposal that insulin induces and maintains a substantial redistribution of surface receptors into the cytoplasm. The acid wash technique used by Draznin *et al.*, (1984) for removing bound ligand from heptocyte membranes proved very disruptive to adipocytes. Although this method was effective in removing cell associated ^{125}I-iodoinsulin, the very low pH (2.5) coupled with a high salt content (NaCl = 0.5M), resulted in destruction of the integrity of the cell membranes, as evidenced by the large increases in non specific binding (Fig 5.14).

A more moderate low pH (glycine/HCl) buffer proved to be quite effective in not only removing initial cell surface insulin but also did not appear to affect the non-specific binding and indeed allowed complete rebinding of ^{125}I-iodoinsulin to the cells, thus indicating that membrane integrity remained intact (Fig 5.15). Using this method it was determined that maximally stimulating insulin concentrations (100nM) induces loss of ~37% of surface receptors (Fig 5.16). Since in the basal state the plasma membrane contains 90% of the total cellular receptors (Arsenis *et al.*, 1985; Marshall 1985a, Knutson *et al.*, 1983) then in the insulin stimulated state the total intracellular receptor pool would be ~45% of the total receptor number

in agreement with Marshall (1985a) and Arsenis *et al.*, (1985). This demonstration of receptor redistribution further supports the proposals made in Chapter 4, for the differential action of PhAsO on basal and insulin stimulated adipocytes (Fig 4.9).

The Scatchard plots for both basal and insulin stimulated isolated plasma membranes were very curvilinear (Fig 5.17). Such concave graphs are a common occurence for Scatchard analysis of insulin binding (De Metys *et al.*, 1973, 1976; Etherton & Walker, 1982; Calderwood & Hahn; 1973) and suggest the existence of multiple classes of binding sites or the existence of negative cooperativity (discussed in Chapter 1, section 1.4.2.). Some researchers have suggested that high and low affinity sites could be extrapolated from such curved lines (Etherton & Walker 1982; Bonen *et al.*, 1984). However although curvilinear graphs may suggest that existence of high and low affinity sites, in view of the shortcomings of Scatchard analysis (Klotz, 1982; Klotz *et al.*, 1984; Jones *et al.*, 1987) it is difficult to justify such a procedure, and no attempt will be made here to extract any such parameters from the curves.

It is interesting that while the Scatchard plot for basal plasma membranes were extremely concave, a similar plot for intact rat adipocytes in the basal state, at the same temperature (15°C), yielded a straight line (Fig 5.7). This observation further implies that fundamental differences exist in the receptor state in intact cells and membrane preparations. It is possible that during membrane preparations some amount of receptor clumping might occur thus giving rise to the concaved graphs (Fig 5.17), while in whole cells, freedom of receptor movement may serve to minimize microaggregation and hence negative cooperativity.

Finally, both Scatchard and Klotz analyses of the binding data (Fg 5.17) indicate that substantial differences exist between receptors in the basal and insulin stimulated states and while receptors in the latter state might be approaching saturation, those in the basal state appear to be a long way off saturation. Fig 5.17.a further implies that while the basal state contains a large amount of low affinity binding sites, the insulin simulated state consists of a smaller number of high affinity receptors.

Effects of Monensin/PhAsO on Insulin Binding and Degradation

Monensin has been shown to interfere with the acidification of lysosmal and prelysosomal compartments, thereby inhibiting dissociation of internalized ligand-receptor complexes and in so doing exerts a block on lysosomal ligand destruction and receptor recycling. (Maxfield, 1982; Mellman *et al.*, 1986; Wolkoff *et al.*, 1984). It has been reported that $10\mu M$ to $100\mu M$ monensin caused a considerable amount of ^{125}I-iodoinsulin activity to accumulate in isolated rat adipocytes within 30 min (Ueda *et al.*, 1985) while Whittaker *et al.* (1986) showed that monensin ($25\mu M$ for 15 min at $37^{\circ}C$) produced a 50-60% decrease in surface binding accompanied by a similar decrease in internalization and total inhibition of insulin degradation.

Certainly over short time periods (<30 min) a considerably reduction in ligand destruction occured in the presence of monensin as compared to untreated cells (Fig 5.18). In particular in the presence of $100\mu M$ monensin no loss in cell associated radioactivity occured within the first half hour (Fig 5.19). This observation was in some agreement with Ueda *et al.*, (1985) and Whittaker *et al.*, (1986), however

it is quite clear that with increasing time monensin could only partially restrain ligand destruction and raises the possibility that only partial inhibition of receptor recycling might also be occuring with increasing time.

This property of monensin to incompletely inhibit ligand destruction probably reflects the inadequacy of this ionophore to completely deacidify the endosomal compartments. Endosomes lower their internal pH by an ATP-dependent mechanism (Mellman *et al.* 1986). Treatment of cells with monensin result in the ionophore inserting into the endosomal membranes, where it preferentially exhanges Na^+ for H^+ ions with consequential quashing of the endosomal acidity. Because of the inability of cells to further process internalized ligand-receptor complexes extensive vacuolization occurs, which may be the result of osmotic forces, resulting in a time dependent enlargement of endosomes (Carpentier *et al.*, 1986b; Mellman *et al.*, 1986).

However since the monensin concentration is constant, with increasing time it may become less effective in deacidifying increasingly enlargening acidic compartments with resulting dissociation and subsequent degradation of hormone. It might be envisaged though, that if the monensin concentration in the medium is increased, the greater will be the ability to cause deacidification as time increases, resulting in prolonged accumulation of the ligand as is observed in Fig 5.19. The dose response curve for the effect of monensin on insulin binding (Fig 5.20) further supports this proposal. Insulin degradation is minimal in the presence of $100 \mu M$ monensin after a 2 hour incubation at 37^oC. However even this concentration of monensin is not absolute in its action (Fig 5.19).

It has been reported that monensin not only inhibits dissociation of H-R complexes but also prevents cycling of unoccupied receptors to the membrane (Wileman *et al.*, 1984; Mellman *et al.*, 1986). Though it cannot be accurately determined from Figs 5.18-5.20 whether "free" receptors are recycled, if it is that monensin inadequately deacidifys endosomes with increasing time resulting in subsequent ligand processing (Fig 5.18; 5.19), then it is possible that some degree of recycling might occur. Hence it is suggested that the maximal effect of monensin occurs after only brief exposure (< 30 min), after which some degree of H-R dissociation occurs as evidenced by increased ligand destruction (Figs 5.18, 5.19) and it might also be speculated that some amount or receptor recyling occurs.

In Chapter 4, the inhibitory effect of low PhAsO concentrations on glucose uptake was found to be transient. (Fig 4.8). This recovery of transport activity remained unchanged when adipocytes were treated with $25\mu M$ monensin for 20 min suggesting that the internalized complexes were allowing signal transduction (Fig 4.10). The data presented here supports this proposal. Monensin $(25\mu M)$ appears to be quite effective in accumulating cell associated radioactivity over short time periods (~30 min). In addition insulin stimulated cells contain ~45% of the cell receptors localized intracellularly. Therefore treatment of insulin-stimulated cells with monensin should result in a considerably build up of I-R complexes within the cells. Hence the ability of insulin stimulated, monensin treated cells ($25\mu M$ for 20 min), to fully recover from PhAsO $(1\mu M)$ inhibtion of transport strongly implies that the internalized complexes are biologically active.

C H A P T E R 6

Conclusion

In recent years a number of major advances have been made in understanding the mechanism of insulin activation of the glucose transport system. The realization that insulin can recruit intracellular hexose carriers and the elucidation of the amino acid sequence of both the insulin receptor and the D-glucose transporter must be recognised as significant contributions to insulin studies. However despite these efforts many aspects of insulin enhancement of sugar transport remain unsolved and no suitable mediator for this process has been identified.

The system used here to measure D-glucose uptake was quite consistent and appeared to be very reliable as evidenced by the low levels of non-specific (passive) diffusion and the extreme sensitivity of the transport activity to cytochalasin B. Routinely insulin enhanced D-glucose uptake 3- to 7-fold and although some researchers have claimed much higher levels of stimulation (Martz et al., 1986 Simpson & Cushman, 1986), other have reported stimulatory levels comparable to those obtained here (Table 3.1). Further since insulin is thought to enhance transport by increasing the V_{max} without altering the transporter Km (Whitesell & Gilemann, 1979; Taylor & Holman, 1981), then it is significant that the levels of stimulation shown here compares well with the 4- to 5-fold increase in transporter number that occurs upon insulin stimulation.

PhAsO has previously been reported to inhibit protein internalization in a variety of cell types (Wallace & Ho, 1972; Walker-Smith & Payne, 1983, Draznin et al., 1984) and indeed this agent was found here to be a potent inhibitor of protein internalization in rat adipocytes, as shown by its ability to completely block insulin induced receptor mediated endocytosis at $37^{o}C$.

However, in addition PhAsO was found to markedly inhibit stereospecific D-glucose uptake at concentrations where protein internalization was blocked. PhAsO reacts with vicinal sulphydryl groups to form stable cyclic dithioarsenite complexes. That the formation of such a complex is directly responsible for transport inhibition is suggested by the fact that inhibition could be significantly reversed by the reducing agent DTT.

The inhibitory effect of the arsenical was very rapid, apparent within 30 sec, and both basal and insulin stimulated states were affected. Since sulphydryl groups have been shown to have important structural and functional roles in both insulin receptors and D-glucose carriers, it is feasible that both these proteins may interact directly with PhAsO with subsequent inhibition of hexose transport. At the concentration of PhAsO examined, the arsenical was found to be a slightly less potent inhibitor of glucose transport than cytochalasin B and probably reflects the broad specificity of PhAsO.

Although PhAsO did not inhibit insulin binding at concentrations where it inhibited D-glucose transport, it does effectively inhibit internalization at this concentration, indicating direct interaction between receptors and PhAsO. Furthermore, high PhAsO concentrations markedly inhibited specific binding while at the same time significantly increased non-specific binding, thus raising the possibilty that this agent might interact adversely with membrane proteins even at lower concentrations.

Monofunctional sulphydryl reducing agents are not capable of reversing PhAsO action (Walker-Smith & Payne 1983   ; 1984) while bifunctional agents can cause reversal. This reflects PhAsO's ability

to react specifically with paired thiol groups. DTT was found to partially reverse PhAsO inhibition of both insulin binding and D-glucose transport. This partial reversibility was probably due to : 1) the establishment of a competition equilibrium between PhAsO and DTT for reaction with sulphydryl groups and 2) the disruptive effect of DTT on insulin binding to isolated adipose cells.

The binding isotherms for insulin to isolated adipocytes and plasma membrane preparations at 15°C are not identical. Additionally, DTT ($\leq$ ImM) has been reported to enhance insulin binding in isolated rat adipocyte plasma membranes (Jarett & Smith, 1983; Czech *et al.*, 1981; Schweitzer *et al.*, 1980). In contrast, DTT was found here to severely inhibit insulin binding, within this concentration range, in isolated adipocytes. It might be suggested that the receptor state in intact cells and isolated plasma membranes are not identical. Additionally, it is speculated that a DTT sensitive component associated with, but not an integral part of, the insulin receptor is involved in insulin binding in intact cells.

Treatment of insulin stimulated rat adipocytes with PhAsO resulted in rapid inhibition of D-glucose transport within 1 min. Such a sharp response to the arsenical indicates that the glucose carriers were being directly inhibited, since it might be envisaged that any inhibitory effect on receptors or putative mediators would result in a slower time dependent inhibition of transport as the transporters are slowly withdrawn into the cytoplasm. This however does not occur. Further, dose response curves for PhAsO inhibition of stereospecific D-glucose uptake was found to be dissimilar at low PhAsO concentrations; insulin-stimulated cells were more severely affected. It is proposed that the large number of transporter units in the plasma

membrane of insulin-stimulated cells is available for direct inhibition by low doses of PhAsO, while in basal cells the bulk of the carriers are intracellular and hence protected from these relatively small concentrations of PhAsO.

At low PhAsO concentrations inhibition of insulin stimulated D-glucose uptake is transient and recovery of stereospecific uptake occurs after approximately 20 min. Transport recovery is probably due to internalized receptors, protected from PhAsO, recycling to the plasma membrane, binding insulin, and facilitating recruitment of latent carriers, in accordance with the recruitment hypothesis. Furthermore, Oka & Czech (1984) have stated that a small but significant fraction of transporters remain unexposed to the extracellular medium after insulin treatment. However the data clearly demonstrates that an apparently large intracellular transporter pool exists and indicates that sulphydryl groups on the transporter are critical for glucose uptake, in agreement with May (1985).

Though the recovery of transport activity could be explained in terms of sequestration of transporters to the plasma membrane, the actual mechanism involved in this process is unknown. A number of substances have been suggested to mediate insulin action, including Ca^{2+}. Although much controversy surrounds the role of this ion in insulin action, substantial indirect evidence have implicated Ca^{2+} as having an important part in the enhancement of glucose transport (Pershadsingh and McDonald, 1984), and some of the data presented here are consistent with the involvement of Ca^{2+} in the activation of D-glucose transport activity.

Monensin was found to allow considerable accumulation of intracellular ^{125}I-iodoinsulin in a dose dependent fashion. Furthermore, $25\mu M$ and $100\mu M$ monensin effectively restricted intracellular destruction of insulin over short (~30 min) time periods. The ability of cells to undergo transitory inhibition of D-glucose uptake by low concentrations of PhAsO in the presence of monensin, implies that the internalized insulin receptor complexes might retain some biological activity, as suggested by Ueda et al., (1985).

During the course of this work Frost & Lane (1985) reported on the inhibition of hexose transport by PhAsO in 3T3 L1 cultured adipocytes and suggested that this agent interrupts the coupling system between the insulin-receptor and the glucose transporters. In a subsequent publication from the same laboratory, Bernier et al., (1987) reported that insulin induced phosphorylation of a 15KDa cytoplasmic protein when 3T3 L1 adipocytes were treated with PhAsO. This 15KDa protein was proposed as having an intermediary role in insulin action. However the data presented here is consistent with a direct inhibition of the arsenical on the D-glucose transporter. Furthermore, recent work done by colleagues in our laboratory have revealed that PhAsO inhibits cytochalasin B binding to the hexose carriers, demonstrating that PhAsO interacts directly with the transporters and possibly at the substrate binding site.

In summary PhAsO has been shown to be a potent inhibitor of : a) stereospecific D-glucose uptake, b) protein internalization and c) insulin binding (at sufficiently high concentrations). The data on inhibition of sugar transport is consistent with direct interaction with glucose carriers and implies that vicinal sulphdryl groups on the transporters are essential for proper functioning. The action of

PhAsO is partially reversed by DTT and this reducing agent is a potent inhibitor of insulin binding in intact cells. The internalized insulin-receptor complexes may continue signal transduction and there is evidence of a large intracellular transporter pool. It is also tentatively suggested that Ca^{2+} is involved in the activation of the transport system. Finally, it has been demonstrated that PhAsO can be a useful tool in the investigation of hormonal action.

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