The kinetics of hexose transport and the insulin response in adipocytes.

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The Kinetics of Hexose Transport and the Insulin Response in Adipocytes

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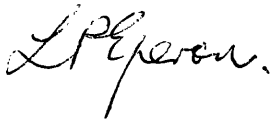

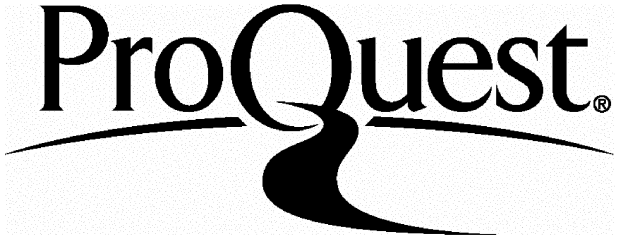

To Ian

...We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time
Through the unknown, remembered gate
When the last of earth left to discover
Is that which was the beginning...
Not known, because not looked for
But heard, half-heard, in the stillness
Between two waves of the sea.

from "Little Gidding", by T.S. Eliot

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Abbreviations

Standard abbreviations and symbols recommended by the IUPAC-IUB Commission on Biochemical Nomenclature have generally been used. Other abbreviations have been described as they appear in the text.

The term "affinity constant" has been used throughout the thesis to describe the Michaelis constant (K_m) or equivalent derivatives (K_{ee}, K_{zt}^{oi}, K_{zt}^{io}, K_{ic}^{io}, K_{ic}^{oi}). In the latter instance, the superscripts o and i and subscripts zt and ic refer to outside, inside, zero <u>trans</u> and infinite <u>cis</u> conditions, respectively.

CONTENTS

Abstract

THE KINETICS OF HEXOSE TRANSPORT AND THE INSULIN RESPONSE IN ADIPOCYTES

Lucy Pauline Eperon

The facilitated diffusion of glucose in the rat adipocyte was examined. Kinetic parameters were determined for cells in both the insulin-stimulated and basal states. Transport was studied in both isolated intact cells and partially purified membrane sacs or "ghosts". Comparisons between the transport in adipocytes and human erythrocytes were made, particularly with respect to inhibition by xanthine derivatives.

The external and internal kinetic parameters were determined by the use of zero trans, infinite cis and equilibrium exchange experiments. Initial rates were determined by direct measurement over short (one to two second) time intervals, critical for accurate estimates, and verified by integrated rate analyses of longer time courses for the sugar transport. Transport was measured using radiolabelled glucose analogues; the reaction was terminated with specific inhibitors followed by rapid separations of the cells from the extracellular medium.

The transport of 3-O-methyl-D-glucose in isolated intact rat adipocytes was shown to be symmetrical in entry and exit. The results were compared to previous studies by a determination of the equilibrium exchange parameters. The procedure used to isolate adipocytes was such that the cells were highly responsive to insulin. 3-O-methyl-D-glucose transport in the insulin-treated adipocytes was also symmetrical.

From the comprehensive kinetic determinations it was concluded that insulin stimulated hexose transport in adipocytes by increasing the maximum rate parameters equally for both influx and efflux, whilst both the external and internal affinity constants were unchanged. The significance of this

finding for models of the insulin-mediated activation of transport was discussed. Some possible mechanisms for the activation were tested.

In ghosts prepared from insulin-treated cells, the stimulation of transport was variable and at most a four-fold increase in initial transport rates was observed. Estimates of the kinetics parameters suggested that the transport symmetry of whole cells may not have been lost in ghosts. It was also shown that pink human erythrocye ghosts (from which 95% of the haemoglobin had been removed) retained the asymmetrical D-glucose transport found in the intact erythrocyte. The implications of these results for models of hexose transport were considered.

The specific inhibition by xanthine derivatives of transport in adipocytes and the insulin-insensitive human erythrocyte ghosts was compared. There were differences in the kinetics of inhibition by 3-isobutyl-1-methylxanthine. In adipocytes the inhibition of zero *trans* entry and equilibrium exchange transport was competitive, whereas in human erythrocyte ghosts zero *trans* entry parameters were unaffected by 3-isobutyl-1-methylxanthine, although the efflux of D-glucose into sugar-free solutions was diminished. These differences were discussed.

Chapter 1: Introduction

1.1. General Introduction.

The specific importance of insulin in regulating mammalian carbohydrate metabolism was first shown in 1922 by Banting and Best (Banting & Best, 1922). They purified a heat-labile substance produced by the pancreas and showed that it reduced blood sugar and sugar excretion in diabetic dogs. This substance, insulin, is a small polypeptide hormone with a molecular weight of 6000 daltons. It is secreted from the β-cells in the pancreatic islets of Langerhans into the blood stream, in response to high blood glucose levels. Insulin is an anabolic hormone, acting on adipose tissue, muscle and liver for its major effects, where it increases the synthesis of glycogen, lipid and protein whilst preventing the degradation of these compounds. The regulatory enzymes in the pathways for the production of storage products are well characterized (reviewed by Pilkis & Park, 1974; Czech, 1977). It has been demonstrated that insulin activates, for example, glycogen synthase (Lawrence *et al.*, 1977; Lawrence & Larner, 1978), pyruvate dehydrogenase (Coore *et al.*, 1971) and acetyl coA carboxylase (Halestrap & Denton, 1973) in adipose tissue. In addition to activating these enzymes within the cell, insulin increases the net rate of entry of D-glucose into muscle (Clausen, 1975) and adipose tissue (Avruch *et al.*, 1972), but not liver cells, mediated by the hexose transporter in the plasma membrane.

In spite of an enormous body of literature published on the subject, the exact mechanism by which insulin achieves these and other effects is still unclear. It is generally agreed that insulin acts at the target tissue by binding to a specific receptor in the cell membrane and that entry into the cell is unnecessary for the acute hormone response. Binding to the receptor must induce secondary changes which affect the relevant enzymes and

transporter, but the mechanism of these secondary effects is in dispute. It has been suggested that one or more "second messengers" may transmit signals from the activated receptors (see Robison et al., 1969). In the action of glucagon in liver and adrenalin in muscle, cyclic 3′,5′-adenine monophosphate (cyclic AMP) modulation of protein phosphorylation is involved as a second messenger (Butcher et al., 1966). Cyclic AMP has therefore been suggested as insulin's second messenger. Other candidates for this role have been Ca^{2+} (Fraser, 1975), and cyclic 3′-5′-guanine monophosphate (cyclic GMP) (Fain & Butcher, 1976). Small peptide fragments of insulin or a peptide formed internally by insulin-receptor activated cleavage, have also been suggested (Seals & Czech, 1980). For the activation of the glucose transporter, direct intramembrane modulation initiated by the insulin-receptor complex is another possibility. This might be caused by membrane protein aggregation (Jarett & Smith, 1974) or altered phospholipid fluidity (Melchior & Czech, 1979).

The mechanism by which insulin activates glucose transport has considerable biochemical importance. One way of analyzing this mechanism is to measure the kinetics of sugar transport in the presence and absence of insulin. Simple kinetic analyses have previously been applied to the glucose transport step of adipose tissue, when stimulated by insulin, and compared to the uptake in the absence of the hormone. However, a thorough kinetic characterization of the transport has not been described. The major part of the research described in this thesis was undertaken to provide a detailed kinetic picture of the glucose transporter from rat adipose tissue.

In the first chapter, a review of the molecular nature of insulin and its receptor, together with a survey of their interaction will be presented. The introduction to the monosaccharide transporter will begin

with a brief consideration of the identification and isolation of the membrane translocator. General aspects of the transport will then be described, followed by a detailed consideration of the kinetic studies on the glucose transporter in the most extensively studied system, that of the human red blood cell. Several models developed to explain the observed data will be introduced. The universality of the type of monosaccharide tranport system found in the intact human erythrocyte is analysed. The available data on sugar transport in mammalian muscle, an insulin-sensitive system is reviewed; it is found that the paucity of kinetic data precludes a full analysis of this mechanism in terms of the models orginally proposed for the transport in human red blood cells. In liver, a similar review leads to the conclusion that the details of this insulin-insensitive sugar transport differ from those for the prototype (red blood cell) system; in particular, recent work shows that transport is symmetric. In an introduction to the sugar transporter of adipose tissue, emphasis is placed on the methodological aspects of the studies reviewed, since the techniques used to measure the kinetics, in particular the determination of initial rates, is of crucial importance to the calculation of accurate kinetic parameters. A comprehensive survey of the parameters found in previous studies for both intact cells and "ghosts" (i.e. resealed cells from which intracellular contents, such as the fat globules in adipocytes or haemoglobin in erythrocytes, have been expelled by lysis) with the different kinetic procedures, will be left until the research described in this thesis is discussed in Chapter 4.

The second chapter reports the materials and methods used for preparing adipose tissue, cells and ghosts, and erythrocyte ghosts for transport measurements. The techniques used to assay transport are also described.

Chapter 3 documents the quality of these preparations and the results

of rapid kinetic measurements in preparations from rat adipose tissue, both with and without added insulin. Some kinetics of hexose uptake in adipocyte ghosts are described, together with electron micrographic work on these membrane preparations.

The third chapter also contains a description of kinetic studies on human erythrocyte ghosts. Xanthine derivatives have well documented effects on cell metabolism. Their specific, short-term effect is the inhibition of cyclic 3′,5′-nucleotide phosphodiesterase (Butcher & Sutherland, 1962). An interesting observation was that this type of compound could inhibit D-glucose transport in human red blood cell ghosts. A comparison of the rapid inhibitory effect in erythrocytes and adipocytes should highlight the differences or similarities between the two transporter mechanisms. Some of the kinetics of this inhibition on both erythrocyte ghosts and rat adipocytes are described.

The final chapter is a discussion of the significance of the results described in this thesis in the light of models for sugar transport and the mechanism of action of insulin.

1.2. Insulin and Its Receptor.

1.2.1. The Structure of Insulin.

Insulin is initially synthesized as a precursor, proinsulin, from which the connnecting "C-peptide" is cleaved, leaving an A and B chain interlinked by two disulfide bridges. The gene sequence (Lomedico *et al.*, 1979) and secondary and tertiary peptide structures of insulin have been determined. The amino-acid sequence was elucidated by Sanger and his colleagues (Sanger & Tuppy, 1951a & b; Sanger & Thompson, 1953a & b), and the three-dimensional structure analysed at 2.8Å and 1.9Å by x-ray crystallography (Adams *et al.*, 1969; Blundell *et al.*, 1971a, 1971b, 1972). The insulin crystals were

composed of insulin hexamers, co-ordinated by two zinc atoms. The similarity of the hexameric and monomeric insulins was demonstrated with circular dichroism: The incidence of circularly polarized light did not show significant differences in the α-helical content of the monomeric (solution) and the crystal forms (Pullen et al., 1976). At physiological concentrations (≅10 to 100 μUnits/ml or 10^{-10} to 10^{-9}M), most insulin is in the monomeric form.

This globular polypeptide has a hydrophobic core and two predominantly hydrophobic surfaces, thought to interact to stabilize insulin dimer and hexamer formation. Many of the hydrophobic residues in one of these areas, together with more polar surface residues, have been defined as the receptor binding region by analysis of the receptor binding capabilities and biological potencies of insulins from several species and chemically modified insulins (Pullen et al., 1976). Receptor binding could involve an antiparallel sheet structure in this region, similar to that seen between dimerised insulins.

1.2.2. The Distribution of the Receptor within the Plasma Membrane.

The extracellular location of the specific insulin binding site or "receptor" was indicated by Kono & Barham (1971) by binding studies on intact adipocytes with radiolabeled insulin. This was confirmed by Bennett & Cuatrecasas (1973), who showed that the inside surface of the plasma membrane did not exhibit insulin binding, as demonstrated with inverted fat cell membrane vesicles.

The distribution of the receptors on the cell surface has been studied; morphological evidence suggested that it may not always be uniform (Kahn, 1976). In fat (Jarett & Smith, 1974) and liver cells (Orci et al., 1975), the binding sites were found at approximately 90 receptors per μm^2, using

ferritin-linked insulin. Freeze-etching of liver cells showed that the external binding sites correlated with intramembrane particles within the bilayer (Orci et al., 1975). At 4°C, the insulin-receptor complexes seen in fat cells were randomly scattered had over the cell surface (Jarett & Smith, 1977). However, at higher temperatures receptors were diffusely distributed in some regions, whereas in others, clusters of three to twelve ferritin-insulin conjugates appeared in the electron micrographs (Jarett & Smith, 1974).

In later work (Schlessinger et al., 1978; Schlessinger, 1980), insulin was labelled by the attachment of α-lactalbumin-rhodamine complexes to enable visualization in the light microscope of the dynamic changes in the fluorescent patterns on the surfaces of 3T3-fibroblasts in response to hormone binding. Up to 20°C, there was a random, temperature-dependent movement of the hormone-receptor complexes, during which time patches formed. Internalization of the fluorescent probe required energy and occurred above 23°C, so the initial patching was independent of this process. Patching was also observed in 1M-9 lymphocytes (Schlessinger et al., 1980). Schlessinger (1980) suggested that patching may be important in eliciting the hormone's response. Several mechanisms for inducing patching were ruled out: in particular, the involvement of microfilaments since it was not affected by metabolic inhibitors, cytochalasin B or colchicine inhibitors. Schlessinger et al. suggested that the subsequent larger clustering of the complexes, followed by the appearance of unlabelled patches within the clusters, is associated with the "down regulation" of the hormone (Schlessinger et al., 1980). Jarett & Smith (1979) found a correlation between glucose transport inhibition in adipocytes (by cytochasin B) and disaggregation of receptors visualized by ferritin conjugation in the electron microscope. Again, microfilaments did not appear to be involved,

since cytochasin D had no effect on receptor aggregation or the stimulatory effects of insulin on glucose transport.

1.2.3. Insulin Binding to the Plasma Membrane.

Knowledge of the nature of the interaction of insulin with its receptor has been greatly increased since the hormone has been available in a specifically iodinated form. The labelled hormone can be produced at specific activities as high as 2200 Ci/mmol (Kahn, 1976). The ^{125}I-label, attached to the tyrosine residue A14 by direct iodination with Na^{125}I in the mono-iodinated A-chain form (Springell, 1961), designated ^{125}I-insulin, does not interfere with the insulin binding activity, or the biological response to the hormone (reviewed by Ginsberg, 1977). Another form of radiolabelled insulin has recently been produced with a specific ^{125}I-label in the B-chain, by substitution of phenylalanine B1 by ^{125}I-iodotyrosine (designated ^{125}I[tyr]-insulin) (Assoian & Tager, 1981). It has similar properties to ^{125}I-insulin and should prove to be a useful additional probe for insulin binding and in particular the fate of the B-chain.

Equilibrium binding studies, on liver cells and lymphocytes, designed to measure the numbers and affinities of the receptors, when analysed according to Scatchard (1949), revealed that the binding of ^{125}I-insulin to cell membranes was complex: The Scatchard plots were curvilinear (DeMeyts et al., 1973, 1976; Kahn et al., 1974).

The first explanation of this data was that there was more than one type of binding site for insulin, each exhibiting different affinities for insulin (Gavin et al., 1973; Kahn et al., 1974). Another explanation is that insulin binding exhibits "negative co-operativity", that is, that as the proportion of receptors occupied by insulin increases, there is a

progressive decrease in the affinity of individual receptors for insulin (DeMeyts et al., 1973, 1976). Thirdly, insulin degradation products may account for the pattern by the cumulative effect of a range of partially active insulins contributing to the net equilibrium binding (Donner, 1980).

In addition to the equilibrium binding approach, the kinetics of dissociation of ^{125}I-insulin under varying receptor occupancies have been measured. Acceleration of ^{125}I-insulin dissociation in the presence of excess unlabelled hormone would have appeared to be good evidence in favour of the negative co-operativity explanation. However, recent observations show that this accelerated dissociation cannot alone be used as incontrovertible evidence for negative co-operativity. There are several examples of hormones showing this phenomenon when dissociating from cell membranes, when non-cooperative binding (Hill coefficients of 1.0) occurs at the relevant ligand concentrations (Pollet et al., 1980; Limbird et al., 1975). There are also the interesting instances of ^{125}I-insulin dissociation from talc being enhanced by the native hormone (Cuatrecasas & Hollenberg, 1975) and ^{125}I-abrin (a lectin) dissociation from neuraminidase-treated fetuin bound to Sephadex beads, increasing in the presence of unlabelled lectins (Sandrig et al., 1978).

Although enhanced dissociation alone should not be taken as conclusive proof of negative cooperativity, it is nevertheless a useful indicator when used in conjunction with other analyses. By combining measurements of dissociation rates of ^{125}I-insulin with increasing unlabelled hormone and the equilibrium binding measurements, DeMeyts et al. (1978) have defined a specific invariant region of the insulin molecule within the putative receptor binding region that appears to be particularly responsible for inducing negative cooperative interactions. They screened twenty-four insulin analogues, which were synthetically modified or found in different

species. They varied in sequence and up to 1000-fold in their receptor affinities and biological potencies. Not all of the insulins showed negative cooperativity. By comparisons of the insulins the domain responsible for negative cooperativity was found to contain the terminal A21 asparagine, A19 tyrosine and eight residues near the C-terminus of the B-chain (particularly B23 to B26).

It had been suggested that negative cooperativity between insulin and its receptor could be due to insulin dimer formation. It was proposed (Cuatrecasas & Hollenberg, 1975) that insulin-insulin binding would seclude the receptor binding region and therefore the number of insulins bound to the receptor would decrease at high insulin concentrations where dimerization is more favourable (Goldman & Carpenter, 1974). However, using two analogues that do not dimerize, DeMeyts et al. (1978) showed that negative cooperativity was not lost at high insulin concentrations.

Although dimerization is not important in facilitating insulin-receptor binding, thermodynamic analyses show that the "hydrophobic effect" may be involved (Waelbroeck et al., 1979). Blundell and coworkers (Pullen et al., 1976; DeMeyts et al., 1978) first suggested that the hydrophobic effect, ie., the sequestration of surface non-polar residues within the hormone-receptor interface, might be the major contributor to complex formation. In Waelbroeck's thermodynamic analyses, this was implicated by the non-classical nature of the insulin-receptor binding in IM-9 lymphocytes when analysed with van t'Hoff plots (the affinity constant for the high affinity state of the receptor was plotted as a function of the reciprocal of the absolute temperature). The enthalpic and entropic changes found in this analysis of the hormone-receptor complex formation decreased signficantly as the temperature was lowered. A large negative heat capacity change was observed on insulin-receptor binding at 25°C. However,

although the data may be explained by the hydrophobic effect, the superimposition of plots describing the temperature dependence of the affinity constants (classical or non-classical) of several steps in a hormone-receptor binding event could also account for the observation of curvilinear van t'Hoff plots.

The negative cooperativity effect described by thermodynamic and kinetic measurements is affected by pH (Gavin et al., 1973), and insulin concentration as well as temperature (DeMeyts et al., 1976). These workers find that increasing the temperature to 37°C diminishes the effect with IM-9 lymphocytes, although Olefsky & Chang (1978) show an increase in negative cooperativity at higher temperatures.

There is, however, some dispute as to whether there is an enhancement of ^{125}I-insulin dissociation in the presence of excess native hormone. Both DeMeyts et al. (1976) and Sonne & Gliemann (1980) find this phenomenon in IM-9 lymphocytes but in direct contradiction, Pollet et al. (1977) find no such enhancement. These latter authors find that the rate of dissociation of ^{125}I-insulin is independent of receptor occupancy and apparently find the reverse effect to that expected with a negatively cooperative system: namely, that the enhanced dissociation occurs as the total number of receptors occupied decreases.

The negative cooperativity model and the multiple affinity receptor hypotheses have been combined by Olefsky & Chang (1978), whose dissociation data on rat adipocytes was adequately described by this combined model. In the model proposed, high affinity, low capacity and low affinity, high capacity receptors were described, in addition to negatively cooperative interactions.

A specific interpretation of the insulin binding data as revealing more than one type and affinity of binding site, with no negatively cooperative

interactions has recently been put forward by Hersberg et al. (1980). With studies on human erythrocytes, two classes of binding site of high and low affinity, with one site of high affinity per cell, fitted the data well. It appeared that only the high affinity site was blocked by concanavalin A. The model would be invalid if the different sites occurred on different cells, which is a reasonable argument in view of the heterogeneity in age of a red blood cell population (Levitski, 1981). In the study, the location of one high affinity site per cell was not proven.

Studies on both human red blood cells and IM-9 lymphocytes may be criticised since neither cell exhibits a biological response to physiological concentrations of insulin, in spite of specifically binding to the hormone. Although this binding in IM-9 lymphocytes shows similar characteristics to those shown by insulin's target cells (Gavin et al., 1973), there are specific differences (Sonne & Gliemann, 1980). One similarity is that the relative affinities of insulin analogues are ranked as for adipocyte receptors (Gavin et al., 1973). Also, the apparent dissociation constant (K_d) is of the same order of magnitude in both cells. However, although K_d is similar, when the kinetics of dissociation are studied under physiological conditions (pH 7.4, 37°C), Sonne & Gliemann (1980) have shown that the rate constants vary considerably. The turnover of insulin was ten times greater than in isolated fat and liver cells, even at low receptor occupancies. They also reported that the negative cooperativity phenomenon (described in kinetic terms) was more pronounced in lymphocytes than in the target cells.

Studies on rat adipocytes have shown that the phenomenon of insulin receptor binding cannot be studied in isolation from the processes of hormone degradation (Gliemann & Sonne, 1978). In the study of Waelbroeck et al. (1979) conditions were used where degradation and internalisation of

insulin were insignificant. Controlling the conditions such that degradation does not interfere with the attainment of equilibrium binding, or the kinetics of dissociation of fully active insulin, is particularly important in the light of the recent finding by Donner (1980), when studying rat hepatocytes. He found that insulin degradation products show a range of binding affinities and that the combination of their binding patterns would lead to non-linear Scatchard plots. When true intact ^{125}I-insulin concentrations have been calculated, linear Scatchard plots were found, representing 36000 binding sites per cell, $K_d \cong 0.4$ nM. When no such correction was made, Donner described the binding data in terms of two classes of binding site. About 30000 sites per cell were a high affinity type ($K_d \cong 1.0$ nM) and many more (600000 per cell) were of low afffinity, $K_d \cong 60$ nM, although a negative cooperativity explanation was equally tenable. In this study, the ^{125}I-insulin fragments assayed were extracted with trichloroacetic acid precipitation from membranes to which they were bound. In contrast, Terris & Steiner (1975) measured the levels of partially degraded ^{125}I-insulin in the medium to assess the possible interference in the binding and degradative processes. Since they found no measurable intermediate degradation products, they assumed that only low levels of, or transient, intermediates were produced.

1.2.4. The Degradation of Insulin.

In their study of isolated hepatocytes, Terris & Steiner (1975) found a first order dependence of degradation on the total concentrations of insulin bound at the steady state over a wide range of insulin concentrations. The degradation was specific for ^{125}I-insulin (the oxidized A- and B-chains did not compete for degradation), and this specificity was determined primarily by the specificity of the binding; the extent of competition for

degradation by analogue insulins was directly related to their binding affinities. The simplest explanation of their data was that insulin bound to heterogeneous sites, with a K_d for a saturable component of 3.5 X 10^{-9}M. About 60% of this bound ^{125}I-insulin was subsequently released, the rest was degraded. The temperature dependence, and time lag observed before degradation occurred led Terris & Steiner to propose that degradation was distinct from receptor binding, although it was dependent on binding for its initiation.

Working with liver plasma membranes, Freychet et al. (1972) had previously suggested that the binding and degrading activities were separate. This was based on evidence that ^{125}I-desalanyl-desasparaginyl-insulin, although possessing only 2% of the binding affinity of insulin, was degraded to a similar extent as the native hormone. Terris & Steiner noted that there were methodological short-comings in this study, not the least of these being a lack of direct assessment of the ^{125}I-insulin degraded. It was also possible that non-specific degradative enzymes had been released from the liver and were contaminating the plasma membrane preparation.

The liver's function with respect to insulin differs from that of other tissues in that it is required to degrade a high proportion of the insulin in the bloodstream (Izzo et al., 1967). In the light of this difference it may not be surprising that differences exist between the degradation system of this and other cells. In IM-9 lymphocytes there was no evidence of a specific degradation system for insulin mediated by receptor binding, although a receptor-independent degradative process was present (Sonne & Gliemann, 1980).

Degradation has also been studied in adipocytes. Gammeltoft & Gliemann (1973) showed that most of the degradation of insulin by rat adipocytes

occurred by a process unrelated to receptor binding: the apparent K_m for the overall degradative process was 100-fold greater than the dissociation constant for binding, and the rate of degradation was much higher than the total turnover of insulin-receptor binding. Under different conditions, notably where non-specific protease activity was significantly inhibited by high albumin concentrations, Gliemann & Sonne (1978) demonstrated that a receptor-mediated process also contributed to insulin's degradation. Any partially degraded, labelled products were rapidly digested by the cell, and no partial products were measurable externally, as was the case for hepatocytes (Terris & Steiner, 1975). About 50% of the bound ^{125}I-insulin was degraded, regardless of the receptor occupancy. With this model, the rest would dissociate as ^{125}I-insulin. This is very similar to the model proposed previously for hepatocytes (Terris & Steiner, 1975), but does not agree with that put forward by Olefsky et al. (1979). Olefsky et al. suggested that insulin initially bound to low affinity receptors where insulin was not degraded and only intact ^{125}I-insulin was released; a minority of these complexes were converted into a high affinity (slowly dissociating) state. These high affinity receptor-bound ^{125}I-insulin molecules were efficient substrates for an insulin-degrading system so once they were formed, both intact ^{125}I-insulin and degradation products were released into the medium.

Unlike Gliemann & Sonne (1978), Kahn & Baird (1978) found measurable quantities of partially degraded iodioinsulin, which could be resolved, by gel filtration, into two types of species, of high and low molecular weights. Later, Goldstein & Livingston (1980) were to suggest that the high molecular weight product was probably artefactual, since it was not necessarily dependent upon cell material for its formation. The binding characteristics described by Kahn & Baird also differed, in that steady

state binding was not observed at 37°C, but the amount of ^{125}I-insulin bound to the cell rapidly decreased after about fifteen minutes: Gliemann and Sonne found that the steady state of bound insulin was maintained under apparently similar conditions, for at least 140 minutes.

Protease digestion suggested that important parts of the degrading system(s) were located in the plasma membrane. Crofford et al. (1971) showed that adipocyte plasma membranes prepared from trypsin-treated cells did not degrade insulin. Hammond & Jarett (1975) and Terris & Steiner (1975) treated fat cells with trypsin and thereby reduced degradation. Receptor binding was reduced concommitantly in all these cases, possibly reflecting that this is the important membrane-situated trigger for degradation, but not excluding the membrane location of other parts of the degrading system.

In a detailed and systematic study of ^{125}I-insulin degradation by adipose tissue, adipocyte, fractionated membranes and cytosol, and combinations of the latter, Goldstein & Livingston (1980) suggested that most of the initial breakdown occurred in the cytosol, plasma membranes serving to accelerate proteolysis of the small fragments produced. Small peptides and/or amino acids were then very rapidly ejected from the cell. Interestingly, the plasma membrane fraction did not appear to be involved in the initial cleavage of ^{125}I-insulin.

1.2.5. The Isolation and Purification of the Insulin Receptor.

Insulin receptors from at least ten different tissues are similar in binding thermodynamics and kinetics, pH optimum, characteristics described by negative cooperativity, ranking of analogue specificity and sensitivity to anti-insulin antibodies (Ginsberg, 1977; Kahn, 1976). Thus the specificity and potency of the interaction of an insulin molecule with the

receptor is determined by the species origin of the insulin, and not that of the receptor: the receptor structure must be highly conserved (Blundell & Humbell, 1980; Mueggeo et al., 1979). A molecular description of the receptor may thus be constructed from studies of receptors from many tissue sources.

The structural features of the insulin receptor have recently been a subject of much interest. Many different approaches have been taken to purifying the putative insulin receptor from a variety of tissues. The type of approach may be divided into two classes: (A) those which involve neutral detergent solubilization followed by a combination of differential centrifugation, molecular sieving and specific ligand, lectin or antibody affinity and ion exchange chromatography, and (B) those where cross-linking probes are used.

When searching for a specific hormone receptor it is important to use a sensitive assay of binding. In the earliest studies (Cuatrecasas, 1972; Ginsberg et al., 1976), the functional purity of the "receptor" was defined by an insulin binding assay of native insulin displacement of ^{125}I-insulin binding. In one study, (Maturo & Hollenberg, 1978) non-specific insulin binding could variably account for up to 75% of the total. Particular specificity for insulin was measured by assessing competition for binding to receptor with other members of the insulin family. This assay has been criticised (Harrison & Itin, 1980) when insulin has itself been used as a selective affinity purification step where "leakage" from the insulin-agarose (Jacobs et al., 1977) or insulin-Sepharose (Maturo & Hollenberg, 1978) affinity column might interfere with the binding assay. An additional assay of purity was developed, wherein autoantibodies (B2 and B3) to the insulin receptor were used to measure receptor yield. Criticisms may also apply to the use of this assay after an immobilised autoantibody or

insulin affinity column purification (Harrison & Itin, 1980).

The overall receptor yield was initially estimated solely in terms of protein concentration (Cuatrecasas, 1972). In subsequent studies, radioactive iodination of the membrane proteins prior to receptor solubilization was used, enabling the efficency of successive purifications to be quantified (Heinrich et al., 1980). Differential isotope labeling (^{125}I and ^{131}I) was employed, in order that the ^{125}I-insulin displacement assay was not interfered with (Harrison & Itin, 1980).

The main criticism of the early methods of purification is that the purity and overall yield of receptor were very low (e.g. Cuatrecasas, 1972; Jacobs et al., 1977; Ginsberg et al., 1976). A partial purification of the receptor from rat liver and fat cell membranes by Cuatrecasas (1972) demonstrated insulin binding in aqueous solvents containing 0.1% Triton X-100. The detergent was necessary to prevent extensive protein aggregation. Gel filtration on Sepharose 6B gave a single broad peak with specific insulin binding corresponding to a Stokes radius of 70Å, and a minor non-specific peak. Insulin binding was unaffected by low concentrations of denaturants (SDS, urea & guanidinium chloride) or high concentrations of salt. The capacity of the "receptor" fraction to bind insulin was destroyed by low concentrations of trypsin. A sedimentation constant of 11S compared to ^{14}C-labeled standards, was found on sucrose gradients. These data suggested that the receptor had a molecular weight of 300000, assuming that it was highly asymmetric.

This partial purificiation technique was applied to avian erythrocyte membranes by Ginsberg et al. (1976). Without insulin, a single fraction (Stokes radius = 72Å), lacking negative cooperativity characteristics of insulin binding, was eluted, but insulin pre-incubation caused a smaller peak (Stokes radius = 40Å) to occur. Insulin allowed interconversion of the

forms, and Ginsberg et al. suggested that a tetrameric complex with four 75000 dalton subunits could dissociate with a concomittant loss of negative cooperativity. Krupp & Livingston (1978) working on fat cell membranes, showed two components (I & II), on SDS gels with different binding affinities for insulin. Unlike the crude avian receptor of Ginsberg et al., II could not be converted into I, although the reverse could be induced, by insulin. It was suggested that sites of different affinity for insulin existed.

Jacobs et al. (1977) introduced two new separation steps for rat liver microsomal membranes bound to ^{125}I-insulin, before a Sepharose 6B filtration. A seven-fold enrichment of ^{125}I-insulin binding was obtained from DEAE cellulose. Insulin-agarose affinity chromatography was used for the second step; 85% of the binding "activity" was adsorbed to the column. Urea as the elutant gave a 2000-fold increase in the specific activity of the insulin binding affinity, even though only 12% of the protein in the fraction bound to insulin. After the third step a fraction with a Stokes radius of 72Å was again found as the major species. Only a half of this fraction showed specific ^{125}I-insulin binding, and the overall yield, on the basis of protein concentration was very low (≅10%). On an additional purification on a lectin column (concanavalin A linked to agarose, with α-methyl mannopyranoside as the elutant) up to 60% of the insulin binding activity was lost. SDS gel electrophoresis, under reducing conditions, of the material from the Sepharose 6B column showed a major band with an apparent molecular weight of 135000. Since the Stokes radius (72Å) was consistent with a molecular weight of 300000, it was suggested that the receptor was composed of two or more subunits. It was noted that the SDS binding of the receptor, being a glycoprotein, could be anomalous.

The structure of the receptor purified by this procedure was analyzed

in more detail on SDS gels (Jacobs et al., 1980). Reducing gels showed bands of M_r = 135000 and 45000. Minor bands at 90000, 75000 and 56000 were also seen. Chymotryptic peptide maps indicated that the major smaller species was not a breakdown product of the larger. Non-reducing gels had major, M_r = 310000 and minor, M_r = 180000 bands. Two-dimensional gels indicated that the two species were found in the same ratios and it was suggested that the native receptor was composed of two of each component, associated by interchain disulphide bonds and noncovalent associations. Neuraminidase-treated complexes showed bands at M_r = 125000 and 48000, possibly more truly reflecting the subunit molecular weights, by reducing SDS binding anomalies.

Maturo & Hollenberg (1978), when studying rat liver and fat cell membranes, compared the purified products from Sepharose 6B chromatography and an insulin affinity chromatography separation. In the latter method the pellet obtained by high speed centrifugation of detergent-solublized membranes, was chromatographed on an insulin-Sepharose 4B gel, and the bound fraction eluted with urea in sodium acetate as described previously (Jacobs et al., 1977); the major purified fraction had a Stokes radius of 38Å. The results of the Sepharose 6B chromatography conflicted with those of Cuatrecasas (1972): two peaks of Stokes radius 70Å and 38Å were found. A glycoprotein fraction obtained from an additional concanavalin A-agarose purification of the residual material from the Sepharose 6B column, when combined with the affinity column purified material, caused the Stokes radius to increase to 70Å. Leakage of insulin from the affinity column was discounted on analysis of the competitive binding properties of the eluted fractions.

A much improved yield was obtained in the combination of steps devised by Williams & Turtle (1979). A 2000-fold increase in the specific activity

of the receptor was achieved. Each successive step was monitored by gel electrophoresis: initially thirteen bands were shown from a Sepharose 6B column fraction with a Stokes radius of 72Å; the second stage, phosphate gradient elution from a hydroxyapatite column, gave six SDS gel bands after; the final step, adsorption to a Concanavalin A-agarose column and elution with a hexose, the receptor appeared as a single band on acrylamide gels in the presence of 0.1% Triton X-100, with no reductant, but low percentage SDS acrylamide gels and reductant gave a major band (M_r = 75000) and a very minor, M_r = 80000, band. On rechromatography on Sepharose 6B, the receptor complex had an apparent molecular weight of 300000 daltons. "Insulinase" activity was excluded early in the purification, thus artefacts due to its action were minimized, unlike the method of Jacobs et al. (1977). The necessity of diminishing proteolysis was later shown by Harrison & Itin (1980). In an earlier study (Lang et al., 1980) on human lymphocytes (IM-9), four bands on SDS gels were demonstrated. When the purification was repeated with protease inhibitors present, only two gel bands (M_r = 130000 and 90000) were found (Harrison & Itin, 1980).

Williams & Turtle (1979) found that pH < 7 drastically affected the insulin binding activity, and they suggested that the elutants of the affinity column used by Jacobs et al. (1977) could have partially accounted for their low yield. This is consistent with the work of Harrison & Itin (1980), also studying the human placental receptor. These workers tested potential elutants on a crude receptor fraction prepared as the first stage of a more extensive purification, revealing that urea caused an irreversible loss of >80% of the insulin binding. The first stage of this purification, from membranes previously radiolabelled as whole cells, was application to a lectin (wheat germ agglutinin) column and displacement with N-acetyl glucosamine. Almost complete recovery, a twenty-fold purification and

exclusion of insulinase activity was obtained, a distinct improvement on the previous 30% elution from a similar system (Jacobs et al., 1977). The eluant was then purified on a column of anti-insulin IgG and autoantibodies to the insulin receptor, and the receptors were removed with 2.5 M $MgCl_2$ at pH 6.5. Unfortunately an irreversible 80% loss of binding was shown when this elutant was tested on the crude receptor, but none better could be found. This step gave a twenty-fold increase in binding and a 40-fold increase in immunoreactivity. 8% SDS gels, under reducing conditions showed three major bands at 126000, 90000 and 42000 and sometimes a 116000 minor species, thought to correspond to the asialo-derivative of the largest species.

The last major development in preparative procedures for the isolation of the insulin receptor was the introduction of chemical cross-linking in attempts to covalently attach ^{125}I-insulin to its receptor in order to stabilize the interaction prior to purification and analysis of the receptor subunits. Both general cross-linking reagents and specific photoreactive ^{125}I-insulin derivatives have been used.

In the first method (Sahyoun et al., 1978), the chemical cross-linking of ^{125}I-insulin to rat liver membranes was done under harsh conditions; prolonged incubation with glutaraldehyde and sodium borohydride reduction at pH 9.6. After homogenization and detergent solubilization, a Sepharose 6B separation gave four peaks. The major peaks had Stokes radii of 72Å and 38Å, which was consistent with previous studies without cross-linkers (Maturo & Hollenberg, 1978). All four peaks were slightly decreased by unlabeled insulin in the cross-linking reaction, although only the 72Å peak was significantly affected. The apparent lack of specificity was probably due to the harsh conditions used for cross-linking (Pilch & Czech, 1979).

Less extreme measures were adopted by Pilch & Czech (1979) who cross-

linked ^{125}I-insulin to rat adipocyte membranes with disucccinyl suberate (DSS) activated by photolysis. The advantages of this reagent were: cross-linking was rapid even at low temperatures and under physiological conditions; a stable amide bond was formed; and the membranes remained susceptible to solubilization. General cross-linking to the membrane proteins of intact cells was observed in subsequently prepared isolated membranes. Although cross-linking of intact cells retained three major bands on SDS gels (M_r = 100000, 78000 and 42000), autoradiography of cross-linked membrane preparations showed that ^{125}I-insulin was only specifically retained by DSS on a M_r = 125000 gel band.

Further studies (Pilch & Czech, 1980) gave similar data on whole fat cells and plasma membranes, and liver cell plasma membranes, although a minor band (M_r = 225000) and higher molecular weight, gel impermeant material was also specifically labelled. The unreduced receptor complex was a 300000 species. A more detailed analysis, on very porous gels, of the unreduced receptor of rat fat, liver, lung, kidney and human placenta (Massague et al., 1980) resolved this 300000 species into three distinct components (M_r = 350000, 320000 and 290000). After sequential reduction with dithiothreitol, it was concluded that three ubiquitous insulin receptor complexes exist, composed of three types of disulphide linked, distinctive subunits (α, 125000; β, 90000; β_1, 49000) in the stochiometries $(\alpha)_2(\beta)_2$; $(\alpha)_2(\beta)(\beta_1)$; $(\alpha)_2(\beta_1)_2$. However further work suggested that a single class of receptor $(\alpha)_2(\beta)_2$ was present in the intact fat cell plasma membrane, and that the occurence of the other two complexes was due to limited proteolytic cleavage of β mediated by a lyzosomal associated enzyme to give β_1. Elastase mimicked this conversion (Massague et al., 1981).

In spite of the apparent success of Czech and coworkers using

disuccinyl suberate, non-specific cross-linking of insulin to non-receptor surface proteins could introduce artefacts. In another approach, (Yip et al., 1978, 1980; Yeung et al., 1980) photosensitive ^{125}I-insulin derivatives of (N-hydroxysuccinimide esters of 4-azido benzoic acid) were synthesized and used to probe the specific insulin-receptor interactions by photolytic cross-linking, with an analysis of the unpurified membranes by autoradiography on 10% gels.

In a preliminary study of rat adipocyte membranes, with a mixture of azidobenzoyl insulin derivatives (Yip et al., 1978), many proteins were cross-linked, but only one (M_r = 130000) was specifically reduced by excess native insulin, although subsequent work (Yip et al., 1980) suggested that an M_r = 90000 component was masked by competition for binding with unmodified ^{125}I-insulin in the crude mixture of the "photoprobes". The binding of the photoprobe and photolysis was rapid and carried out at pH 7.4. The method was refined by separation and analysis of the photoprobes (Yip et al., 1980): rat, mouse or guinea pig liver membranes with mono- (at B29) or di- (at A1 and B29) substituted insulins, gave two labelled SDS gel bands (M_r = 130000 and 90000). The labelling intensity was dependent on the dose of native insulin and competition with insulin analogues reflected their biological potencies. Yeung et al. (1980) found that with an α-amino B1 derivative only a M_r = 130000 band was specifically labelled and suggested that spatial arrangements of the insulin-receptor complex prevented the tagging of the 90000 species. In a similar study on rat liver membranes (Wisher et al., 1980), aryl azide photoprobes at insulin residues A1, B1 or B29 gave an M_r = 130000 and a minor variable 100000 to 110000 band. But SDS gel mobility was shown to be anomalous, the invariant species had an apparent molecular weight of 130000 on 7% gels, but 110000 to 100000 on 8% gels. Assuming that the Ferguson analysis (Ferguson, 1964) was valid

in this instance, an adjusted M_r of 90000 for the invariant component was given.

Jacobs et al. (1979) combined the use of an aryl azide photoprobe with their previously developed purification procedure (Jacobs et al., 1977). The labelling of membrane preparation (from rat liver and human placenta) was compared with that of purified receptors from rat liver. In all three cases, under non-reducing conditions, a 310000 species was found. In the presence of 10 mM dithiothreitol, 7.5% SDS gels showed bands at 135000 in all cases, but a variable minor 90000 band appeared in the last instance. During receptor purification, a minor 75000 band, seen in the insulin-affinity column eluate was removed by the lectin affinity step. Cultured 3T3 cells also showed a 135000 species with this photoprobe.

A recently developed technique has been used to probe the structure of the receptor in situ. This is in the study of Harmon et al. (1980) where "radiation inactivation" gives a quantitative measure of target size by controlled tissue damage with high energy radiation. Components of 87000 ± 13000 daltons which bind insulin and ≅350000 which seems to regulate receptor affinity were found in rat liver cells. There was a discrepancy in Triton X-100 solubilized membranes, which showed 104000 and 850000 binding and affinity components respectively.

In conclusion, there is considerable agreement that the receptor complex is a glycoprotein with an apparent molecular weight of 300000 to 350000 daltons, and a Stokes radius of 72Å. It may exist in multiple forms. The nature of the disulphide linked subunits is still in dispute. This problem is considerably exacerbated by anomalous gel mobility: even the conclusion that there is at least one subunit of 125000 to 135000 (Pilch & Czech, 1979, 1980; Jacobs et al., 1977, 1979, 1980; Yip et al., 1978, 1980; Yeung et al., 1980; Harrison & Itin, 1980) is doubtful in the light of the

corrections made by Wisher et al. (1980). It is possible that the subunits combined as $(\alpha\beta)_2$, M_r = 350000 found by Massague et al. (1980, 1981) represent a definitive description of the receptor, and that these subunits are selectively purified, modified (for example through specific conversion of β to β_1), or excluded by different preparative procedures.

1.3. The Mechanism of Transport Activation by Insulin: Some Current Hypotheses.

1.3.1. Multivalent Insulin-Mimetic Agents.

There are so many compounds and conditions that mimic the action of insulin on glucose transport that, taken individually, they are of limited use in providing evidence for insulin's mechanism of action. However, compounds in certain categories have given some notable insights into the mechanisms of transport activation.

One such class includes agents which bind to multiple sites on the plasma membrane; this leads to transport activation which can be independent of binding to specific insulin receptors. In this class are lectins: concanavalin A and wheat germ agglutinin were shown to stimulate fat cell glucose uptake (Cuatrecasas & Tell, 1973). Cuatrecasas showed that lectins bound to cell surface insulin receptors (Cuatrecasas, 1973a,b), but Czech & Lynn (1973) found that trypsinised adipocytes remained concanavalin-sensitive, implying that a non-insulin-receptor-mediated stimulation was occurring. This process may be complicated by the stimulatory effect of trypsin itself at low concentrations, shown in fat cell plasma membranes by Seals & Czech (1980). The ability of the lectins to agglutinate and initiate patching in fat cells led Czech (1980) to speculate that these phenomena were important in transport activation.

Other multivalent compounds used to probe the mechanism of transport

activation were antibodies. Studies with autoantibodies found in patients with acanthosis nigricans and insulin resistance showed that although these antibodies blocked ^{125}I-insulin binding, they mimicked insulin's action (Jarrett et al., 1976; Flier et al., 1975; Kahn et al., 1976; 1977). Antibodies (raised in rabbits) to the purified insulin receptor also activated hexose transport in fat cells (Jacobs et al., 1978). Although ^{125}I-insulin binding was not prevented by antibodies raised to the fat cell plasma membrane or intrinsic membrane proteins, hexose transport and lipogenesis in adipocytes were stimulated by these antibodies (Pillion & Czech, 1978; Pillion et al., 1979). It was shown that aggregation (minimally, the formation of dimers) was important in this insulin mimetic response, by demonstrating that monovalent fragments of either of these antibodies (to human insulin receptors, Kahn et al., 1978; or adipocyte membranes, Pillion et al., 1979) were ineffective in elevating transport rates, but that cross-linking with antibodies to these monovalent fragments elicited stimulation of both glucose uptake and inhibition of lipolysis. This insulin-like response of multivalent agents indicates that aggregation of binding "sites" in the membrane may be involved in the activation of transport. It is pertinent to recall that insulin binding appears to induce aggregation of receptors previously scattered in the membrane observed with the electron microscope (Section 1.2.2.). Inferences from these multivalent compounds to insulin are interesting, but the former results may reflect the general effects of membrane binding agents rather than the specific manner in which insulin activates transport under physiological conditions.

1.3.2. The Role of Sulphydryl Reagents.

In another hypothesis of transport activation by insulin, it was proposed that the oxidation of certain membrane sulphydryls to the

disulphide form was the signal by which transport rates were elevated. In this model, the number of these relevant sulphydryls in the activated, disulphide form at any time determined the hexose transport rate exhibited by the adipocyte. Activation occurred when the hexose transport system, or a regulatory component of it, previously in the reduced, inactive form was converted into the oxidised, active state, thereby catalysing facilitated diffusion (Czech, 1976a).

The relevant data was briefly reviewed by Czech (1977; 1980). It was consistent with this hypothesis that agents such as hydrogen peroxide and diamide activated sugar transport in fat cells, whilst reductants (such as dithiothreitol) inhibited transport (Czech et al., 1974a; Czech, 1976b; Mukherjee & Lynn, 1977; Mukherjee et al., 1978). NEM treatment of fat cells prevented stimulation by insulin, and reversal of the order of treatment (ie insulin prior to NEM) protected the activated transport from inhibition by the sulphdryl reagent (Czech et al., 1974a; Czech, 1976b).

This "thiol redox" model has been neither proven nor disproved and may be important in insulin's activation of transport, although there is no direct evidence for this hypothesis.

1.3.3. Soluble Peptides as Intracellular Mediators.

It has been suggested that an intracellular polypeptide acts as insulin's mediator of glucose transport elevation. In this model, the activation of a trypsin-like protease by insulin is responsible for generating the intracellular peptide fragment from a membrane-bound precursor molecule.

The evidence suggesting that the an intracellular polypeptide is important as a mediator of the post-receptor action of insulin is quite compelling. Polypeptide factors have been found in several tissues. With

regard to tissues with an insulin-responsive hexose transport system, Larner and his colleagues (Larner et al., 1979; Cheng et al., 1980), working on skeletal muscle from insulin-treated rats, detected a factor capable of inhibiting cAMP-dependent protein kinase, and of stimulating phosphoprotein phosphatase: mitochondrial pyruvate dehydrogenase (PDH_a) was activated by the gel filtration fraction containing this factor. From adipocytes previously treated with insulin, an isolated factor also with this latter activity (Kiechle et al., 1980) stimulated the low K_m cAMP phosphodiesterase from rat liver (Kiechle & Jarett, 1981). A factor extracted from rat liver showed sensitivity to insulin (increasing the factor's activity) and adrenalin (diminishing its activity). That both of the factors from adipose and muscle tissue could be degraded by several proteolytic enzymes (Seals & Czech, 1980), and the muscle tissue factor reacted with ninhydrin (Larner et al., 1979; Cheng et al., 1980) suggested that these factors were polypeptides.

Thus there is indirect evidence that an intracellular polypeptide can modulate insulin-sensitive enzymes. However, the role of such a polypeptide in stimulating glucose transport has not yet been described.

1.3.4. The Involvement of Calcium Ions.

Yet another mediator of the post-receptor actions of insulin has been suggested in calcium ions. In this hypothesis, it is the change in compartmentalisation of the putative second messenger which is thought to be affected by insulin. Fraser (1975) has proposed that insulin changes the plasma membrane conformation in such a way as to rapidly displace calcium ions from their high affinity membrane binding site, diminishing calcium efflux and increasing net glucose transport into the cell. The increase in calcium ions in the cytosolic compartment would promote dephosphorylation

and thereby inhibit enzymes such as triglyceride lipase and glycogen phosphorylase, in conjunction with activation of glycogen synthase and pyruvate dehydrogenase. In addition, Cheung (1979) has proposed that calcium flux could be controlled by binding to calmodulin, a 16700 dalton, calcium-binding protein.

Although it has been reported that calcium ions are necessary for hexose transport in muscle and fat tissue (briefly reviewed by Czech, 1980), elimination of these ions does not lead to complete abolition of insulin's stimulatory effect. Some studies on adipocytes do suggest that calcium ion binding (McDonald et al., 1976a) and flux within the cell (McDonald et al., 1976b) are affected by insulin. In purified adipocyte plasma membranes the calcium ion concentration does not affect basal or insulin-elevated D-glucose transport, although the size of the insulin-stimulated increase in transport was dependent on the calcium ion content of the buffer (Ludvigsen & Jarett, 1980a). As yet there are no clear conclusions to be drawn about the involvement of calcium ions in the mechanism of transport activation by insulin.

1.3.5. Other Mechanisms for Insulin-Induced Transport Stimulation.

The role of temperature changes in providing evidence for different mechanisms of transport and its stimulation by insulin was considered in the work described in this thesis. The results of this and previous studies will be discussed in Chapter 4.

Insulin effects on protein phosphorylation have been demonstrated in several cell types (Avruch et al., 1976; Benjamin & Clayton, 1978; Seals et al, 1979) and have been implicated in the post-receptor actions of insulin.

Potent inhibitors of glucose transport and comparisons of the type of inhibition observed in basal and the insulin-stimulated state in adipocytes

can probe the type of transporter responsible for basal as compared to insulin-activated transport. In this context, methyl xanthines were examined (see later chapters).

It has recently been proposed that hexose transport in adipoycytes is enhanced in response to insulin by the movement of extra transporters from an intracellular location to the plasma membrane, where they are inserted and act as fully functional transport glycoproteins (Suzuki & Kono, 1980; Cushman & Wardzala, 1980). In theory, this process could involve the synthesis of new transporter molecules, or post-translational modification, such as glycosylation, of precursor molecules. Signals for the movement of these molecules to the cell membrane can be considered by using potential inhibitors of the process. In the work described in this thesis glycosylation was investigated in this way using the inhibitor tunicamycin (Takatsuki et al., 1975).

1.4. The Identification and Isolation of the D-Glucose Transporter.

In the identification and isolation of the membrane components responsible for mediating D-glucose transport, contradictions similar to those found in the analysis of the insulin receptor were apparent. For example, in the apparently specific cross-linking of high molecular weight subunits, which do not copurify with a functional transport unit. A complete molecular description of the translocator has yet to be described.

Most of the studies have been carried out on the human erythrocyte. A summary of this work is described below. In contrast, relatively little is known about the molecular nature of the other transport system considered in this thesis, that of the adipocyte.

1.4.1. The Erythrocyte Transporter.

In the first studies of the structure of the glucose transporter in human red blood cells, attempts were made to find the transporter by radiolabelling it *in situ*, using SDS-polyacrylamide gel electrophoresis to identify specifically labelled bands. Three types of approach were adopted: general membrane protein inactivation with potentiation of the labelling of the transporter by transporter-specific compounds; differential labelling by protection with transporter-specific compounds; and specific labelling with "site-directed" inhibitors.

With the first type of approach, Jung & Carlson (1975) used a general reagent to inactivate membrane proteins, 1,fluoro-2,4-dinitrobenzene (FDNB). The presence of D-glucose significantly enhanced the reactivity of a fraction with an apparent molecular weight of 180000, relative to the dinitrophenylation of the bulk membrane. Shanahan & Jacquez (1976) also used this reagent, but observed bands labelled at even higher molecular weights.

Attempts were made to improve the recognition of the relatively small number of specific transport sites against the large background of non-specific sites. Differential labelling with the sulphydryl reagent, N-ethylmaleimide (NEM) was used, with initial protection of the glucose transport sites by a compound specifically binding these sites, for example, D-glucose (Kahlenberg *et al.*, 1971) or cytochalasin B (Lin & Spudich, 1974; Taverna & Langdon, 1973b). Using cytochalasin B, LeFevre *et al.* (1975) also found high molecular weight bands labelled, which were either the same weight, or heavier than spectrin (220000 to 250000 daltons). Batt *et al.* (1976) used D-glucose and cytochalasin B to protect the outside surface of the human erythrocyte from alkylation by another impermeant maleimide, glutathione-maleimide (Abbott & Schachter, 1976). By removing the D-glucose or cytochalasin B, and treating with radioactive glutathione maleimide, one

polypeptide component, migrating between Bands 4.2 and 5 (M_r = 65000 to 70000) was specifically labelled. Partial purification of the cross-linked fraction by elution from slab gels was undertaken, and comparisons with an unmasked preparation showed that only 24% of the material of this molecular weight had been specifically labeled.

Taverna & Langdon (1973a) treated human red blood cell membranes with a site-directed inhibitor, radioactive D-glucosyl isothiocyanate, which indicated that bands with apparent molecular weights of 75000 to 100000 were specifically labelled by the inhibitor (Bands 4 and 3, according to the nomenclature of Fairbanks et al., 1971). Another site-directed inhibitor, ^{14}C-maltosyl isothiocyanate was found by Mullins & Langdon (1980a,b). This analogue inhibited zero trans influx of glucose with a concommittant binding to Band 3 proteins. The binding was prevented by maltose or cytochalasin B, but not by L-glucose or sucrose which do not specifically interact with the transporter. Some other isothiocyanates which react with Band 3 proteins had no effect on D-glucose transport showing that this transporter was not the same protein as the anion transporter (also found in Band 3).

In order to decide which of these bands identified by differential labelling and cross-linking were definitely involved in glucose uptake mediation, it was important to isolate a functional transport molecule. This isolation has proved difficult: a particular problem has been the low yield of transporter.

Kasahara & Hinkle (1976, 1977) encountered this difficulty during an attempt to isolate a glucose transporter from protein-depleted human red blood cell membranes. In this procedure, the preparation was progressively assayed by measurement of D-glucose transport in the extract, when reconstituted in soybean phospholipid liposomes. In the earlier experiments

(Kasahara & Hinkle, 1976) a crude protein fraction was obtained by solubilization (with Triton X-100 or octylglucoside) from red cell ghosts, washed with EDTA and NaCl to remove about half of the protein and to convert the ghosts into small vesicles. The protein fraction, found in the supernatent after a 100000g centrifugation was freed from the Triton X-100 by treatment with polystyrene beads (Biobeads SM-2) at 4°C, or from octylglucoside by diafiltration to exclude molecules smaller than 50000 daltons. The protein was then added to the phospholipids and reconstituted.

In the modified procedure (Kasahara & Hinkle, 1977) one additional step, freeze-thawing, was introduced after adding the protein fraction to the phospholipids. This improved reproducibility and increased the extent of D-glucose transport. An additional step, DEAE cellulose column chromatography, was used to purify the Triton X-100 extract. The specific activity of the pure fraction was 85 nmol/min/mgPr with 0.2 mM glucose, ten-times higher than that found in the previous experiments with the crude Triton X-100 extract. In the earlier method (Kasahara & Hinkle, 1976) both Triton X-100 and octylglucoside solublized several proteins (70% and 56% of the total protein in the vesicles respectively) of which the main protein was Band 3 (M_r = 95000), four glycopeptides (observed with periodic acid-Schiff stain on SDS gels), and some phospholipids. The pure preparation (Kasahara & Hinkle, 1977) contained one major polypeptide (96% of the total protein) with an apparent molecular weight of 55000, and a small amount of a lower molecular weight band. In both these cases (Kasahara & Hinkle, 1976, 1977), the band at M_r = 55000 was diffuse.

Gorga *et al.* (1979) demonstrated that the broadness of the band was due to heterogeneous glycosylation of a single polypeptide: they found that only 70% of the glycosides (previously labelled with galactose

oxidase/NaB[^{3}H]$_4$) from the purified transporter were digested with β-galactosidase; only half of the transporter molecules were retained by a sugar-specific agglutinin during chromatography on R. communis agglutinin I-agarose (as assessed by cytochalasin B binding). Furthermore, the removal of sugar molecules with the endo β-galactoside caused the apparent molecular weight to decrease to 46000, and the coomassie blue stained band to sharpen. Additional evidence for the glycoprotein nature of the transporter had been provided by Sogin & Hinkle (1978) who demonstrated with a fraction with a molecular weight of 55000 (purified by detergent extraction and DEAE cellulose chromatography, and gel filtrations) that the pure transporter contained 17% carbohydrate by weight.

Kasahara & Hinkle (1976) showed that their crude protein fraction contained the D-glucose transporter by demonstrating that D-glucose was rapidly and stereospecifically taken up into the reconstituted liposomes: D-glucose was taken up thirty-six times faster than L-glucose over an "initial" fifteen second period. Inhibition of D-glucose transport with the unlabeled sugars, 2-deoxy-D-glucose, 3-O-methyl-D-glucose, D-mannose and D-galactose demonstrated the specificity of transport. Both mercuric ions and cytochalasin B inhibited the uptake in a manner similar to that seen in human red blood cells, but other inhibitors of D-glucose uptake in erythocytes and their ghosts, stilboestrol (LeFevre, 1961; Jung et al., 1971) and 10 μM phloretin (see Bowyer, 1957) did not inhibit the system, suggesting that some factors involved in transport inhibition were not present in either reconstituted system (Kasahara & Hinkle, 1976, 1977). That the liposome assay represented D-glucose transport and not binding was indicated in several ways, for example, by the inhibition of uptake after sonication for longer than three minutes, addition of non-ionic detergents, and an analysis of the binding capacity of the major protein. In the

modified procedure, electron microscopy was used to show that the liposomes were unilamellar. Antibody studies showed that the major protein was accessible to the inner membrane surface. The protein yield was about 1% of the total red blood cell membrane protein.

In a similar preparation, Kahlenberg & Zala (1977) report a thirteen-fold purification of the transporter. Reconstitution of glucose transport was obtained when a purified fraction from DEAE cellulose chromatography, with a molecular weight of about 55000 (Region 4.5) on SDS gels, was rapidly inserted into total membrane lipid liposomes.

Instead of monitoring the capacity for D-glucose uptake of the membrane extracts, Zoccoli et al. (1978) characterized the purification of the glucose transporter by its affinity for cytochalasin B (documented as a potent, reversible inhibitor of the monosaccharide transporter (Taverna & Langdon, 1973b)). In several respects, their purification (Zoccoli et al., 1978) and the improvements described by Baldwin et al. (1979) were similar to the methods developed by Kasahara & Hinkle. Zoccoli et al. (1978) removed all but a single set of high affinity sites for cytochalasin B by treatment of human red blood cell membranes with dilute EDTA and concentrated NaCl. The association of these sites with the specific D-glucose transport mediator was shown by competitive inhibition of cytochalasin B binding by D- and not L- glucose. After solubilization with Triton X-100 and removal of the residue by centrifugation, in the presence of dithiothreitol to prevent "-SH inactivation", Triton X-100 was removed with BioBeads SM-2 (Holloway, 1973); Triton X-100 was a potent inhibitor of cytochalasin B binding. Some of the molecular characteristics of the cytochalasin B binding fraction (assayed by equilibrium dialysis) were determined by Sepharose 4B gel filtration in H_2O and D_2O. The Stokes radius was determined as 6.6 nm, the partial specific volume as 0.85

cm^3/g, and the molecular weight as 225000 daltons. Zoccoli et al., discussing the variation between this molecular weight and that found by Kasahara & Hinkle (1977) concluded that Kasahara and Hinkle could have been measuring the transport activity of a minor contaminant of the purified protein (M_r = 55000), since Zoccoli et al. calculated that only 0.3% of the total protein was responsible for sugar uptake. Alternatively, they may have found reduced transport because the artifical membrane vesicles had a different phospholipid environment from intact cells, or because the extensive purification had damaged the transporter. Other explanations for the discrepancy in molecular weights is that the Triton X-100 in the Sepharose 4B filtration could lead to anomalously large complexes, or that a tetrameric complex was seen after Sepharose 4B filtration, with a monomer molecular weight of 55000 daltons, as observed on SDS gels.

In a brief report, Baldwin et al. (1979) described another procedure in which cytochalasin B binding was used to monitor purity. In this case, one major glycoprotein, with an apparent molecular weight of 55000 was purified. The dissociation constant for cytochalasin B from this pure preparation was 1.3 X 10^{-7}M, very similar to that for the intact erythrocyte. The initial depletion of protein from the membrane was achieved by alkali and EDTA treatment at 4°C. Again, Triton X-100 solubilization and DEAE cellulose column chromatography, followed by Triton X-100 removal from the protein with BioBeads SM-2 were used: fifty percent of the applied protein and phospholipid material was recovered from the column. The cytochalasin B binding fractions were mixed with acetone-washed soybean phospholipids in order to decrease the adsorption of the protein to the polystyrene beads. Specificity for D- over L-glucose binding was found in the purified fraction, demonstrating that the cytochalasin B binding fraction was associated with D-glucose transport. The K_d (from

Scatchard analyses) for cytochalasin B binding was similar for the crude Triton X-100 extract and the purified component. There appeared to be 0.4 cytochalasin B sites per polypeptide, suggesting that, in the native state, the cytochalasin B binding component, and by implication, the glucose transporter, was a dimer.

Morphological studies have been undertaken: freeze fracture electron microscopy showed particles of 62Å in diameter (corresponding to a molecular weight of 110000) and consistent with dimer formation from 55000 dalton monomers (Sogin & Hinkle, 1978); radiation target size techniques have suggested that the transporter could be a tetramer in the whole cell, with a monomer molecular weight of 50000 (Jung et al., 1980).

It may be concluded from these reconstitution and morphological studies that the isolated D-glucose transporter could be a glycoprotein dimer or tetramer, with a monomer molecular weight of about 55000 daltons. The exact involvement of species with higher apparent molecular weights has not yet been established; in particular, the involvement of a Band 3 protein convincingly suggested by the site-directed inhibitor studies has not been explained.

1.4.2. The Adipocyte Transporter.

Brenner & Kahlenberg and Brenner et al. (1977) studied the glucose transporter in human omental adipocytes. In this study, rather than isolating the glucose transporting component and inserting it into an artifical system to measure transport, a large proportion (70%) of the membrane protein was removed from the adipocytes with 2,3-dimethylmaleic anhydride (DMMA), and the transport activity in the remaining fractions was assessed. Only two major bands on SDS gels of molecular weights approximately 30000 and 80000 daltons, the heavier band being associated

with a PAS-staining band, were not extracted to any extent. The glucose transport was not decreased by the extraction procedure, so Brenner & Kahlenberg imply that either or both of these polypeptides may be mediating sugar uptake.

Shanahan & Czech (1977) used a similar principle to identify membrane components associated with rat adipocyte glucose transport. Here, up to 80% of the membrane proteins were removed with sodium hydroxide, hyperosmolar sodium iodide, or DMMA. The remaining fractions consisted almost entirely of two glycoproteins of 78000 and 94000 daltons on SDS gels. Small vesicles formed by sonication of these purified membrane preparations had a high affinity for [^{3}H]-cytochalasin B. The dissociation constant (K_d = 5 x 10^{-7}M) was similar to that in intact cells for a high affinity component: low affinity sites were also present. Cytochalasin B retarded both influx and efflux, showing that transmembrane fluxes, and not solely glucose binding was being observed. Furthermore, disruption of the vesicles resulted in a complete loss of glucose retention. Vesicles prepared by DMMA treatment took up glucose with a distinct stereospecific preference for the D-isomer. Unlike the purified human red blood cell transporter inserted into artifical membranes by Kasahara & Hinkle (1977), phloretin inhibited the D-glucose uptake, as did other inhibitors of glucose transport in intact cells, phlorizin and dipyridamole. The competitive inhibitors of transport, 3-O-methyl-D-glucose and unlabelled D-glucose, both decreased the uptake, and L-glucose and sucrose had almost no effect. Shanahan & Czech therefore proposed that D-glucose transport was associated with either or both glycoprotein fractions with apparent molecular weights of 78000 and 94000 daltons.

Benjamin & Clayton (1978), finding a phosphoprotein (M_r = 130000 on SDS gels), considered whether phosphorylation of this band was directly

related to modulation of 2-deoxy-D-glucose uptake, and by implication, whether this phosphoprotein was involved in sugar translocation. However, they found that inhibition of the insulin-stimulated sugar uptake by cytochalasin B was not paralleled by a diminution of protein phosphorylation of this band.

It is apparent that a complete purification of the rat adipocyte sugar transporter has yet to be described.

1.5. Hexose Transport in a Model System.

1.5.1. An Introduction to Human Erythrocytes.

In several important tissues (in particular, adipose and muscle), insulin acts on metabolism by stimulating the net uptake of D-glucose from the bloodstream. In order to understand the nature of this action, the characteristics of the D-glucose transport in the basal, compared to the insulin-stimulated state are important. Most information about specific D-glucose transport arises from studies on the human red blood cell. Although this cell is unresponsive to physiological concentrations of insulin (Wilbrandt, 1961) the data on the basal transport and the general way in which stimulation or inhibition can occur should illuminate the understanding of insulin-sensitive systems.

The transport by human erythrocytes has been studied very extensively and used as the testing ground for models of sugar transport. There are several reasons for the choice of this cell for such intensive study. The first is that the cells are readily available from human blood banks. The preparation of the robust cells for transport studies is very straightforward. Superfically, the cell appears simple from a metabolic viewpoint, in that storage and retrieval of glucose metabolites is not a major function of the cell, and also because the interior of the cell is not lined with complex systems of internal membranes so that

compartmentalisation of substrate does not interfere with the interpretation of transport data.

1.5.2. The Characteristics of Facilitated Diffusion.

About seventy years ago it was discovered that human red blood cells showed selective permeability to monosaccharides over disaccharides (Kozawa, 1914). This suggested that the rate of uptake of the former did not proceed by direct diffusion through the lipid of the membrane, limited only by their solubility in lipid. Neither was the flow of monosaccharides directly proportional to the difference in sugar concentration across the membrane, dependent only on the overall diffusion coefficient of the plasma membrane: experimentally, the results of transport do not obey Fick's First Law of Diffusion (eg. Klinghoffer, 1935 and Miller, 1969).

The rapidity of the specific transport of monosaccharides, when compared to the non-specific permeation of neutral molecules across the cell membrane is remarkable. Lieb and Stein (1971) predicted a theoretical value for a "non-specific permeability constant" of the human erythrocyte membrane for D-glucose, and Jung (1971a,b) and Jung et al. (1971) calculated the permeability constant from the observed speed of equilibration of D-glucose, and found that it was four orders of magnitude higher than the theoretical non-specific permeability constant. Indeed, Stein (1967) showed that just such a slow, non-specific "leak" pathway for D-glucose was discernable in human red blood cells, in addition to the specific, rapid transport mechanism.

The importance of the rapid, specific transport system to the red blood cell was adequately demonstrated by the observation that the normal rate of glucose metabolism was 8×10^{-20} moles/s/cell (Laris, 1958; McManus & Kim, 1968) which Jung (1975) calculated to be almost one hundred times greater

than the rate of uptake of glucose by a non-specific mechanism. By the specific transport system, however, the cell may exchange sugar about 250 times faster than the metabolic rate (Widdas, 1954).

The specific transport system is distinguished from either the leak pathway (Stein, 1967), or active transport accomplished with an energy-requiring pump, by several fundamental features. Firstly, by the manner of its deviation from Fick's Law: the increase in rate of sugar movement is not linear as the concentration changes, but saturates. LeFevre (1954) and Widdas (1954) established that the transport could be described by a Michaelis-Menton type of equation, where J, the unidirectional flux is defined as

$$J = V_{max}S/ (K_m + S) \qquad -(1)$$

The parameters V_{max} and K_m therefore define the transport for a sugar of concentration S.

A more detailed analysis of the kinetics of transport will be discussed below. Suffice it to say here that the saturation suggests that only a limited number of sites in the membrane are responsible for transport, and these sites are said to facilitate the diffusion of the sugar through the membrane.

1.5.3. The Specificity of the Transporter.

Enantiomorphic preference for the facilitated diffusion of certain sugars was shown by Wilbrandt (1947) for D-xylose over L-xylose. LeFevre and coworkers (LeFevre, 1961, 1962, 1972; LeFevre & Marshall, 1958) showed that, in general, L-isomers (except L-arabinose) are not good candidates for the system.

LeFevre (1961, 1962) also established quantitative preferences for many pentoses and hexoses by their affinity constants for transport. The values

varied by three orders of magnitude, although the V_{max} for the sugars tested was essentially invariant. These results suggested that a single type of transporter carried all the sugars across the membrane.

The sugar transport system shows a marked preference for a particular form and conformation of sugar. The pyranose ring form of the hexose (most commonly adopted in solution) is preferred (LeFevre, 1961) and although both $^4C_1$ and $^1C_4$ conformers are stable, the translocator favours the $^4C_1$ conformer, where in the case of D-glucose all the hydroxyl groups are equatorial and the substituents lie in the plane of the ring. This was demonstrated by LeFevre (1961) who found a positive correlation between the increasing stability of the $^4C_1$ chair and the apparent affinity for the transport site.

This specificity requirement suggested to Stein (1967) that, minimally, three-point interaction would be necessary to bind the conformer, and he speculated that hydrogen bonds through the sugar hydroxyls were the means of binding or, alternatively, that a primarily hydrophobic interaction might occur between a postulated hydrophobic area in the transporter and the pyranose ring plane, unhindered by the electronegative equatorial groups.

A more thorough analysis of the structural requirements for recognition of a potentially transported molecule was made by Barnett *et al.* (1973). The inhibition of the transport of a low affinity sugar (L-sorbose) by a wide range of other sugars and halogeno-derivatives was investigated. Although L-sorbose was an atypical substrate for specific transport, the technique enabled the tested sugar to be equilibrated across the membrane, so that it was not necessary to consider compartmentalisation at the inner membrane surface (Naftalin & Holman, 1977). The apparent inhibition constants (K_i) were analysed comparatively to provide a picture of the structure preferably selected by the transport site.

Potential hydrogen bonding interactions were investigated by studies on sugars in which the hydroxyl groups were either positioned axially instead of equatorially, or substituted by hydrogen (to demonstrate hydroxyl involvement) or fluorine atoms (to demonstrate that the oxygen, in particular, was donating electrons to a transporter proton). The C-1, C-3 and C-6 deoxy sugars, α-fluoro-glucose, and sugar epimers of the parent molecules had lower affinities than substituents with fluorine in positions βC-1, C-3 or C-6. The C-2 deoxy- and epimerised sugars do not have this low affinity. The C-4 epimer of D-glucose had a lower affinity than D-glucose, suggesting that the C-4 hydroxyl was probably also involved in hydrogen bonding. It was, therefore, concluded from these affinity comparisons, that hydrogen bonding between the βC-1, C-3, C-6 and possibly C-4 hydroxyls contributed to favourable hexose binding.

The proximity of certain parts of the sugar molecule to the translocator during the initial process of acceptance was examined by the steric hindrance to transport of analogues with bulky substituents attached to the ring carbons. Again, the inhibitory effects were compared, showing that the C-4 and C-6 region was probably exposed, whereas the space around the C-1 and C-2 positions was constrained during the recognition step: O-methyl substituents at the latter positions were inhibitory; at C-3 the affinity decreased with the larger aliphatic substituents.

The interaction at C-6 appeared complex. Both hydrophobic and hydrophilic interactions were implicated as non-polar and polar substituents enhanced the parent sugar affinity, although charged groups at C-1 or C-6 were not accepted.

Thus, successful recognition of the sugar molecule appeared to occur in a pocket, in which the C-1 to C-3 region was found buried within the transport site, but the C-4 to C-6 part initially remains on the outside.

1.6. The Kinetic Parameters Defining D-Glucose Transport.

Although the simple competition studies described above have provided useful structural information about the recognition requirements of transport, it is the refined kinetic studies which have been most useful in the elucidation of the transport step. From the kinetic parameters, models of transport have been suggested in the light of the current understanding of transport molecules within the plasma membrane. There are several comprehensive reviews of the transport kinetics and models (Jung, 1975; LeFevre, 1975; Miller, 1969; Naftalin & Holman, 1977).

1.6.1. An Introduction to Transport Techniques.

A precise, sensitive method of measuring the flux across a membrane is necessary for the determination of the kinetic constants. In 1935, Orskov's densitometry method (Orskov, 1935) superseded an earlier haemolytic method (described by Miller, 1969). In the densitometry technique, the degree of light scattered by the cells was directly proportional to volume changes, which, in turn, were related to the rate of substrate movement (LeFevre, 1954). However, the method was only applicable to the net flux of sugar at concentrations greater than 20 mM.

The more sensitive measurement of the movements of a radioactive sugar across the membrane enables both low concentrations of sugar and exchange flux to be assayed. The actual amount of tracer used is negligible and flux may be measured in both directions. Sampling of the time course of transport is accomplished by the addition of an immediate transport inhibitor, such as mercuric chloride ($HgCl_2$) at 0^oC (Britton, 1956, 1964; LeFevre & McGinness, 1960), effective at higher concentrations, and with the addition of $HgCl_2$ and potassium iodide to stop transport at low concentrations (Levine & Stein, 1966). Separation of the cells from the

extracellular medium may be achieved either rapidly without the addition of the inhibitor solution or, less urgently, after the addition.

Kinetic studies on the human erythrocyte have emphasized the need for a thorough and systematic analysis of transport using several combinations of experimental design. There are five types of experiment described in the literature from which "K_m" and "V_{max}" parameters may be obtained which define the transport from a particular position. There follows a brief description of these protocols (Eilam & Stein, 1974; Jung, 1965; Naftalin & Holman, 1977).

1.6.2. The Zero trans Procedure (Lieb & Stein, 1972).

This procedure measures the radioactive flux of sugar in one direction, as a function of the changing concentration of sugar on that side (cis), into the trans space, where the sugar concentration is initially zero. The entry or exit of a sugar in either direction may be measured. Initial rates for entry, or initial rates and integrated rate equations for exit, have been used to determine the kinetic parameters (see Table 1).

1.6.3. The Infinite cis Procedure.

This was first described by Sen & Widdas (1962). At one face of the membrane (cis) the sugar concentration is kept saturatingly high, whilst the concentration on the opposite (trans) side is varied. As the trans concentration is increased, the rate of change of concentration from the cis face is reduced. In the absence of trans substrate, the rate of change of concentration is maximal and equal to the zero trans maximum velocity. The "infinite cis K_m" (K_{ic}) has been described as the trans concentration for which the rate of concentration change is half the "zero trans V_{max}" (V_{zt}).

As with the zero *trans* procedure, either the inside or outside face may be designated *cis*, so that infinite *cis* entry (K_{ic}^{oi}, V_{ic}^{oi}) and infinite *cis* exit parameters (K_{ic}^{io}, V_{ic}^{io}) may be obtained (Table 1).

1.6.4. The Equilibrium Exchange Procedure.

The movement of the radioactive sugar across a membrane where sugar has previously been equilibrated is measured (Eilam & Stein, 1974). The flux may be followed from either direction. A range of concentrations is studied, and integrated rates or initial rates used to determine the kinetics. V_{ee} and K_{ee} are the maximum velocity and half-saturation concentrations given in the transport equation (1). Either identical sugars, or different glucose analogues (heteroexchange) may be placed on either side of the membrane.

1.6.5. The Infinite trans Procedure.

In an infinite *trans* experiment, the initially unlabelled, sugar on one side of the membrane is at a concentration high enough to saturate the transporter, even when the radioactive sugar concentration on the opposite side is increased. Initial rates are then determined. The maximum rate will be the same as V_{ee}, since V_{it} will apply to the transport only when the concentration is saturatingly high on both sides of the membrane. Although the infinite *trans* experiment may be designed with either the internal or external side of the membrane designated the *cis* side, results from only the infinite *trans* entry (*cis* side outside) parameters have been determined as K_{ee} = 1.7 $\pm$ 0.3mM, V_{ee} = 174 $\pm$ 30 µmol/ml cell water/min for glucose (Lacko *et al.*, 1972) and as K_{ee} = 21 $\pm$ 2.0mM and V_{ee} = 239 $\pm$ 11 µmol/ml cell water/min for galactose (Ginsberg & Stein, 1975).

1.6.6. The Counterflow Procedure (Miller, 1968a).

In Miller's counterflow procedures the movement of a radioactive sugar placed outside of a membrane is followed, when another analogue, at a higher concentration, has previously been placed on the *trans* side of the membrane. The radioactive sugar will move into the cell, to transiently give a higher concentration inside than the concentration at equilibrium. At infinite time, both sugars are equilibrated across the membrane. Rosenberg & Wilbrandt (1957) described a different procedure in which similar "uphill transport" occurred. Erythrocytes were equilibrated with one sugar (radioactive) at a low concentration, and a high concentration of a second sugar was then added to the external medium: the movement of the first sugar out of the cell was monitored. Lacko *et al.* (1972) described an acceleration of D-galactose entry by D-glucose at 0°C. Several other sugars exhibit this phenomenon, for example, mannose and galactose (Miller, 1965a).

1.6.7. The Kinetics of Hexose Transport in Human Erythrocytes.

A summary of parameters obtained with the above procedures is found in Table 1. It is apparent from this Table that parameters defining both the inside and outside sites of transport may be determined in a variety of ways. For example, the affinity and maximum velocity of the internal site may be measured (in the zero *trans* exit procedure) in one experiment, and that of the external site in another (zero *trans* entry). Alternatively, the affinity constant for the inside site and the maximum rate constant for movement from the external to internal space may be obtained from a single type of experiment (the infinite *cis* entry experiment).

These experimental procedures have been emphasized here because of their importance to a full understanding of any transport system. The human

Table 1: The Kinetic Parameters for Sugar Transport in Human Erythrocytes, from Naftalin and Holman (1977), at 20°C.

Procedure		Sugar	K_m (mM)	V_{max} (μmol/ml cell water/min)	Reference
Zero *trans* entry	Measures affinity of external site and V_{max} for net entry.	Glucose	1.6±0.2	36±1.2	Lacko *et al*. (1972)
		Galactose	31.76±4.42	28.6	Ginsberg & Stein (1975)
Zero *trans* exit	Measures affinity of internal site and V_{max} for net exit.	Glucose	25±3.0	129±11	Karlish *et al*. (1972)
		Galactose	240±5.7	255±96	Ginsberg & Ram (1975)
Infinite *cis* entry	Measures affinity of internal site and V_{max} for net entry.	Glucose	2.8±0.6	85±2.6	Hankin *et al*. (1972)
		Galactose	25±17	---	Ginsberg & Stein (1975)
Infinite *cis* exit	Measures affinity of external site and V_{max} for net exit.	Glucose	1.7	83	Sen & Widdas (1962)
			1.86	210	Harris (1964)
			1.8±0.3	104	Miller (1965)
		Galactose	12.0	---	Krupka (1971)
Equilibrium Exchange	Measures K_m and V_{max} for exchange	Glucose	38±3	260±30	Miller (1968a)
			32±1.1	357±10	Eilam & Stein (1972)
			20±1.0	264±42	Lacko *et al*. (1972)
			34±0.6	360±31	Eilam (1975b)
		Galactose	138±57	432±44	Ginsberg & Ram (1975)
			191±17	453±9	Eilam (1975b)
			75±13	180±20	Miller (1971)
Infinite *trans* entry	Measures affinity of external site and V_{max} for exchange	Glucose	1.7±0.3	174±30	Lacko *et al*. (1972)
		Galactose	21±2.0	239±11	Ginsberg & Stein (1975)

red blood cell is unusual in that studies have been so prolific that the original conclusions that the D-glucose transport could be described as a simple "carrier" type of transport, have been shown to be erroneous.

That it is not sufficient to measure a general K_m and V_{max} for the whole transport process by following only one of the procedures is illustrated in Table 1. The value for the "K_m" for D-glucose in the equilibrium exchange experiment shows at least a twelve-fold discrepancy with the zero trans result (Lacko et al., 1972). Other workers find that K_{ee} is even higher (Miller, 1968a; Eilam & Stein, 1974; Eilam, 1975b) and the difference therefore even greater (about 20-fold). This difference in values for the affinity constant of the external site, and the "general" exchange constant was first shown by Miller (1968a,b) who compared infinite cis exit and equilibrium exchange parameters. An infinite cis exit K_m (K_{ic}^{io}) for D-glucose was determined as 1.8mM, with a V_{max} of 104 μmol/ml cell water/min, but the equilibrium exchange K_m (at 20°C), was 38mM with a V_{max} of 260 μmol/ml cell water/min.

1.7. Kinetic Models for D-Glucose Transport.

Useful and accurate models for the facilitated diffusion of D-glucose need to be consistent with the observed facts about the transport, which have been briefly reviewed above. These include saturation of rapid transport, structural specificity, competition between sugars and the countertransport phenomenon. However, it is of primary importance that the differences between the kinetic parameters are adequately explained by a model.

A description of models is necessarily historical as their formulation is directly linked to the accumulation of observations about the transport kinetics, in particular from the testing of the predictions of earlier

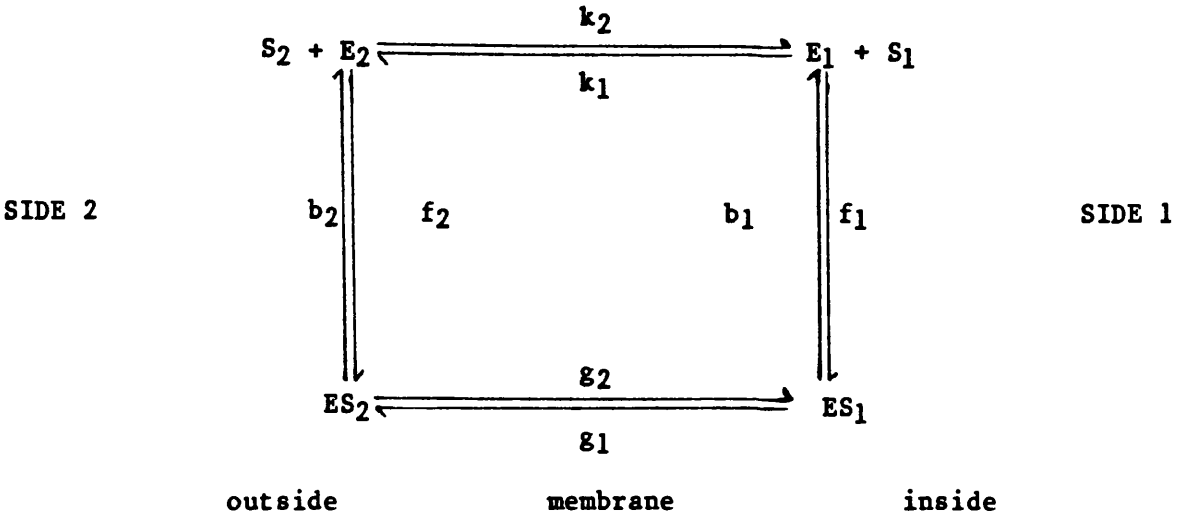
models. They are also dependent upon the prevailing consensus of opinion about the general nature of membranes and their transport facilitators.

If a model is applicable to one tissue where the transport has been vigorously analysed, it will tend to be used, initially, as the standard of comparison for other tissues where perhaps less complete experimental observations have been made. An example of this is the "mobile carrier" hypothesis (Widdas, 1952) which has gained so much credence that transport systems are often known, generically, as "carriers". This does not imply that the mobile carrier hypothesis has been verified for the system so described.

1.7.1. The Mobile Carrier Hypothesis.

In the mobile carrier hypothesis, formulated by Widdas (1952), the key assumption was that the transporter could shuttle through the membrane, both when carrying sugars with it in a carrier-sugar complex, or when "empty". The model was based on the observed similarities of the facilitated diffusion of D-glucose to an enzyme: A selective change in the location of a whole sugar molecule by a membrane component, was seen as analogous to changes in the structure of a specific substrate, catalyzed by an enzyme. The rate of movement of the carrier-sugar complex, and not its dissociation was assumed to be rate determining.

The diagram represents the simple mobile carrier process (Eilam & Stein, 1974).



Scheme 1: E_1 and E_2 represent forms of the carrier as available on either Side (1) or Side (2) of the membrane. S represents the sugar molecule, the subscripts (1) and (2) referring to the side of origin of the sugar. ES_1 and ES_2 denote the carrier-sugar complex within the membrane. Rate constants k_1, k_2, b_1, b_2, f_1, f_2, g_1 and g_2 characterize the process.

The model states that the carrier, E_1 remains within the membrane, but may be exposed at either surface, (1) or (2), in both its free form, and when complexed to a sugar molecule (S_1 or S_2) from either compartment. It is assumed that the carrier-sugar complex is mobile and may diffuse across the membrane, so that, having bound a substrate molecule (eg. S_1) at one side of the membrane, the complex ES_1 may diffuse across the membrane to appear at the other face, where the sugar may dissociate. The "empty" carrier may either remain unloaded and move back across the membrane, or combine with another sugar molecule (S_2 or the translocated S_1) and ferry it back to the *cis* side. The whole cycle may then be repeated.

In this model, the overall rate of sugar transport in either direction is directly proportional to the concentration of the complex ES and its mobility. Several assumptions about the relative size of the rate constants are made. In the simplest form of the model, the constants b_1, b_2,

f_1, f_2, g_1 and g_2 are assumed to be much larger than k_1 and k_2, so the reaction is almost at equilibrium. In addition, the interactions between forms of E and S at both faces were originally thought to be symmetric (Widdas, 1952) when f_2 would equal f_1 and b_1 equal b_2. Also, the mobility of the empty and of the loaded carrier was assumed to be identical, ie. $k_1 = g_1$ and $k_2 = g_2$. The rate of movement from one side to another of the loaded, or of the empty carrier was also assumed to be the same, ie $k_1 = k_2$, and $k_1 = g_2$.

Rigorous mathematical analyses of this model have been described by Miller (1969) and in a shorter manner, by Eilam & Stein (1974). In the latter's description, no single step in the process was specified as rate limiting, or symmetrical. The constraint of equality in the rate of movement of unloaded and loaded carrier was relaxed. The mathematical assumptions and equations pertaining to this "minimal assumption" mobile carrier model are briefly summarized below. For a fuller account, see Eilam & Stein (1974).

The first assumption made is the steady state assumption, when at a particular instant, the rate of change of concentration of the intermediates of the carrier is zero. Since steady state kinetics cannot distinguish between a model with only one form of ES, or forms ES_1 and ES_2, an analysis for only one form of ES is described. Secondly, from the law of conservation of matter, the sum of concentrations of the intermediate forms of the carrier is constant and equal to the total carrier concentration. Thirdly, from the priniciple of microscopic reversibility, $b_1 f_2 g_2 k_1 = b_2 f_1 g_1 k_2$. The fractional concentration of each intermediate E_1, E_2, and ES in terms of these rate constants may then be obtained. Eilam & Stein (1974) used the pool analysis of Britten (1964) for the unidirectional flux. By defining K, R_{00}, R_{12}, R_{21} and R_{ee} in terms

of various combinations of the reciprocal rate constants for each individual conversion of E, where $R_{00} + R_{ee} = R_{12} + R_{21}$, they obtained the equation for the unidirectional flux from side 1 to 2.

$$U_{12} = \frac{S_1(K + S_2)}{K^2R_{00} + KR_{12}S_1 + KR_{21}S_2 + R_{ee}S_1S_2} \qquad -(2)$$

The equation for flux in the opposite direction is in the same form, but the subscripts 1,2 and 2,1 are interchanged.

In all experiments where the movement of sugar in one direction is measured, equation (2) defines the transport. In other experiments, where the net flux in one direction is measured, the difference between fluxes cis to trans, and trans to cis is defined by the subtraction of the two appropriate unidirectional flux equations. By putting S_1 and S_2 equal to the appropriate values in equation (2), the affinity constant (K_m) and maximum velocity (V_{max}) parameters for each of the procedures described in Section 1.6 may be defined in terms of R_{ee}, R_{12}, R_{21}, R_{00} and K. Comparison of the values obtained from the different procedures can then be used as kinetic tests of the mobile carrier hypothesis.

The symmetrical mobile carrier hypothesis can account for many of the observations about monosaccharide transport in human red blood cells. These observations include saturation kinetics, specificity, competition, counterflow and some of the "heteroexchange" results. Until about fifteen years ago, it was thought to be an adequate explanation of transport. However, some serious discrepancies between the predictions of the model, and experimental observations have been found (Miller, 1969; Lieb & Stein, 1970, 1972).

The counterflow phenomenon (described in Section 1.6.6) is one of the

predictions of the symmetrical mobile carrier hypothesis. Observation of counterflow excludes simple diffusion, or fixed combining sites in transport models. According to the model, the addition of a second sugar (S_2), to cells previously equilibrated with another sugar (S_1), allows the second sugar to compete with the first for binding to the carrier and subsequent entry into the cell. Thereby, the entry of the first sugar is diminished. Initially, there is no competition of two sugars for carrier binding from within the cell, so the efflux of the first sugar remains as it was in the equilibrium phase. Therefore, the net effect on the first sugar is overall efflux of sugar, against its concentration gradient, as observed in counterflow (Wilbrandt & Rosenberg, 1961).

Although the counterflow phenomenon has been observed, curves simulated from the simple carrier theory do not always fit the detailed measurements of the time course of counterflow (Miller, 1965b). Miller (1965b) found that time courses for labelled galactose outside the cell, with high unlabelled galactose concentrations inside (labeled galactose against galactose) did not fit well at the early time points, and that labelled galactose against mannose had an even poorer fit. However, not even an approximate fit was obtained for the time course for labelled glucose against glucose. In a later analysis, Lieb & Stein (1972) suggest that inconsistencies occur in Miller's observations, and discredit the result.

The simple mobile carrier hypothesis had to be extended to explain why the unidirectional efflux of glucose was stimulated about two-fold by glucose outside the cells (the equilibrium exchange experiment). If, as is the case in the symmetrical model, the rate of loaded and unloaded carriers is equal (ie. $k_2 = g_2$ and $k_1 = g_1$), this "<u>trans</u>-stimulation" would not be predicted, and its observance would lead to rejection of the model. Thus a modified hypothesis was adopted, where $k_2 \neq g_2$ and $k_1 \neq g_1$

(Levine & Stein, 1966).

A third discrepancy appeared between the observed and predicted data, in a process described as "heteroexchange". In this procedure, cells were loaded with a high concentration (130 mM) of a radioactive sugar and then diluted rapidly, with effective stirring into solutions of the same, but unlabeled sugar, or other sugars, all at 130 mM. The results determined were the amount of label lost from the cells, with time. Initial rates for the process were estimated by extrapolation to zero time (Miller, 1968a,b). Miller found that labeled glucose was lost more rapidly into galactose or mannose solutions, than into glucose. However, galactose flowing into galactose, or mannose into mannose appeared slower than glucose effluxing into glucose. This result did not accord with the carrier model, which would predict that if a galactose- or mannose-carrier complex moves more rapidly than the glucose-carrier complex, as would appear to be the case from the heteroexchange rates, then the stimulation of the unidirectional efflux of galactose present in the external solution should be greater than the stimulation of glucose exit by unlabeled glucose, and likewise for mannose.

Eilam & Stein (1972) reinvestigated this type of experiment, with rates measured at thirty seconds, for galactose and mannose efflux into glucose solutions. Over this period, glucose reduces the galactose and mannose exit rates compared to those found with homoexchange of the later two sugars. However, when integrated rates were used to better estimate the effect of the external sugar on the <u>initial</u> rate, Eilam & Stein (1972) observed that the maximum rates for homoexchange of mannose and galactose were greater than that for glucose, so the hierarchy for heteroexchange would accord with that for homoexchange, and therefore also with the mobile carrier hypothesis. Lieb & Stein (1972) suggested that Miller's measurements of

homoexchange were inaccurate because he had used only one sugar concentration, which was too low to fully saturate the transporter.

The simple mobile carrier model predicts that the maximum rate of all sugar transport, being determined by the movement of loaded carriers through the membrane, should be constant for all transported sugars. Although LeFevre and Miller both observed this phenomenon, Eilam & Stein had results which did not support this hypothesis.

The greatest discrepancy between predictions of the mobile carrier hypothesis and the observed data lies in the measurement of the affinity constant. As has been described above, the parameters of maximum velocity (V_{max}) and the "half saturation constant", K_m can be determined for the transport in at least five different ways. In the late 1960's it became apparent that the size of the parameter depended upon the procedure used, and it did not appear to be constant. Miller (1968a,b) found that, under comparable conditions, the equilibrium exchange K_m (K_{ee}) was over twenty-times larger than K_{ic}^{io} (the infinite <u>cis</u> exit K_m). Both values have been confirmed by other groups (See Table 1). Lieb & Stein (1972) have analysed the simple mobile carrier hypothesis and shown that it predicts that the infinite <u>cis</u> K_m must be greater than or equal to one half of K_{ee}. This is obviously not the case. Lieb & Stein (1972) have also shown that the infinite <u>cis</u> K_m should be greater than or equal to K_m (zero <u>trans</u>), and point out that the infinite <u>cis</u> value has been observed to be at least an order of magnitude less than the zero <u>trans</u> constant.

It may be concluded that the simple mobile carrier hypothesis is not an adequate description of D-glucose transport in human red blood cells, although it may explain the behavior of other transport systems. Before the mobile carrier hypothesis was completely rejected as an explanation of this transport system, several modifying assumptions were made, whilst the

essential features of the carrier were retained. These have been described as the "easy-off", asymmetry and unstirred layer assumptions.

1.7.2. The "Easy-Off" Assumption.

In the "easy-off" modification, ES dissociation was promoted by a second substrate molecule competing for the binding site on the carrier (Lieb & Stein, 1972). The modification was proposed to explain the differences between K_m (equilibrium exchange) and K_m (infinite *cis*) . The predicted unidirectional flux would remain as in the simple mobile carrier model. However, for exchange, if it was assumed that the carrier-sugar complex moved so rapidly through the membrane that the bound sugar would only be released by the attack of a second substrate molecule; the kinetic prediction would be that the exchange K_m would be significantly greater than the infinite *cis* K_m. Although this prediction fits the observations, this modified model does not explain why the K_m values for the infinite *cis* and zero *trans* procedures differ. This "easy-off" mobile carrier hypothesis was therefore unsuccessful.

1.7.3. The Asymmetry Assumption.

In the simple mobile carrier hypothesis, the rate of movement of the sugar-carrier complex was assumed to be the same, whether the complex moved inwards, or outwards. Likewise the movement of the empty carrier was considered to be symmetrical. The simple model was modified such that the rate of movement of empty carrier was not constrained to be the same as that of the carrier-sugar complexes. This modification was made in order to explain the "*trans*- stimulation" phenomenon (Jung, 1975).

In another attempt to extend the simple model to account for the different kinetic parameters, all the partial rate constants were considered

as independently variable, and in consequence, the movement of all forms of carrier and sugar was potentially asymmetric. Lieb & Stein (1972) found that measured values for the affinity constant and maximum rate parameters, when substituted in an equation relating them to the individual rate constants, gave products that were about an order of magnitude higher than the values predicted for an asymmetric carrier scheme.

Geck (1971) also developed the kinetics for an asymmetric carrier model. The scheme could account for some of the observations which the symmetrical model had failed to explain. The asymmetry was consistent with the high K_m and V_{max} for net exit, and low K_m and low V_{max} for net entry. The high values for the K_m and V_{max} for equilibrium exchange were also acceptable. However, the model does not account for the low internal K_m found in the infinite *cis* entry experiment (Hankin *et al.*, 1972; Ginsberg & Stein, 1975), since only one type of inside site was predicted, and this was to be of low affinity.

Baker & Widdas (1973) formulated a similar asymmetric carrier model, to explain the assymetric inhibitory effect of 4,6-O-ethylidene glucose which, when it remains outside the cell, inhibits the exchange of sugar more than when it is inside. They also found that the time course of counterflow fitted predictions of the model.

1.7.4. Unstirred Layers.

Naftalin (1971) found that vigorous stirring of human erthyrocyte suspensions increased the zero *trans* efflux of D-glucose, at 20°C. In order to account for this phenomenon which was not explained by the simple mobile carrier hypothesis, he proposed that external "unstirred layers" existed which could account for some of the asymmetries observed in the transport, in particular, the acceleration in exchange.

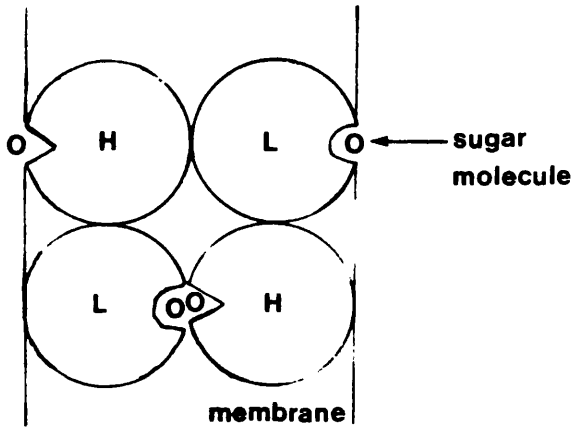
Lieb & Stein (1972) pointed out that the resistance to permeation of a glucose molecule by an external unstirred layer would be negligible unless the layer was impossibly large. If, however, the layer was partly within the membrane (Naftalin, 1972; Regen & Tarpley, 1974), the diffusion coefficient might have been lower than that in the free solution, and the unstirred layer thickness might be small enough to be credible. However, other workers (Hankin & Stein, 1972; Miller, 1972), repeating Naftalin's efflux experiments, found that, provided that the stirring was efficient enough to disperse the initial suspension of cells, the efflux phenomenon observed by Naftalin was undetectable. External unstirred layer effects in conjunction with the mobile carrier hypothesis were therefore dismissed (Miller, 1972).

Small unstirred layer effects at the inner membrane surface have been proposed (Edwards, 1974; Regen & Tarpley, 1974). Naftalin & Holman (1977) consider that both internal and external layers may be important in glucose transport in human erythrocytes. Regen & Tarpley (1974) used the modified asymmetric carrier model (Regen & Morgan, 1964) as the basis for their addition of unstirred layers. They envisaged a rather thick unstirred layer within the cell, and a thinner layer outside. The internal transport site was assumed to have a low affinity, and the outside, a higher affinity. The model successfully explained the exchange, zero _trans_ exit and entry, and infinite _cis_ exit parameters. Miller's heteroexchange phenomena (Miller, 1968a,b) were accounted for. Unlike the simple mobile carrier hypothesis only the movement of unbound carrier across the membrane from the inside to the outside was rate determining. Its movement in this direction was thirty times slower than in the other direction, inhibited by some component within the cell. These modifications did not lead to a complete description of the kinetics: the low K_m for infinite _cis_ entry of glucose or galactose was

not explained since the model assumes that the inside site always has a low affinity.

Thus, none of the subsidiary hypotheses designed to modify the simple mobile carrier hypothesis to explain the observed transport results have been successful. Other models which are not based on a rotating facilitator have been proposed to explain the asymmetric maximum rates and affinity constants and the accelerated exchange phenomenon.

1.7.5. The "Internal Transfer" or Tetramer Model.



Scheme 2: The high (H) and low (L) affinity sites in the tetramer model. (O) represents a sugar molecule. From Lieb & Stein (1972).

This model was devised by Lieb & Stein (1970) in order to account for the high K_m for exchange transport compared to the zero *trans* sugar movement, and the V_{max} for exchange which was higher than that for zero *trans* exit. In the tetramer model, unlike the mobile carrier model, sugar carrying components of the membrane do not shuttle back and forth across the membrane, ferrying sugar molecules. Instead, Lieb & Stein have postulated

that a tetrameric protein embedded in the membrane facilitates transport. Of the four subunits, two have high affinity (H) and two low affinity (L) binding sites for sugar. The subunits are arranged such that H and L sites are accessible to both the outside and inside of the cell. Within the tetramer is an internal cavity, inaccessible to the external and internal media, but large enough to be occupied by four sugar molecules and enabling two sugar molecules on one axis to bypass each other. The tetramer may exist in two energetically favored conformations (see Scheme 2). Binding of a substrate molecule is necessary to induce the tetramer to change conformation. The tetramer transports a sugar molecule across the membrane by a series of events. Firstly, a molecule must bind to one of the subunits, inducing the conformational change to the second form. This translocates the sugar into the internal cavity. There, depending upon energetic considerations, the sugar may bind to the opposite subunit, or to its original partner. This binding induces a second sugar-translocating conformational change, whereupon the sugar may be released on the _cis_ or _trans_ side of the membrane; a transport event is deemed to have occurred when release is on the _trans_ side.

Values for the kinetic parameters were predicted from a consideration of the transport procedures described above (zero _trans_, infinite _cis_ etc.). For example, in the zero _trans_ procedure, binding to a low affinity site and translocation to the high affinity site would be unfavorable at low concentrations, when the adjacent, _cis_ high affinity site would bind the sugar. But in this instance, translocation by transfer to the opposite L site would be unfavorable and net transport would not occur. At higher concentrations, when the _cis_ H site is already occupied, the L site will be rate determining and the K_m value predicted to be high.

Similar considerations of the substrate concentrations at *cis* and *trans* faces, and the degree of binding to H and L sites, leads to predictions that the K_m (equilibrium exchange) will also be high, and that the infinite *cis* K_m for either side of the membrane would be low. These predictions fit the observed data (Table 1) for all except the zero *trans* entry K_m. Although Lieb & Stein's prediction of two sites of high and low affinities is borne out by the observed data for the internal site, it does not account for the observation of only low K_m (high affinity) sites outside for glucose.

Differences in the V_{max} values found in the equilibrium exchange and zero *trans* experiments can also be explained by the maximum number of molecules bound (four in the former instance, and two in the latter), which is proportional to the transition rate for glucose transport where the activation energy is high. However, the tetramer model predicts that the zero *trans* entry and exit V_{max} values should be similar: for both glucose and galactose the rate of exit is five- to ten-fold faster than entry (Lacko *et al.*, 1972; Karlish *et al.*, 1972), an effect enhanced at lower temperatures (Hankin & Stein, 1972; Lacko *et al.*, 1972).

Although the model can account for some of the varying kinetic parameters, it does not explain the counterflow phenomenon, or the disputed initial rate of heteroexchange found by Miller (1968a,b).

1.7.6. The Modified Tetramer Model.

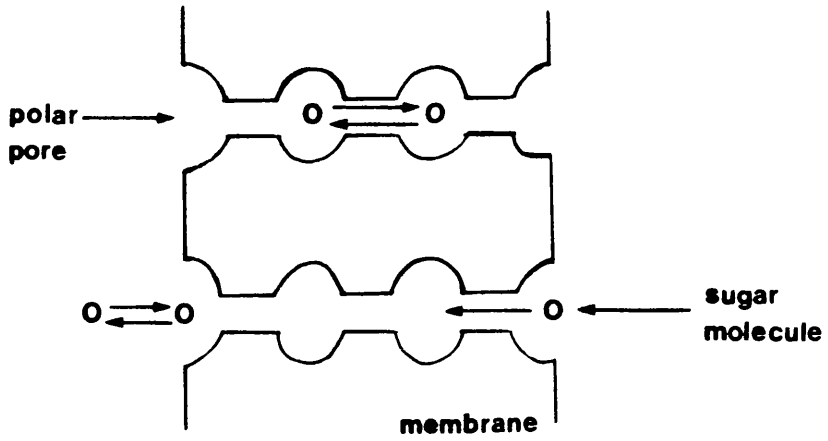
In the tetramer model devised by Lieb & Stein, the transposition of the two pairs of subunits occurs simultaneously. Eilam (1975a,b) described a modification of this model, in which each of the subunit pairs spanning the membrane is able to move independently of the others. Transport of two

sugars need not therefore occur simultaneously. This model differs from its predecessor in that for each set of "α" subunits (low affinity outside, opposite high affinity inside), there are ten "β" subunits (where the high affinity site is outside, low affinity inside). The model requires the ratio of the affinity constants for each pair of spanning subunits to be ten.

Many of the kinetic parameters are explained by this model: the two zero <u>trans</u> K_m's (low for entry and high for exit), the low K_m's for both infinite <u>cis</u> entry and exit, the high K_m for equilibrium exchange and the low infinite <u>trans</u> entry K_m. Differences between the V_{max} values for zero <u>trans</u> entry and exit, and the high V_{max} for exchange also fit the model. Eilam finds that the exchange results are more appropriately described by her model than by the original "internal transfer tetramer model", in which non-Michaelis-Menton kinetics would be observed over a wide concentration range due to contributions from both H and L sites.

1.7.7. The Lattice Membrane Model.

A model proposed by Naftalin (1970) differed from the tetramer and modified tetramer models, in that it described binding sites of only one affinity. This model, the "lattice membrane" model was a revision of an earlier model described by Zierler (1961). He proposed that pores spanned the membrane, through which one substrate at a time could "creep" to effect transport; saturation kinetics being observed because the number of pores was limited. In Naftalin's model, membrane spanning polar pores lined with sugar binding sites facilitate transport. Unlike the tetramer models, it was envisaged that sugar molecules may move between neighboring pores, either by exchange with occupying molecules, or by movement into vacant sites, during the process of diffusing through the pores.



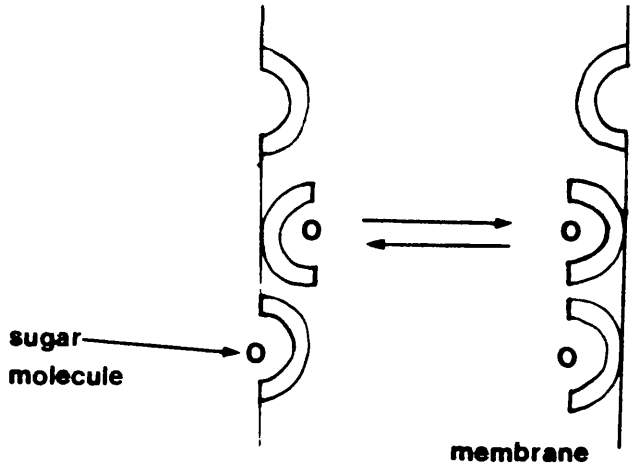
Scheme 3: The Lattice-Pore Membrane Model, from Naftalin & Holman (1977).

This model, whilst explaining saturation, competition between substrates, and countertransport, does not satisfactorily explain the K_m differences between infinite cis and exchange procedures, or accelerated exchange, except by proposing that sugar molecules must pass through an inordinately thick external "unstirred layer", or a thinner layer within the outer part of the membrane (Naftalin & Holman, 1977).

1.7.8. The Introverting Hemiport Model.

Refinements to the lattice pore model were introduced by LeFevre (1973). LeFevre reduced the number of available pores in the "matrix" of a transporter to two, but retained the feature of a single affinity for

substrate. Like the tetramer models, it has two components with sugar binding sites each being accessible to the bathing medium or cell interior, and an internal compartment within the membrane.



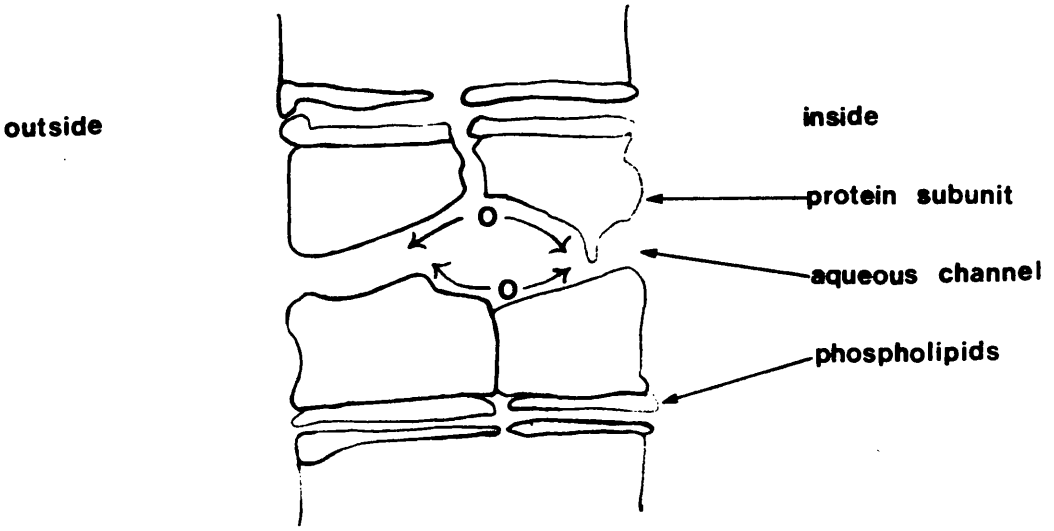
Scheme 4: The Introverting Hemiport Model. The sugar induces conformational changes in transporters with a single affinity.

An essential feature of the model is that substrate binding induces a conformational change in the binding component or "hemiport". This translocates or "inverts" the sugar attached to the hemiport into the internal aqueous compartment. Here, the sugar may diffuse to an opposite or adjacent hemiport, where binding and another conformational change moves the sugar either to the *trans* or *cis* side of the membrane. The rate determining step is not considered to be the initial binding, but the substrate-induced conformational change. The probability of this event is dependent on the affinity of the sugar for the transporter.

This model does explain the low infinite *cis* K_m's, high equilibrium exchange K_m and V_{max}, and high zero *trans* exit K_m. However, this model, having only one class of binding site, is symmetrical and so, like the lattice pore and tetramer models, it does not explain the asymmetry

observed between the V_{max} values for zero <u>trans</u> entry and exit.

Jung (1975) considered the molecular implications of LeFevre's introverting hemiport model (see diagram).

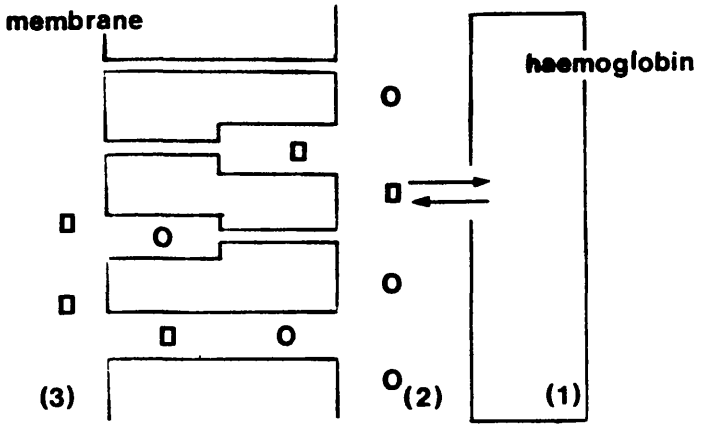


<u>Scheme 5 :</u> A physical postulate of the Introverting Hemiport model.

The asymmetric nature of some of the transport phenomena was acknowledged, but it was considered to be a reflection of the non-identical composition of the bilayer leaflet (Guidotti, 1972) and not of the protein structure, which was thought to be symmetrical.

<u>1.7.9. The Gating Pore Model.</u>

Naftalin & Holman (1977) modified the proposals for the introverting hemiport model and described a "gating pore" model.



Scheme 6: The Gating Membrane model with intracellular glucose-haemoglobin complex formation.

Like LeFevre, Naftalin and Holman deduced that the rate of transport within the membrane was as fast as free diffusion, given the limited number of transport mediators. They therefore also based their model on a water filled pore. The model was described as a pore bounded by binding sites or "gates". The gates could be opened independently of one another by substrate-induced conformational changes. Once opened by a sugar molecule, the gate may remain in the "opened" state even when the sugar molecule has moved to the centre of the pore. The gates are therefore said to be "metastable". This feature endows the unidirectional and exchange fluxes with different properties. Net transport involves conformational change, but exchange between two open sites differs in that the sugar does not need to induce further opening of *trans* gates in order to pass through the membrane. This facet of the model may account for the lower exchange than zero *trans* activation energy. Differences between the exchange and zero *trans* V_{max} values are also explicable. The model also explains the lower activation energy for infinite *trans* entry over zero *trans* entry. The varying effects on the exchange and zero *trans* parameters may be explained

by the differential effects of the activation energies.

The other parameters so far determined also fit this model if an internal sugar-haemoglobin complex, contributing to the transport, is postulated.

1.7.10. The Allosteric Pore Model.

A further variation of this water-filled, gated pore model for sugar transport was recently proposed by Holman (1980). In this model, the so-called "allosteric pore" model, a crucial difference is that a sugar-haemoglobin complex is not required to explain the transport kinetics. Instead, substrate entering the central aqueous space (pore), moves through gates which initially have a high affinity, low maximum rate, site for sugar binding. The presence of sugar within the pore can induce this gate site to change to a low affinity site, thus decreasing the likelihood of other sugars binding at this gate. However, if a sugar does bind, the *trans* gates are also "destabilised", and the process has a high maximum rate constant.

Like the gating pore model, the allosteric pore satisfies the previous kinetic observations. Some of its predictions were first tested by the inhibition studies described in Chapter 3. The allosteric pore model will therefore be discussed in detail when the implications of this inhibitor study is considered in Chapter 4.

1.8. Sugar Transport in Muscle.

Because of the structural nature of muscle tissue, it has not been possible to prepare the type of isolated cell suspension which is ideally suited to analyses of rapid transport kinetics. Attempts have been made to produce muscle cell ghosts, but rapid transport studies were technically poor: for the D-galactose "binding kinetics" attempted, unsuitably long

time intervals were used and efflux was not inhibited (Kono & Colwick, 1961). Thus the studies of sugar uptake into muscle have been performed on isolated sections of muscle tissue or perfused tissue (eg. Randle & Smith, 1958a,b). However, a major criticism of all such preparations is that the heterogeneity and complexity of the membranes surrounding the muscle cell and the limitations imposed by extracellular diffusion of substrate to the site of uptake into the cell have not been sufficiently accounted for (Narahara & Ozand, 1963).

In spite of these methodological shortcomings, and in the absence of more precise data on transport into individual cells, the results of such studies will be briefly surveyed, since, like adipose tissue, sugar transport in muscle is of regulatory importance. A more complete review has been given by Clausen (1975).

Lundsgaard (cited by Morgan et al., 1964) first suggested that transport through the muscle cell membrane might be important in metabolic rate determination in perfused hind limbs, since the uptake of sugar reached a maximum concentration and no free glucose was found within muscle tissue. Previously, Goldstein et al. had put forward the view that glucose uptake by muscle was rate determining for metabolism and that insulin acted at this point of regulation (Goldstein et al., 1953; Levine & Goldstein, 1955; Park et al., 1955). Park and his colleagues (Park et al., 1959) demonstrated that transport was freely reversible and accelerated in both directions by insulin.

Randle & Smith (1958a,b), with photometric assays of D-glucose and D-xylose uptake into isolated rat diaphragm, confirmed the stimulatory effect of insulin on glucose uptake, with high (0.1 U/ml) insulin concentrations, although 10^{-3} U/ml of insulin had no effect on this preparation: both "hemidiaphragms" and "intact" diaphragms were used in this study. The

diaphragm is well-suited to *in vitro* uptake studies in so far as it is a thin sheet of tissue, easily extracted from the animal, that may be rapidly oxygenated and equilibrated with substrates. There are variations in the methods of preparation, where the integrity of the fibres is preserved to varying degrees. For example, Kipnis & Cori (1957) and Kono & Colwick (1961), who, wishing to preserve as many muscle cells intact as possible, left adhering tendon, cartilage and intercostal fibres attached. However, these extra structures give potential errors in the estimation of internal spaces in sugar uptake studies (Clausen, 1975). The sensitivity of the diaphragm to insulin had been shown by Walaas & Walaas (1952) who stimulated the rat hemidiaphragm with insulin at concentrations of 0.1 U/ml.

Morgan *et al*. (1959) demonstrated a similar acceleration of D-glucose and arabinose transport in perfused rat hearts. In perfused tissue the integrity of individual cells is preserved but accurate measurement of intracellular space is difficult and substrate accessibility is more of a problem. However, insulin sensitivity and the function of the tissue were retained.

While probing the regulation of glucose uptake in perfused rat hearts, Morgan *et al*. (1961) found that, over a physiological range of D-glucose concentrations, transport was rate determining but that at higher concentrations, glucose phosphorylation became predominantly rate-controlling. The inward transport conformed to Michaelis-Menton kinetics.

Battaglia & Randle (1960) went on to study both the perfused rat heart and isolated diaphragm. Photometric and radioactive methods were used to estimate substrate taken up. Sorbitol was used as an extracellular marker. Saturation and stereospecificity was shown in the uptake of several sugars over long incubation times (thirty to forty-five minutes). D-glucose

and D-xylose were taken up into the diaphragm whilst the perfused rat heart took up D-glucose and D-arabinose. Competition for uptake between sugars was demonstrated. The results appeared to suggest that there was more than one type of transporter and preliminary conclusions as to their specificity were drawn. In view of the long time interval over which uptake was measured, and the shortcomings of the preparations in giving accurate quantiative kinetic data, these conclusions appear premature. Insulin (0.1 U/ml) increased D-glucose uptake under anaerobic conditions, but glycogen stores were not mobilized, suggesting that there was no direct glycogen involvement in the stimulation of uptake. Sulphydryl reagents (pCMB and NEM) inhibited insulin-stimulated D-galactose uptake by approximately 40%. Other groups report sensitivity to phlorihizin inhibition (Morgan et al., 1959; Park et al., 1959).

Fisher & Zachariah (1961) showed, with D-xylose uptake, that pentose uptake in perfused hearts would fit a "carrier" equation. This was in contrast to the conclusion of Kipnis & Cori (1957), with studies of the diaphragm, whose results appeared to fit a simple diffusion hypothesis. They did, however, assume that half of the intracellular water was inaccessible to the pentoses. In another paper, Morgan et al. (1961) showed a 75% penetration of intracellular water in the rat heart by L-arabinose after finding apparently constant sugar concentrations after one hour of incubation. Fisher & Gilbert (1970) pointed out that, although slow, accumulation of substrate would still be continuing at that time if the uptake fitted an equation describing simple facilitative diffusion. These authors measured the arabinose content in the heart and found it to be equal to that in the perfusing medium after a sufficiently long incubation.

Park et al. (1959) used counterflow in the rat heart to demonstrate the applicability of the mobile carrier model. Morgan et al. (1964)

demonstrated counterflow in tissue from starved rats, with 3-O-methyl-D-glucose as the counter sugar to D-glucose. Perfusions were over thirty minutes and this time interval was used to determine initial rates, apparently assuming that uptake was sufficiently slow. They also confirmed that no free diffusion of L-glucose occurred, and that transport of D-glucose for stereospecific.

Attempts at kinetic analyses gave conflicting results. In the isolated heart, Post et al. (1961) showed an insulin-induced six-fold increase in the V_{max} for D-glucose uptake. Morgan et al. (1964) appear to show a three-fold increase in K_m for D-glucose transport from 9 to 25 mM. The "K_g" values obtained from Morgan's counterflow experiments (Morgan et al., 1964) suggested that skeletal and cardiac muscle exhibited similar affinity constants for sugar uptake.

Fisher & Gilbert (1970) measured the kinetics of L-arabinose and D-xylose penetration in the rat heart with and without insulin between 0.2 and 4 mU/ml. No care was taken to ensure that initial uptake rates were measured, and under such conditions, the K_m appeared to increase with insulin treatment, as did V_{max}, although a slight decrease in rate was observed with low insulin. Previously, Bilher et al. (1965) had noted wide discrepancies in uptake parameters. These authors used 1 mU/ml insulin which induced a maximal response within fifteen minutes in the rabbit heart. The total intracellular space was available to sugar, and inulin was used as an extracellular space marker. With fifteen minute perfusions, the product of the maximum rate and the affinity constant (VK), for D-arabinose uptake increased six-fold with insulin. Other groups found a decrease in the apparent K_m for sugar transport on insulin treatment (Norman et al., 1959; Guidotti et al., 1966; Chaudry & Gould, 1969).

From this brief review, it is clear that although the stimulatory effect of insulin on the sugar transport in muscle has been demonstrated, the kinetics of the transport have not been reproducibly described.

1.9. Sugar Transport in the Liver.

Sugar transport in the liver provides a particularly interesting comparison with muscle and adipose tissue because it is not stimulated by insulin.

Cahill *et al.* (1958) showed that D-glucose transport into liver, *in vivo*, was very rapid and apparently insensitive to insulin. However, from their limited data, they suggested that a significant passive uptake of the sugar occurred. This was later refuted by more detailed studies. In spite of the considerable difficulties in using perfused tissue to study rapid uptake, Williams *et al.* (1968) demonstrated that D-glucose transport in isolated rat liver was stereospecific, and had other properties characteristic of facilitative diffusion. Although the rates of entry of D- and L-glucose were significantly different, L-glucose was apparently partially transported by facilitated, in addition to passive, diffusion. The transport was temperature sensitive over the range 17 to 37°C, and was inhibited by phloridzin. A simple kinetic analysis gave a minimum value of 17 mM for the K_m and a V_{max} of 345 μmol/min/ml cell water. High permeability of the liver to glucose prevented demonstration of countertransport.

Two groups working on perfused livers found rather different values for the transport parameters of D-galactose. Keilding *et al.* (1976) calculated a K_m of 0.12 to 0.30 mmol/l plasma water, and a V_{max} of 0.34 to 0.57 mmol/min/kg liver for D-galactose elimination in isolated pig liver, using a model which assumed that saturation kinetics applied. In contrast, Sestoft

& Fleron (1974) found a K_m of 67 mM and a considerably higer V_{max}, 30 mmoles/min/kg liver in rat liver, where membrane transport was shown to be physiologically rate limiting. The discrepancies between the two studies may be due to inherent difficulties in the analysis of the contributions of metabolism and facilitated diffusion to the overall fructose uptake, and in indirect measurements of transport.

In vivo perfusion studies of the livers of anaethatised dogs (Goresky & Nadeau, 1974) showed that phloridzin and galactose competitively inhibited D-glucose facilitated diffusion, in an approximately symmetrical fashion. β-methyl-D-glucoside had a very low affinity for the facilitated system. In contrast to the results of Williams et al. (1968), L-glucose did not compete for the transporter and entered very slowly. Kinetic modeling suggested that, unlike D-galactose (Goresky et al., 1973), D-glucose was not sequestered inside the cell. Very high values for both K_m and V_{max} for D-glucose uptake were calculated by Goresky & Nadeau (1974) (K_m = 120 mM; V_{max} = 28.5mmoles/sec/l cell water). The K_m for D-galactose was lower (28 mM; Goresky et al., 1973).

Hexose uptake into the livers of conscious sheep was measured by the same "reference indicator" method (Goresky & Bach, 1970) as was applied to dogs: glucose uptake was inferred from alterations in the ratio of glucose to the impermeant indictor, sucrose, during perfusion. In addition to stereospecificity (D-glucose>D-galactose>>D-fructose>>>L-glucose) and saturability of D-glucose, D-galactose and D-fructose uptake, sucrose and methyl-α-D-glucoside inhibition of D-gluocse and D-galactose were shown. Hooper and Short (1977) later confirmed Goresky & Nadeau's result that mediated uptake of L-glucose was negligible.

Some characteristics of the liver transport system were demonstrated in monolayer cultures of rat liver parenchymal cells, isolated by collagenase

perfusion (Kletzien et al., 1975): 3-O-methyl-D-glucose was transported by rapid facilitated diffusion which was inhibited by phloretin (0.1 to 1 mM) at 4°C and 25°C. Accelerated exchange was demonstrated, but the kinetics of the process were not described.

Other studies were performed on collagenase-treated isolated hepatocytes with a direct measurement of transport assessed by an oil-centrifugation method (Bauer & Heldt, 1977). For both D-glucose and D-galactose, transport greatly exceeded the metabolic rate, thus transport was not rate limiting for metabolism. In this preparation, L-glucose showed 5% of the transport activity when compared to D-glucose. D-glucose uptake was saturable and competitively inhibited by methyl-D-glucose>2-deoxy-D-glucose>D-galactose > D-fructose. D-glucose uptake was more rapid than D-galactose (which was also saturable) but closely mimicked by 3-O-methyl-D-glucose. Transport of D-glucose, which was Na^+ independent, was inhibited by cytochalasin B, phloretin and phlorizin, and was not stimulated by 0.1 μM insulin. The biphasic temperature dependence of uptake had a transition temperature of about 18°C. Activation energies of 92 kJ/mol for the slower phase (between 4°C and 18°C) and 29 kJ/mol between 18°C and 37°C were observed. D-galactose showed similar but slower uptake, D-fructose transport was even slower, had a non-mediated component and was potentially metabolically rate limiting. Kinetics for D-glucose entry were determined from rates measured from uptake differences at ten seconds and twenty or thirty seconds. The K_m at 20°C was 30 mM, and V_{max} was 110 nmol/μg protein/min, both values being significantly lower than those found in perfused liver by Goresky & Nadeau (1974), although the K_m (17 mM) found by Williams et al. (1968) was of the same order.

Using a slightly modified hepatocyte preparation, Craik & Elliott (1979) described a thorough kinetic characterization of the transport of 3-O-

methyl-D-glucose. They determined the zero *trans* and equilibrium exchange parameters for both entry and exit of the sugar. Their results demonstrated that methylglucose transport was symmetrical and showed no evidence of *trans* acceleration. Initial rates of uptake were estimated by eye as tangents to progress curves. In addition, integrated rate plots confirmed the exchange parameters.

TABLE 2

Experimental Design	K_m (mM)	V_{max} (mmol/lcellH_2O/min)
	3-O-methyl-D-glucose	
Equilibrium exchange entry	18.1 ± 5.9	86.2 ± 9.7
Zero *trans* entry	20.2 ± 2.7	81.9 ± 4.6
Equilibrium exchange exit	17.6 ± 3.5	78.8 ± 5.3
Zero *trans* exit	16.8 ± 4.6	84.1 ± 8.4
	D-galactose	
Zero *trans* entry	174 ± 48	288 ± 48
	D-fructose	
Zero *trans* entry	212 ± 32	291 ± 26

Table 2. Kinetic parameters obtained by Craik & Elliott (1979; 1980) for methylglucose, D-galactose and D-fructose transport in isolated rat hepatocytes, at 20°C. Values are ± S.E.M. The analysis developed by Hoare (1972) to test various transport mechanisms illustrated that this transport system was univalent and was of a single type.

It is noteworthy that there are discrepancies between the affinity constants and maximum transport rates measured by Craik & Elliott and those found by Baur & Heldt (1977) for D-glucose. Craik & Elliot suggested that the rapidity of the hexose transport causes systematic inaccurancies to occur in the measurement of initial rates by Baur & Heldt. These inaccuracies and consequent underestimation of transport rates also apply to the determination of the kinetics for D-galactose and D-fructose in isolated hepatocytes (Baur & Heldt, 1977), where a K_m of 100 mM and V_{max} of 250

nmol/mg protein/min. for the former, and a K_m > 100 mM, V_{max} > 250 nmol/mg protein/min. for the latter were found. Unlike Baur & Heldt, Craik & Elliott (1980) showed that the transport of D-fructose was by facilitated diffusion (see Table 2 for K_m and V_{max} values), although they agreed that D-fructose was transported more slowly than D-galactose. The single, univalent transport system also appears to transport these two sugars: 3-O-methyl-D-glucose inhibits the uptake of both with inhibition constants (K_i) consistent with the K_m for 3-O-methyl-D-glucose uptake. Both D-galactose and D-fructose competitively inhibit the uptake of each other.

In the work of Craik & Elliott, a thorough kinetic characterization of the facilitated, rapid sugar transport system of mammalian tissue of major metabolic importance, has been achieved. Emphasis has been placed on the need to determine initial transport rates accurately: comparisons with other work have highlighted this necessity.

1.10. Sugar Transport in Adipose Tissue.

Amongst the acute actions of insulin, the acceleration of D-glucose uptake in muscle and fat tissue has been well documented (Morgan & Whitfield, 1973; Avruch *et al.*, 1972; Clausen, 1975).

Early indications that glucose transport might be rate limiting for the metabolism of adipose tissue were found by Herrera & Renold (1960). In these experiments, the intact tissue (rat epididymal fat pads) was treated with insulin, and then homogenized. The rate of glucose phosphorylation was even faster than the maximum rate of glucose use, thus the preceeding step, glucose transport into the cell, might be rate limiting. The results of Hernandez & Sols (1963) supported this conclusion. Hernandez & Sols studied the specificity for glucose, mannose and fructose of the hexokinase reaction, under conditions of insulin stimulation. They compared this to

the overall relative efficiency of utilization, assayed manometrically by CO_2 production, of these sugars by the intact adipose tissue. The requirements for the two processes differed, and they therefore concluded that phosphorylation did not control the overall rate of metabolism. Further evidence to support this view was the observation that phlorihizin (1 to 2 mM) inhibited fructose metabolism but did not affect the hexokinase activity of fat pad homogenates.

These authors found that insulin did not affect the rate of the hexokinase reaction. The rate, measured in homogenates from tissue not pretreated with insulin, was not different from that measured in homogenates of insulin-prestimulated tissue. DiPietro (1963) similarly concluded that the fat pad hexokinase was not the rate determining enzyme for glucose uptake since glucose uptake increased with increasing external glucose concentrations, when glucose was well in excess of that required to saturate the hexokinase. Froesch & Ginsberg (1962) studied the inhibition of fructose metabolism by glucose in intact tissue. They concluded that the intracellular glucose level was low, since hexokinase has a far greater affinity for glucose than fructose, and inferred that glucose transport was rate determining. These results contrasted with those of McLeod *et al*. (1960) who appeared to show that insulin could stimulate the production of glucose 6-phosphate.

One factor that was not considered by Hernandez & Sols (1963), and DiPietro (1963) was the diffusion of glucose through the serosal membrane and extracellular space within the adipose tissue. In contrast to muscle tissue, for fat tissue, the ratio of extracellular to intracellular water is greater than one (Crofford & Renold, 1965a). With sorbitol as a marker for extracellular space, Crofford & Renold established that, under conditions promoting adequate diffusion, transport across the cell plasma membrane was

the major rate determining step for metabolism. Also, transport was the major site of insulin's action. Glucose phosphorylation became rate limiting at very high rates of uptake (ie. with high insulin and added glucose concentrations).

Baker & Rutter (1964) tested the sensitivity of glucose uptake to various substances. Insulin preincubation for thirty minutes gave an eight- to ten-fold stimulation of uptake. Although this was poorly reproducible, it was significantly greater than the two- to three-fold effects reported by Winegrad & Renold (1958). Chloride, but not zinc, ions appeared to be necessary for the response. As the pH was increased from 6 to 8, glucose uptake in insulin-treated tissue increased to a maximum at pH 7 and thereafter it remained constant; the basal level was unaffected by pH up to 7. This effect was irreversible. The insulin effect was least apparent at the lower temperature in the range 15 to 42°C. The temperature coefficient (Q_{10}) for uptake was significantly greater than that expected for simple diffusion.

Further indications that the transport might be by facilitated diffusion were given by Crofford & Renold (1965b). The nature of the system was analysed by measuring $^{14}CO_2$ evolution over three hours from radioactive glucose, and by determining the loss of glucose from the medium by assessing the intracellular spaces for glucose minus sorbitol (Crofford & Renold, 1965a). Preferential enantiomorphic specificity for D-glucose over L-glucose was demonstrated. It was established that phlorrhizin and phloretin inhibited glucose metabolism at the level of transport, whereas the inhibitory site for mannose was elsewhere. 3-O-methyl-D-glucose appeared to compete competitively with the glucose transporter. An important characteristic of a facilitative diffusion mechanism, countertransport, was demonstrated for 3-O-methyl-[^{14}C]-D-glucose in a

glucose concentration gradient. 3-O-methyl-^{14}C-D-glucose was not accumulated by the tissue: its distribution space did not exceed the $^{3}H_2O$ space.

Since it had, therefore, been established that glucose transport was an important rate determining step in adipose tissue metabolism, was sensitive to insulin and appeared to operate by facilitated diffusion, simple kinetic analyses of the transport mechanism were attempted. Comparisons between basal and insulin-treated tissue were made.

Baker & Rutter (1964) found an apparent K_m change from 60 mM to 7 mM for overall glucose uptake, and by inference, for transport, on insulin-treatment of fat tissue. The V_{max} (2 μmoles/100 mg tissue/hr) did not appear to be affected by insulin. Crofford & Renold (1965b) also suggested that the major effect of insulin was to decrease the apparent affinity constant to a similar value.

Intact adipose tissue has proved to be of limited use as a material to which kinetic analyses may be applied. Most of the studies in which glucose transport kinetics were determined indirectly from measurements of metabolism were performed under steady state conditions, for periods of several hours (Crofford & Renold, 1965b; Hernandez & Sols, 1963). Since the hexose must diffuse through the tissue before transport into the cell may occur, it would not be feasible to measure uptake over very short time intervals. This would be necessary if initial rates of transporter activity were to be measured.

In order to overcome this problem, fat cells have been freed from their surrounding matrix by digestion with a crude bacterial collagenase-protease mixture (Rodbell, 1964). Rodbell (1964) compared the metabolism of glucose by intact adipose tissue and isolated adipocytes. The enhancement of metabolism caused by increasing glucose concentrations in the bathing medium

was substantially greater in isolated cells than in the intact pad. However, the proportion of glucose converted to CO_2, glyceride glycerol and fatty acids was similar in starved and fed rats, for both the basal and insulin-stimulated conditions.

Kuo (1968) measured glucose oxidation by free fat cells, and found that insulin stimulation was reflected in a higher V_{max}, and lower K_m than the basal state. A V_{max} effect was also observed when the insulin stimulation of isolated cells was lost by omission of sodium ions from the bathing medium, but in this instance, the K_m was unchanged (LeTarte & Renold, 1967).

It therefore appeared that these early free cell preparations qualitatively retained their hormone sensitivity, although there was some doubt as to whether the kinetic parameters for D-glucose uptake remained unchanged during the isolation process. It seems unlikely that the cells were altered by collagenase in the light of a recent report of cells isolated by a method not involving this pad digestion step, which, when compared to collagenase-digested cells showed no difference in the pattern of membrane protein phophorylation either in the presence and absence of insulin (Belsham et al., 1980). However, the length of the collagenase incubation and the concentration used were both one half of that used in the early methods, so it was still conceivable that the different conditons for the collagenase treatment altered the hormone response of glucose uptake.

The collagenase-treated isolated fat cell preparation has become very widely used as a convenient preparative method for in vitro studies such as insulin binding and cell response to lectins and oxidants (Czech, 1977). The retention of these characteristics suggests that the cell membrane remains essentially unchanged although quantitative measurements of particular phenomena (eg. the sensitivity of glucose uptake to insulin) do

show variation between laboratories and different cell preparations.

Several groups (Czech, 1980; Gliemann, 1967) have shown that the size of the insulin response varies considerably with different batches of collagenase. In a detailed study on the optimal conditions for cell isolation, Vega & Kono (1979) suggested that cell damage could be considerably reduced by the prevention of cell starvation during the preparation, and by extreme care in the handling of cells. Gliemann (1967), whilst also optimizing preparative conditions, even developed a special pipetting device to minimize cell damage. In any systematic study, of glucose uptake, the monitoring of cell viability and hormone sensitivity is obviously of crucial importance. Gliemann (1967) has measured the extent of insulin stimulation by assaying the oxidation of [1-^{14}C]-glucose by the isolated cells. He found that free fat cells were sensitive to insulin concentrations between 0.25 and 1.0 μU/ml, with a maximum response (8- to 30-fold increase) at 20-30 μU insulin per ml.

In the early experiments, glucose oxidation was used to infer rates of glucose transport. As in whole tissue, the oxidation was assayed manometrically, by the production of ^{14}C-carbon dioxide from radioactive glucose, or from the incorporation of ^{14}C into a "lipid fraction" (Dole, 1956). Various refinements of such indirect measures were developed. In one instance (Taylor et al., 1976; Taylor & Halperrin, 1979), the conversion of [1-^{14}C]-glucose into $^{14}CO_2$ was monitored. Glucose transport was made rate limiting by enhancement of the rate of the pentose phosphate pathway with phenazine methosulphate, an agent that rapidly reoxidizes NADPH (Katz & Wals, 1970). Apparent rates of glucose transport in the isolated cells were reportedly increased by several agents, including insulin, which showed a two-fold stimulation under optimal conditions. This type of method for estimating "apparent" rates of glucose transport is of limited use in

measuring steady state kinetics becasue of the very fact that the transport rates are indirectly inferred from the rate of a complex metabolic pathway. The addition of agents such as phenazine methosulphate (Taylor & Halperrin, 1979) may introduce artefacts into the system, by direct or indirect effects on the transport: measurements made under such conditions do not necessarily accurately reflect the physiologically relevant "basal" and insulin-stimulated transport rates. Some of the apparent kinetic constants determined from the glucose concentration dependence of the rate of glucose metabolism in adipocytes are shown in Table 3 (from Vinten et al., 1976).

TABLE 3

V_{max} (insulin stimulated)/V_{max} (unstimulated)	Half-saturation constant (mM)		Reference
	Insulin Absent	Insulin Present	
2.2	0.74	0.26	Rodbell (1966)
3.0	5.55	5.5	Kuo et al. (1967)
1.8	4.0	0.6	Chang & Cuatrecasas (1974)
3.0	3.4	0.74	[a]Clausen et al. (1970)
1	2.1	0.4	Livingston & Lockwood (1974)

[a]Insulin concentrations submaximal (5 μU/ml)

Techniques were therefore developed to measure sugar transport in isolated cells, directly. In white fat cells, where more than 90% of the internal cell volume is unavailable to the sugar, the successful use of conventional centrifugation or filtration for cell recovery is precluded by the amount of medium (20-30%) trapped between cells. Two types of methods were devised. One was a rapid filtration method where the separated cells were extensively washed with an ice cold solution to minimize extracellular sugar contamination (Czech et al., 1974); the other was a centrifugation technique in which an inert, non-aqueous substance was used to clear the

bathing medium from between the cells (Gliemann et al., 1972). Both methods employed radioactive D-glucose analogues which were not fully metabolized: 3-O-methyl-D-glucose, which is not metabolised by the cells (Crane, 1960), and 2-deoxy-D-glucose which is phosphorylated but not converted into the furanose ring form (see Chapter 4 for a discussion of the merits of this sugar as a D-glucose analogue in transport studies).

The Millipore filtration method was originally developed with brown fat cells, in which the cytoplasmic water volume is greater than that of white fat cells, (Fain et al., 1967) as a model system. When applied to white fat cells, the equilibration of 3-O-methyl-D-glucose across the cell membrane took five minutes or longer (Czech, 1976c) even in the presence of insulin. In a later review of this work, the long equilibration time, and concommittant linearity of uptake through thirty seconds, was accredited to the particular batch of collagenase used (Czech, 1980) (see Chapter 4 for a more detailed discussion of this point). The transport inhibitor, cytochalasin B, markedly inhibited the initial rate of 3-O-methyl-D-glucose uptake, but a residual passive diffusion did occur in the presence of cytochalasin B. Phloridzin (an inhibitor of glucose transport in erythrocytes, LeFevre, 1948), partially inhibited the net entry of sugar.

A rather limited analysis of initial rates of zero trans uptake (measured at thirty seconds, and adjusted to allow for a substantial passive diffusion), yielded K_m values of between 1 and 3 mM which was not altered by insulin, but a V_{max} which increased in the presence of insulin (Czech, 1976).

The filtration method, and therefore the results obtained with it, may be criticised on several accounts. Firstly, the addition of an ice-cold solution to the cells is a documented method for breaking fat cells prior to their fractionation (Rodbell, 1967a). It therefore seems likely that errors

would be incurred by the cell lysis on the addition of the cold diluting solution at the end of the transport period. Secondly, even with Czech's cell preparation, which showed unusually slow equilibrium times (Czech, 1980), the ten to thirty seconds taken for the cell/medium separation represents a considerable fraction of the actual time interval of transport.

In the "oil centrifugation" method (Gliemann et al., 1972) a completely different approach was used for the cell/medium separation in the measurement of initial rates of sugar transport. Transport in small volumes of suspended fat cells was stopped with a solution containing an inhibitory concentration of phloretin in a sufficient volume of buffer to effectively dilute the extracellular radioactivity. The cells were then separated within two seconds from the bathing medium by centrifugation up through an oil which had a specific gravity intermediate between that of the fat cells and the aqueous medium. The method relied on rapid manipulations, and accurate timing of intervals as short as one second, accomplished with the aid of a metronome. This study showed that unbiased measurements of the intracellular water space could be obtained, and that the method could be used successfully to measure the time course of efflux of 3-O-methyl-D-glucose, where the shortest time point was taken at thirty seconds. It was subsequently used, with minor modifications, to study the concentration dependence and effect of insulin on the exchange of 3-O-methyl-D-glucose at intervals as short as three seconds (Vinten et al., 1976). Rapid efflux was measured and the curves obtained were treated as simple hyperbolae to give rate constants graphically from a simple logarithmic analysis. At 37°C, the half saturation constant (K_t) for the exchange in both the presence and absence of insulin was 5 mM; the maximal exchange rate (V_{max}) showed a degree of variability, between 0.07 and 0.2 $mmols^{-1}$ (1 cell water)$^{-1}$

without insulin, but rose to 1.7 $mmols^{-1}l^{-1}$ in the presence of insulin. The slight deviation from linearity was tentatively attributed to variations in the transport ability of individual cells. This study (Vinten et al., 1976) also demonstrated that of 3-O-methyl-D-glucose was not concentrated by the cells, by the equality of the equilibrium methylglucose distribution space and the ^3H_2O space; insulin had no effect on the methylglucose equilibrium space. Stereospecificity was shown by the fifty-fold difference in the rates of millimolar L-glucose and methylglucose self-exchange; the major part of the L-glucose transport was inhibited by D-glucose or 3-O-methyl-D-glucose. Phloridzin was a simple competitive inhibitor of the exchange process.

Initial rates of entry or exit were not measured directly in this methylglucose exchange study. The principle of the method, following efflux curves and graphically estimating rate constants, made this unnecessary. This technique was not applicable to influx measurements. However, Olefsky (1978) attempted to use a similar method to measure transport into sugar-free cells (zero trans entry) and inhibition constants (K_i) for 3-O-methyl-D-glucose and D-glucose on 2-deoxy-D-glucose transport. In this case, no sugar transport inhibitor was used to stop the out- or inflow of labeled sugar at the end of the transport interval, thus necessarily introducing timing errors. Also, rates of uptake were assumed to be constant for as least as long as the ten second interval used to determine initial rates of entry.

Whitesell & Gliemann (1979) described a method in which time intervals as short as one second were reproducible. They used it to find inhibition constants and exchange kinetic parameters of methlyglucose influx in adipocytes. Transport, initiated by the rapid injection of the cell suspension into small volumes of the labelled sugar solution, was stopped by

an eighty-fold excess of a phloretin-containing "stopping solution". Cells and medium were separated by centrifugation through oil and the cells collected with an absorbant material for the estimation of retained radioactivity. The efficacy of the stopping solution and stability of the cell preparation were assessed; contributions of unstirred layers and binding components to the apparent uptake were negligible. Non-mediated diffusion did not contribute significantly to the transport. Countertransport with methylglucose or glucose against ^{14}C-methylglucose was seen.

The responsiveness of this cell preparation to insulin was analysed. At 37°C, maximal activation was achieved within one and a half minutes with 1 μM insulin; maximal activation was found at approximately 2 x 10^{-10} M insulin. Lowering the temperature from 37°C to 22°C halved the maximum permeability of insulin-stimulated cells, whereas a small increase in uptake was observed in cells in the "basal" condition.

Values for the rate constants (K_{ee}^{io} and V_{ee}^{io}) were determined graphically via the initial rates calculated from uptake curves. A value of 3.5 mM for the K_m (exchange) was obtained which was unaltered by insulin treatment, and V_{max} increased five-fold on insulin stimulation. An assumption was made that the transport system was symmetrical (defined by the equality of zero <u>trans</u> and equilibrium exchange K_m values) and it was shown that a theoretical zero <u>trans</u> uptake curve at a single, high concentration, based on this assumption, fitted experimental data well.

That the inhibition constant (K_i) of methylglucose net uptake by methylglucose was similar to K_{ee}^{io}, also suggested that the system was symmetrical. The K_i for glucose on methylglucose uptake was twice as high (Whitesell & Gliemann, 1979). These suggestions of symmetry were not tested further, but using the same transport technique an investigation of the

symmetry of both the basal and insulin-stimulated transport is reported in this thesis and will be discussed later.

This review of previous studies of sugar transport in intact adipocytes shows that the exchange parameters for 3-O-methyl-D-glucose transport have been measured by rapid transport techniques (Vinten et al., 1976; Whitesell & Gliemann, 1979). However there are discrepancies between these results and those obtained in different laboratories. Those of Whitesell & Gliemann differ, particularly in the greater rapidity of transport, from those obtained by Czech (1976). Calculated initial rates of uptake of low (0.1 mM) methylglucose appear to be 100-fold greater in Whitesell & Gliemann's study compared to Czech's study (Czech, 1976). Czech (1980) accounts for this by the use of different collagenase preparations; Whitesell and Gliemann dispute this, since they find a maximum difference of three between rates using different batches, and suggest that the filtration technique (Czech, 1976) underestimates initial rates. To resolve some of the differences between studies, it is necessary to demonstrate that initial rates could be measured. In this thesis, the use of integrated rate equations (Eilam & Stein, 1974) to confirm rapid measurements of initial rates is documented. With rates that are shown to be initial, the exchange parameters are calculated and compared to the earlier results.

The unidirectional flux of 3-O-methyl-D-glucose in both directions has not previously been measured using rapid transport techniques. For this thesis, unidirectional measurements were made not only with the zero trans experimental designs, but also with confirmatory infinite cis measurements. In studies of a transporter in fat cells where variations in transport occur between laboratories, it is important to describe transport parameters on cells prepared in a single laboratory to completely describe the transport, and in particular, its symmetry. Of necessity, separate cell

pools must be used, but variations between pools can be assessed by using experiments of more than one design on one pool, and by statistically analysing data from experiments of the same design obtained from several pools. This work is the subject of the next few chapters.

Chapter 2: Materials and Methods.

2.1. Materials.

Male rats (Wistar strain) were provided by the University of Bath Animal House, fed ab libitum on normal laboratory feed. Outdated human blood was obtained from the Wessex Regional Transfusion Service, Bath. Crude collagenase was from Sigma, PL Biochemicals and Worthington Enzymes, Millipore (UK) Ltd. Bovine serum albumin (fraction V) and isobutylmethylxanthine were from Sigma. 3-O-methyl-D-glucose was obtained from Koch-Light and Sigma. Porcine crystalline mono component insulin was a gift from Novo Laboratories; bovine insulin was also from Novo Laboratories Basingstoke, and from Sigma. Phloretin was obtained through Kodak Ltd. from K&K Laboratories, Liverpool and cytochalasin B from the Aldrich Chemical Company. Dinonyl phthalate and silicone oil (100cs) were from Hopkins & Williams, Chadwell Heath, Essex.

3-O-(^{14}C)-methyl-D-glucose (specific radioactivity > 50mCi/mmol) was purchased from the Radiochemical Centre, Amersham, or was synthesized from (^{14}C)-methyl iodide (>500mCi/mmol) (the Radiochemical Centre, Amersham) following the method of Barnett et al. (1975). Isotope from both sources behaved identically. 3-O-methyl-[I^{3}H]-D-glucose (specific radioactivity 2.3 Ci/mmol, and [U^{14}-C]-D-glucose was also from the Radiochemical Centre, Amersham.

Tunicamycin (Takatsuki et al., 1975) was a gift from Dr. J.P.R. Herman of Glaxo Laboratories, Stoke Poges. Pipecleaners were supplied by Tranters, tobacconist of Bath and Trowbridge and used in 1.5cm sections. Nylon mesh (250μm diameter) was from Henry Simon.

All other chemicals were of analytical grade, from normal laboratory

suppliers.

2.2. Preparative Methods.

2.2.1. The Preparation of Adipose Tissue.

Epididymal fat pads were dissected from male rats (200-250g), recently killed by cervical dislocation. Each pad was cut into pieces weighing approximately 50 mg, and one piece was placed in a mesh-base tube. These mesh-base tubes were prepared from a 2ml disposable syringe, nylon mesh (mesh size, 250μm) and a polypropylene washer. The tube was placed in a 10ml conical polypropylene tube containing 3mls of albumin-free Krebs Ringer Bicarbonate buffer (see standard data), gassed with 95% O_2 / 5% CO_2 to pH 7.4, at 37°C.

Prior to the measurement of 2-deoxy-D-glucose transport, the tissue pieces were pre-incubated for 15 minutes at 37°C, with or without additions of insulin.

Several methods were used to estimate tissue weight, the most reliable one was to take the difference in weight of a mesh-base tube within the 10ml conical tube containing the 3mls of solution, with and without the adipose tissue.

2.2.2. The Preparation of Adipocytes, method (a).

Isolated adipocytes were prepared from rat epididymal fat tissue from rats weighing 140 to 170g. The method used was that of Foley et al. (1980) with small modifications. The fat pads were dissected from animals freshly killed by stunning and cervical dislocation, with or without decapitation. The tissue was washed in about 10mls of Hepes-saline buffer (Na^+, 140mM; K^+, 4.7mM; Ca^{2+}, 2.5mM; Mg^{2+}, 1.25mM; Cl^-, 142mM; $H_2PO_4^-$ /HPO_4^{2-}, 2.5mM; SO_4^{2-}, 1.25mM; 4-(2-hydroxyethyl)-1-piperaze-ethanesulphonic acid, 10mM) with 1% w/v albumin (extensively dialysed and

filtered as described in Section 2.2.7) and chopped into 0.5mm cubes with a McIlwain tissue chopper (Mickle Laboratories, Gomshall). The tissue from one rat was placed in 3mls of Hepes buffer containing 3.5% albumin, 0.5mM glucose and 1.5mg collagenase (0.5mg/ml, Worthington Enzymes, Lot number 40B011P) at 37°C, and saturated with humidified oxygen and carbon dioxide (19:1). Rapid stirring for thirty to fifty minutes ensured complete digestion of the tissue, which was then filtered through a nylon mesh (mesh size, 250μm). The fat cells were washed five times with twenty mls of 1% albumin-Hepes-saline buffer; the cells were allowed to float to the surface for several minutes and the subnatent was discarded by aspiration with a siliconized and flamed Pasteur pipette, attached to a 20ml disposable syringe. The cytocrit was adjusted to give the required suspended concentration (not > 50%), by the capilliary method (see Section 2.2.3), and the cells were incubated for thirty minutes at 37°C with occasional gentle shaking by hand. This stabilisation time was concurrent with hormone stimulation or methylglucose pre-equilibration when appropriate.

Cells that were stimulated with insulin were treated with 10nM insulin (except where noted otherwise) for 15 minutes at 37°C before the transport was measured.

The adipocyte preparations were studied under a phase contrast microscope to assess the purity of the cell population.

2.2.2. The Preparation of Adipocytes, Method (b).

In this earlier series of experiments, adipocytes were prepared by isolation with 1mg/ml collagenase. The fat pads were rapidly removed from 180-250g male rats, killed either by decapitation or stunning and cervical dislocation. The fat tissue was roughly minced in a small volume of 37°C tris-phosphate-saline buffer (125mM sodium chloride, 5mM potassium chloride,

2.5mM magnesium chloride, 1.0mM potassium dihydrogen orthophosphate, 25mM tris-Cl, 0.5mM calcium chloride) at pH 7.4, or Krebs Ringer bicarbonate buffer (see standard data) pH 7.4, either buffer containing 2% w/v bovine serum albumin which had been prepared as described in section 2.2.7. The tissue was then transferred to polypropylene vials (2cm x 5cm) containing 2.5mls of buffer at 37°C. The buffer had been saturated with humidified 95% O_2 / 5% CO_2. Each vial contained two pads. Tissue was incubated with or without insulin at 24 or 10mU/ml at 37°C, in a water bath shaking at 30cycles per second.

Adipose tissue was digested with crude collagenase, by the addition of 5mg-collagenase dissolved in 2.5mls of buffer to give a final concentration of 1mg/ml, for one hour at 37°C, shaking as above. Digested material was forced through the nylon mesh (two fat pad's tissue over an area of 2.2cm^2) into a 10ml conical plastic tube. The cell suspension was collected by flotation at 1000xg for 30 seconds, spooned into another tube, and washed twice by resuspension and centrifugation in 10mls of tris-phosphate-saline containing 2% albumin.

The cells were packed by centrifugation at 1000xg and kept in a humidified atmosphere of 95% O_2 / 5% CO_2 at 37°C, prior to, and during the transport assay.

2.2.3. Estimation of Cell Number for Isolated Adipocytes.

(a) Cytocrit under microscope (Hirsch & Gallian, 1968).

An aliquot (20µl) of the fat cell suspension was added to 1ml of osmium tetroxide in 50mM cacodylate buffer containing 125mM sodium chloride, at pH 7.4. An aliquot of this was then placed on the grid-slide, and the number of cells per grid square counted. This gave the number of cells x 10^4, per ml. The fixation was not entirely successful since it caused clumping and an

uneven cell distribution. Therefore in some cases the fixative was not used, and the estimation performed rapidly to minimise the errors introduced by the tendency of the cells to lyse under the warmth of the microscope.

(b) Capillary Method.

A 20 µl capillary was placed in a well-suspended vessel of cells. The end of the capillary was plugged and then it is spun at 1200xg for 20 seconds. The distance occupied by the cell layer relative to the aqueous phase was measured and the percentage of cell to total volume calculated (Gliemann et al., 1972).

2.2.4. The Preparation of Adipocyte Ghosts.

The method used was essentially that devised by Rodbell (1967): adipocytes were isolated from fat tissue with crude collagenase and "ghosts" (membrane sacs formed from a whole cell or smaller vesicles formed from membrane fragments, containing some cytoplasmic components, but empty of fat droplets), prepared by hypotonic lysis, and resealed in medium of approximately physiological ionic strength.

Type I Ghosts. Adipocytes prepared by method (b) (described above in Section 2.2.2.) were used for the preparation of these ghosts. The cells were lysed in 10mls of 2.5mM magnesium chloride, 1.0mM potassium hydrogen carbonate, 2.0mM Tris-Cl at pH 7.4, or in 12.5mM sodium phosphates at pH 7.4. Lysis was affected by vigorous shaking by hand: 25 strokes per two fat pads' tissue. The pellet and infranatent were collected by centrifugation at 1000xg for 30 seconds and stored at 4°C; the lysing procedure was repeated four times with the remaining fat cake. The membranes were pooled by centrifuging all the pellets and infranatents at 1500xg for five minutes, the supernatent was removed and the pellet resuspended in a suitable volume of tris-phosphate-saline, or Krebs'-Ringer

bicarbonate buffer.

When insulin effects were studied, insulin was added to the washing and lysing solutions.

Type II Ghosts. The detailed experimental technique, but not the essence of this method differs from that described for Type I ghosts. The present method was developed to give a more reproducible yield of ghosts.

All glass- and plastic-ware was washed in 6M HCl prior to use, and doubly distilled water was used throughout. Before use, the bovine serum albumin was dialysed for $\geq$24 hours at 4°C against double distilled water (5g albumin/1 of water).

Immediately after removal, four epididymal fat pads (from 180-210g male rats, Wistar strain) were placed in 20mls tris-phosphate-saline (containing 1% w/v albumin, 0.5mM $CaCl_2$, and 2mM glucose) at 37°C in a water bath. Care was taken in the dissection, not to remove large blood vessels or glandular tissue with the fat tissue. The tissue in solution was continuously aerated with humidified 95% O_2 / 5% CO_2, throughout the preincubation and collagenase digestion. Preincubation with or without insulin was as described in Section 2.2.4., but for 30 minutes. Fat tissue was then roughly minced with scissors and 5mg crude collagenase added in a 200µl aliquot of calcium chloride-free, tris-phosphate saline. Digestion was complete within 30 minutes if the surface area of the tissue suspension was $\cong 20cm^2$ and shaking was vigorous (70 cps): the shaking and surface area are critical to the length of time needed for the digestion. The completion of digestion was assessed by inspection.

The digested tissue suspension was collected by centrifugation at 1500xg for 15 seconds, and the infranatent and pellet aspirated away by means of a Pasteur pipette. The material was gently redistributed in 30mls (per 4 or 6 pads) of calcium chloride-free, tris-phosphate saline containing

2mM glucose, at 37°C, before being gently forced through a nylon mesh (pore size, 250μm); 4 to 6 fat pads' tissue was squeezed over an area of 3.4cm^2, into a 30ml round bottomed tube. The adipocytes were collected by flotation at 1500xg for 30 seconds; the infranatent and pellet were removed by suction. The adipocyte layer was gently resuspended by swirling whilst adding 10mls of the lysing buffer (tris-phosphate saline, containing 1% dialysed albumin, diluted ten times with water) at 37°C and the lysate immediately collected by centrifugation and transfer into 10ml conical tubes after removal of the upper clear fat layer by suction. The membrane pellet and infranatent were stored at room temperature whilst the remaining adipocytes were lysed four more times, firstly in tris-phosphate lysing buffer further diluted by the addition of 5mls water at 37°C to 10mls of lysing buffer, and subsequently in the initial tris-phosphate lysing buffer.

The total lysates were spun at 1500xg for 12 minutes, the supernatent discarded and the pellets pooled, and resuspended in tris-phosphate saline (containing 1% albumin, no glucose, and no calcium chloride), and the membranes collected by centrifugation at 800xg for 5 minutes.

The membranes were resealed at 30°C for one hour in tris-phosphate saline (containing 1% albumin) for zero *trans* entry and infinite *cis* experiments, and as described below for zero *trans* exit and equilibrium exchange entry experiments.

2.2.5. The Preparation of Adipocyte Ghost for Electron Microscopy. Fosdyke (1979).

1ml of ghosts suspended in tris-phosphate saline (with 1% albumin) with or without additions of 0.5mM 3-isobutyl-1-methylxanthine, 1mM ATP and 10mU/ml insulin, were packed in a microfuge for one minute at 10000xg. The

supernatent was aspirated away, leaving approximately 50µl of packed ghosts. These ghosts were washed twice with 1ml of 0.15M cacodylate buffer and once in 2.5% glutaraldehyde. The ghosts were again washed with cacodylate buffer, then left in it for one hour. The pellet was stained in a solution of osmium tetroxide in 0.15M cacodylate buffer for one hour. After two further washes with cacodylate buffer, the ghosts were stored overnight in cacodylate buffer, at 4°C.

Ethanol was used for dehydration: 70% ethanol for 30 minutes, 96% for 30 minutes, then 100% for three changes of 20 minutes' duration each. The ghosts were then embedded in resin. Sections of the material were mounted on copper grids and examined under an electron microscope.

In earlier preparations, 0.25M sucrose, 10mM Tris, 10mM EGTA buffer was used throughout, and ghosts were not fixed in glutaraldehyde. After fixing for one hour, and washing in the buffer, the ghosts were stored for several days at 4°C before the ethanolic dehydration. Ghosts were then stained with uranyl acetate, prior to being embedded in resin.

2.2.6. The Measurement of [U^{14}C]-Glucose Oxidation.

The responsiveness of adipose tissue and isolated adipocytes to insulin was tested by monitoring the [U-^{14}C]-glucose oxidation by the tissue and cells.

In the first method, the absorption of ^{14}C-carbon dioxide by a potassium hydroxide saturated paper wick held above the incubation solution was estimated by liquid scintillation counting.

This method was then modified and the incorporation of the label into the cell fraction was measured, the cells being separated from the extracellular medium by oil-centrifugation. In these experiments, the ^{14}C-carbon dioxide evolution was sometimes also estimated, after acidification of the solution and collection with

hyamine hydroxide (Gliemann, 1967).

In this latter instance, the methodological details were as follows: For each sample, 100 µl of an approximately 50% (v/v) cell suspension was incubated ± 50 µl of a 10x concentrated solution of porcine insulin, with 400 µl buffer containing 0.5 µCi [U-^{14}C]-glucose, to give a final concentration of 1mM glucose, at 37°C under an atmosphere of humidified 95% O_2 / 5% CO_2 for fifteen or thirty minutes. 3.5mls of buffer was added to dilute out the extracellular label, 1.5mls dinonyl phthlate was layered onto the suspension and the sample was centrifuged at 2500xg for one minute. The cells were collected with 1/8th pipe cleaner and the ^{14}C estimated by liquid scintillation counting. When the $^{14}CO_2$ evolution was measured, it was collected above the acidified solution, in plastic wells containing paper wicks saturated with 200µl hyamine hydroxide. The ^{14}C was then estimated in 0.5% (w/v) P.P.O. in toluene as a scintillant.

The third method used to monitor [U-^{14}C]-glucose uptake into fat cells was the "Dole Extraction" technique, as developed by Gliemann, J. (personal communication). In this procedure, the incorporation of radioactive label into a "lipid" fraction was measured, after extraction in heptane (Dole, 1956; Dole & Meinertz, 1960).

For this extraction procedure, as with the previously described method, samples of 500µl of the cell suspension in 1mM glucose (radiolabelled) were used. The uptake was allowed to proceed at 37°C in a slowly shaking waterbath for 40 minutes, when the reaction was terminated by the addition of 100µl of 1.5M H_2SO_4 to the sample. 2.5mls of a 4 to 1 mixture of 2-propanol and N-Heptane were added to the vial, followed, after a few minutes, by 1.5mls of N-Heptane. When the phases were almost separated, 2mls of water was added. After each addition, the vials were vigorously shaken by hand. Phase separation having occurred, one ml of the upper

(lipid) phase was collected, 9mls of scintillant was added to it, and the ^{14}C c.p.m. estimated by liquid scintillation counting. The necessary standards contained (a) a 10µl aliquot of the [^{14}C]-glucose solution, 1ml heptane and 9mls scintillant, and (b) a sample treated as above, but containing no cells.

2.2.7. The Preparation of Albumin.

(a) The Chen Defatting Procedure (Chen, 1967).

This preparation of defatted albumin was used during the development of an insulin-responsive adipocyte preparation.

50g albumin (Sigma A4503) was dissolved in 500mls water at 4°C. 25g of activated charcoal was added (the charcoal having previously been freed of impurities by washing in 100mls water and collected by centrifugation at 20000xg). The pH was adjusted to 3.0 with 1M HCl, and the suspension stirred at 4°C for one hour. The charcoal was separated by centrifugation at 20000xg for 20 minutes, and the supernatent collected. The residue was washed at 4°C with 100ml water,and acidified to pH 3 with 1M HCl. The resultant suspension was again centrifuged at 20000xg for 20 minutes. This supernatent was pooled with that previously collected and the solution filtered through a 0.45µm cellulose filter to remove any remaining charcoal. The pH was adjusted to 7.0 with 0.2M NaOH, the solution was freeze-dried and stored at 4°C.

(b) The Dialysis and Filtration Procedure (Gliemann J. personal communication).

This method was used to prepare the albumin for use in the adipocyte preparation described in Section 2.2.2.(a).

100g bovine serum albumin (Sigma A4503) was dissolved by gentle stirring in 500ml distilled water at 4°C. The solution was poured into

two 150cm pieces of dialysis tubing (previously boiled and thoroughly washed in water). The albumin solution was dialysed against 5l to 6l of water for seven hours or more at 4°C. The water was changed and the dialysis continued for a further seven hours. The albumin solutions were washed twice and then emptied from the tubing, and the volume made up to 1000ml to give a 10% (w/v) stock solution. The solution was filtered under pressure, successively through Whatman 41 filter paper, Whatman 42 ashless filter paper, 5μm cellulose filter (Millipore type) then 0.8μm cellulose filter, avoiding as much bubbling and foaming as possible. The pH was adjusted to 7.6 at 22°C with 10M NaOH, and stored at -20°C in 10ml aliquots.

All water was doubly distilled, and stored in glass containers.

2.2.8. The Preparation of Pink Erythrocyte Ghosts (human).

The method used was essentially that of Dodge *et al*. (1963). 2ml aliquots of outdated human erythrocytes (stored at 4°C in citrate) were washed by centrifugation and the pellet resuspended three times in 30mls of phosphate-saline buffer (154mM sodium chloride, 12.5mM sodium phosphate), pH 7.4; any leucocytes were aspirated from the surface of the pelleted erythrocytes before resuspension. The cells were then lysed in 30mls of 12.5mM sodium phosphate, pH 7.4 at 4°C, and the membranes collected by centrifugation at 38000xg for 25 minutes at 4°C. This lysis step removes approximately 95% of the haemoglobin. The membranes were resealed in phosphate-saline buffer, with the addition of sufficient sodium chloride to return the final concentration to 154mM, at 30°C, for one hour, in a gently shaking water bath.

The ghosts were pelleted in a Bench Centrifuge at 2500xg for 10 minutes, the supernatent was aspirated off, and the ghosts resuspended in sodium phosphate buffer for transport measurements.

2.3. Techniques for Measuring Transport.

The experimental descriptions follow the terminology of Eilam & Stein (1974). The *cis* side of the membrane is defined as the side from which the isotope is transported, and the *trans* side is the opposite side of the membrane.

The temperature during the assays was maintained by use of a chiller apparatus.

2.3.1. Measurement of Transport in Adipose Tissue.

The mesh-base tube (Section 2.2.1) was removed from the pre-incubation medium, excess liquid shaken from the end and the vessel immersed in 3mls of solution containing an appropriate concentration of the glucose analogue, 2-deoxy glucose + 1μCi tracer. The incubation was ended by removing the inner vessel and washing it in 6mls of stopping solution I (5mM mercuric chloride, 0.14mM sodium iodide, 1% w/v sodium chloride) twice, for 15 seconds each. The mesh-base tube was then turned upside down and the adipose tissue washed out with 1ml of Krebs'-Ringer bicarbonate buffer. 1ml of 20% trichloroacetic acid was added to the tissue suspension, which was then spun at 2500xg in a Bench centrifuge for 15 seconds and a 1ml aliquot of the supernatent taken in order to estimate the amount of radioactive sugar taken up by the tissue with scintillation counting.

2.3.2. Zero trans Entry Experiments.

In this procedure, initial rates are estimated for a range of hexose concentrations, when the *trans* (internal) concentration is zero.

2.3.2.1. Filtration.

(a) Erythrocyte Ghosts.

10 µl of packed ghosts were suspended in 40 µl of phosphate-saline buffer at 20°C. To start the transport, 50 µl of an appropriate concentration of [^{3}H] or [^{14}C] D-glucose solution was mixed with the ghosts by injection. The transport was stopped by the addition of 1ml of ice cold stopping solution II (154mM sodium chloride, 1mM mercuric chloride, 0.142mM sodium iodide and 0.1mM phloretin in 1% ethanol). Samples were shaken by hand (2 strokes) then immediately filtered through glass fibre filters (Whatman GF/F), at a rate of $\geq$1ml/s; care was taken not to allow the sample to touch the sides of the Millipore tower. The ghosts retained by the filter were washed free of extracellular glucose with 15mls of stopping solution. The filter edges were removed with a cork borer to minimize background radioactivity and the filters dried in air before the sugar was extracted from the ghosts into 1ml of 1% (w/v) trichloroacetic acid, and estimated in a Packard "Tri-carb" Liquid Scintillation Spectrometer.

In order to measure initial rates of glucose uptake (necessary in the zero *trans* entry experiment) an electronic metronome, which gave an auditory and visual signal at specified time intervals was used; the shortest time interval used was one second over a range of glucose concentrations between 0.5mM and 10mM. Ginsberg & Stein (1975) report an accuracy of 10% at one second using a similar system.

For a zero time sample, 1ml of ice cold stopping solution II was added to the ghosts before the glucose solution. Ghosts were incubated with radiolabelled glucose for 15 to 20 minutes to obtain equilibrium isotope levels. The amount of sugar [c] within the ghosts at a given time (t), = $[(cpm_t - cpm_o) / (cpm_{oo} - cpm_o)] \times [S] = c$ in mM, where cpm_x = counts per minute when x = t, zero or infinity.

In inhibitor studies, the inhibitor was present both inside and outside the ghosts; it was added both to the resealing membranes, the resuspending solution, and to the sugar solution.

(b) Adipocyte Ghosts.

The method was essentially that used for erythrocyte ghosts, with the following modifications.

Tris-phosphate-saline (containing 1% or 2% (w/v) albumin) or Krebs' Ringer bicarbonate buffer with 2% albumin, was used as the suspending medium. The non-metabolised glucose analogue [1-^{3}H]-3-O-methyl-D-glucose was used in all adipocyte ghost transport assays. Final sample volumes of 100 µl or 150 µl were used. The stopping solution III contained 154mM sodium chloride, 0.5mM mercuric chloride, and sometimes 0.142mM sodium iodide, with the addition of either 1µM cytochalasin B or 0.1mM phloretin in 0.5% to 1% (w/v) ethanol. The glass fibre (Whatman GF/F) filters were dipped in the albumin containing buffer prior to use. 1ml of 2% sodium dodecyl sulphate or 1ml of 1% trichloroacetic acid were used to extract the labeled sugar from the ghosts: more reproducible data was obtained by not allowing filters to dry in air before tracer extraction.

2.3.2.2. Oil Flotation.

(a) Method 1.

The principle of this method is that transport of a non-metabolisable D-glucose analogue by isolated adipocytes may be rapidly stopped (within two seconds) by microfugation, by separating the cells from the sugar-containing medium through a layer of light oil. The basic technique was devised by Gliemann et al. (1972) and is described here, with the modifications used in the measurement of sugar transport in intact adipocytes, prepared as described in Section 2.2.3.

A 50µl aliquot of packed adipocytes was placed in a 0.5ml Eppendorf tube, where it remained by capillary action. To start the transport incubation, 50µl of labelled 2-deoxy-D-glucose at 0.5mM or 20mM was injected onto the cells. Thirty seconds before the end of each incubation, 100µl of the oil, dinonyl phthalate, was added to the tube, followed by 100µl stopping solution II. At the appropriate time, the adipocytes were centrifuged in a microfuge for 5 seconds at 10000xg; the tube was placed on a glass petri dish and cut through in the centre of the oil layer. The cells were washed by the stopping solution as they travelled up through the oil layer, and the stopping solution travelled downwards. The addition of the stopping solution markedly reduces the non-specific radioactivity associated with the adipocytes at time zero. The amount of radioactivity in this adipocyte layer was estimated by immersion of the top portion of the tube in 1ml of 1% (w/v) trichloracetic acid, followed by liquid scintillation counting.

(b) Method 2.

A later modification of the oil-flotation method does not rely solely upon centrifugation to stop the transport, but employs the hexose transport inhibitor phloretin to block transport at short time intervals. As with the filtration assays described above, rapid sampling is aided by the use of a metronome. Flotation of the cells up through a light oil is used only as the method for rapid separation of the cells from the extracellular medium. The extracellular isotope is diluted 50-fold by the addition of the phloretin solution.

The method adopted here was essentially that of Whitesell & Gliemann (1979). Briefly the method is as follows. The cells were maintained throughout the assay in a 40% (v/v) suspension of Hepes-saline-1% albumin buffer and, except were otherwise noted, 0.5mM sodium pyruvate.

Polypropylene tubes (Sardstedt) were used for this assay. To begin the transport, 40 µl of the cell suspension were injected onto 12 µl Hepes-saline-1% albumin buffer containing 0.15µCi [^{14}C]-3-O-methyl-D-glucose per sample, and an amount of methylglucose such that the final concentration in the sample was 1mM to 40mM, as appropriate. Alternative volumes of sugar solution and cell suspension were 20 µl and 80 µl, or 15 µl and 50 µl respectively. The temperature at which transport was assayed was 37°C. A metronome set at 120 beats per minute was used to accurately assess time intervals of $\geq$ one second. The transport was stopped by the addition of 3mls of Hepes buffer (albumin-free) containing 0.3mM phloretin, previously dissolved in a solution of 0.015% (v/v) ethanol and 0.005% (v/v) dimethylsulphoxide at 22°C. Although ethanol alone had initially been used to dissolve the phloretin, this ethanolic phloretin solution had a tendency to precipitate when mixed with the Hepes-saline buffer; the ethanol/dimethylsulphoxide mixture was found to be more reliable. Within two minutes, 1ml of dinonyl phthalate or silicone oil was layered onto the surface and the sample was centrifuged at 2500xg in a bench centrifuge (M.S.E., Super Minor) for one minute. The separated cells were immediately removed from the surface of the oil with a 15mm piece of a pipecleaner. The radioactivity was released into 1ml of 1% (w/v) trichloroacetic acid and estimated by liquid scintillation counting.

The amount of radioactivity associated with the cells at zero time was measured by adding the cell suspension to the isotope solution immediately after the stopping solution had been added. The amount of radioactivity within the cells at equilibrium was measured by incubating the cells in the radioactive sugar solution at 37°C for an "infinite" time of sixty minutes for the basal condition and twenty minutes for the insulin-stimulated cells. Throughout this incubation the cells were gently resuspended by hand every

five to ten minutes in order to obtain good reproducibility of the replicates. The intracellular concentration of sugar (C_t) was calculated as described in Section 2.3.2.1. for red blood cell ghosts.

2.3.3. Zero trans Exit Experiments.

In this procedure, initial rates of efflux from cells or ghosts pre-loaded with isotope and sugar are estimated for a range of 3-O-methyl-D-glucose concentrations when the trans (external) concentration is zero. Initial rates are either measured directly at an early time point and/or calculated by integrating time courses in the same concentration range of substrate.

2.3.3.1. Filtration.

(a) Erythrocyte Ghosts.

D-glucose (at 40mM or 10mM) was added to the buffer in which the ghosts were then resealed for one hour. [1-^{3}H] or [U-^{14}C]-D-glucose was added and equilibrated across the ghost membrane at 30°C for 10 minutes. The ghosts were microfuged at 10000xg for two minutes and the supernatent removed by suction. A time course was measured at 20°C by rapid mixing of 10 µl of these packed ghosts with one ml of phosphate-saline buffer; the reaction was stopped by adding 1ml of ice-cold stopping solution followed by rapid filtration and washing, as described in Section 2.3.2.1. (a). The concentration of sugar remaining within the ghosts was calculated as described in Section 2.3.3.2.

(b) Adipocyte Ghosts.

Microfuge-packed ghosts became too viscous to allow accurate pipetting of small (5 to 10 µl) aliquots, so a more loosely packed pellet was obtained by centrifugation at 1000xg for one minute. A technique similar to that

used for erythrocyte ghosts was unsuccessful when applied to adipocytes, so a modified method was developed. 100µl of loosely packed ghosts to which an aliquot of labeled methylglucose at an appropriate concentration had been added, 45 minutes after resealing in tris-phosphate saline (with 1% albumin), were further resealed for 30 minutes at 30°C to allow the labeled sugar to equilibrate. The ghosts were then incubated at 25°C for approximately 5 minutes before transport was measured at 25°C. For each sample, 7µl ghosts, loosely packed and in the sugar solution, were placed in a plastic vial and, at zero time 7mls of tris-phosphate-saline (albumin free) was injected onto the ghost aliquot. During transport, the ghost suspension was constantly stirred. The efflux was stopped at an appropriate time by injection of 7mls of stopping solution IV (154mM sodium chloride, 1mM mercuric chloride, 0.2mM phloretin in 0.5% ethanol), stirred for 4 seconds and filtered as before except that the ghosts were washed with stopping solution V (154mM sodium chloride, 0.5mM mercuric chloride, 0.1mM phloretin in 1% ethanol).

Zero time samples were measured by the addition of 14mls of a 1:1 mixture of tris-phosphate-saline and stopping solution IV.

Time courses for efflux at various methylglucose concentrations were measured, as were initial rates of efflux.

The concentration of sugar remaining within the ghosts was calculated as described in Section 2.3.3.2.

2.3.3.2. Oil Flotation.

(a) Adipocytes.

A 50% (v/v) suspension of cells was equilibrated with 3-O-[^{14}C]-methyl-D-glucose at various concentrations, at 37°C, for 60 minutes. The efflux was initiated by adding 50µl of the cell suspension to 5mls of sugar- and albumin-free Hepes-saline buffer, in a 10ml polystyrene tube, at

37°C. The suspension was either shaken by hand or mechanically stirred with a magnetic follower. Transport was terminated by the addition of 5ml Hepes-saline buffer containing 0.6mM phloretin dissolved in 0.015% (v/v) ethanol and 0.005% (v/v) dimethylsulphoxide.

In some initial experiments, 9 mls of the efflux buffer and 1 ml Hepes-saline buffer containing 3mM phloretin were used. These latter cell suspensions were mechanically stirred both during the flux period and during the addition of the stopping solution.

In both instances, cells were spun through 1ml dinonyl phthalate at 2000xg for 1.5 minutes. The radioactivity retained in the cells was estimated as described in Section 2.3.3.2. The amount of radioactivity associated with the cells at zero time and at "infinite" time were estimated and used in the calculation of fractional efflux. Fractional efflux multiplied by the loading sugar concentration gives the concentration remaining (C_t).

2.3.4. Equilibrium Exchange (Entry) Experiments.

Initial rates are measured for a range of sugar concentrations entering the cell when the sugar concentration on the *trans* side is the same as that on the *cis* side, but only the latter sugar solution initially contains isotope.

2.3.4.1. Filtration.

(a) Erythrocyte Ghosts.

10µl of packed ghosts were suspended in 40µl phosphate-saline buffer, and a 50µl aliquot of twice concentrated sugar solution added, after resealing. The sugar was allowed to equilibrate across the membrane for 15 minutes at 20°C, then influx was measured over two seconds in a final

sample volume of 150 μl, as described for zero *trans* entry (Section 2.3.2.1(a).

(b) *Adipocyte Ghosts.*

The experimental details differed from those noted for erythrocyte ghosts only in that the medium for transport was tris-phosphate-saline, containing 1% or 2% (w/v) albumin.

2.3.4.2. Oil Flotation.

A 40% (v/v) suspension of adipocytes was equilibrated at 37°C with a range of concentrations of methylglucose for either ten minutes, in the case of insulin-stimulated cells, or for up to 60 minutes for cells in the basal condition. Initial rates were measured by adding 50 μl of the cell suspension to 10 μl of labelled methyl glucose at the appropriate concentration. The stopping solution, and the method of cell collection for ^{14}C estimation were the same as those used for the zero *trans* entry experiments.

2.3.5. Infinte cis Entry Experiments.

Net flux is measured at a sugar concentration previously shown to be sufficient to produce maximal influx. The measurement of a time course over which backflux occurs can then be used to estimate the *trans* (internal) saturation constant by using an integrated rate equation.

2.3.5.1. Filtration.

(a) *Erythrocyte Ghosts.*

The uptake of 40mM [^{14}C]-D-glucose was assayed over a time course of 4 to 64 seconds, using the technique previously described for the zero *trans* entry experiment (Section 2.3.2.1(a)).

(b) Adipocyte Ghosts.

The uptake of 20mM 3-O-methyl-D-glucose was studied in the same manner as for erythrocyte ghosts.

2.3.5.2. Oil Flotation.

(a) Adipocytes.

The net influx of 40mM 3-O-methyl-D-glucose was measured as described for the zero trans entry experiments (Section 2.3.2.2(b)).

The "microfuge technique" described by Foley et al. (1980) was also used for these experiments. The method was the same as that described by Whitesell & Gliemann (1979) except that transport was stopped with 400µl of 0.1mM phloretin. 400µl of this mixture was transferred to a microcentrifuge tube (550µl) containing 100µl silicone oil (0.99g/l) and within two minutes was centrifuged at 10000xg for 30 seconds in a Beckman microfuge. The tube was cut through the oil phase with a hot knife and the top part, containing the cells, was added to a vial for scintillation counting.

2.3.6. Infinite cis Exit Experiments.

For this type of experiment, cells are equilibrated with a substrate concentration sufficient to saturate the internal sites, and the rate of net exit into varying trans (external) concentrations of sugar, is measured.

2.3.6.1. Oil Flotation.

(a) Adipocytes.

A 40% (v/v) cell suspension was equilibrated with 40mM 3-O-[^{14}C] methyl-D-glucose for 60 minutes. The transport assay was started by adding 50µl of this suspension to a volume of Hepes-saline buffer that has been calculated to give, on dilution of the extracellular sugar in the cell suspension, the desired trans (external) concentration of 3-O-[^{14}C]-methyl-

D-glucose. For external concentrations of 1, 3, 5, 10 and 15mM, the dilution volumes were 1170, 370, 210, 90 and 50µl respectively. The transport was halted by the addition of a volume of stopping solution (Section 2.3.3.2(a)) that brought the final sample volume to 3ml: a constant dilution of the background, or extracellular isotope was thus achieved.

The net exit at zero time was measured by adding 50µl of the cell suspension and 2mls of the stopping solution to 1ml of *trans* buffer, simultaneously. The estimation of the cellular 3-O-[^{14}C]-methyl-D-glucose was as described previously in Section 2.3.2.

2.3.7. Statistical Treatment of Kinetic Data.

For each experiment, 2 to 6 replicates were used for each condition or time interval of transport. The coefficient of variation for replicates was approximately 10%. The kinetic parameters were determined by a weighted regression of S/V vs. S plots. Each data point was weighted for constant percentage error as described by Cornish-Bowden (1976). Other plots were analysed with an unweighted regression.

2.3.8. The Testing of Potential Effectors of 3-O-Methyl-D-Glucose Uptake in Adipocytes.

The isolated cells were prepared according to the method described in Section 2.2.4.II. Both basal and insulin-stimulated cells were treated with the potential effector for a variety of times, at 37°C. The compounds tested are listed below:

Puromycin

Cordecypin

Tunicamycin

Xanthine

Hypoxanthine

Caffeine

Theophylline

3-Isobutyl-1-methylxanthine

The compounds were added in 4µl aliquots to 400µl pools of 40% (v/v) cell suspensions. Transport was then assayed following the initial rate of zero *trans* entry of 1mM 3-O-methyl-D-glucose at one or fifteen seconds.

In all these studies, means were compared using Students' t-test, and a significant difference was taken to have a random probability of $\leq$ 5%.

Chapter 3: Results

3.1. Preliminary Studies on Adipose Tissue and Isolated Cells.

3.1.1. 2-Deoxy-D-glucose Uptake by Adipose Tissue.

In order to investigate the insulin response of sugar uptake in mammalian adipose tissue, the ability of tissue pieces to take up the D-glucose analogue, 2-deoxy-D-glucose was studied. This particular analogue was chosen since the time course for uptake was sufficiently long to permit the simple method devised to be used. The method developed allowed pieces of tissue to be immersed in a radioactive 2-deoxy-D-glucose solution, removed and washed in a solution containing an uptake inhibitor in order to assess the extent of uptake into the tissue. Table 4 shows that the washing method was efficient.

TABLE 4

Radioactivity in Tissue Washing Solutions.

Solution	Radioactivity* in cpm/ml
[^{3}H]-2-deoxy-D-glucose	100%
Wash (a)	0.5%
Wash (b)	0.025%

* The radioactivity was expressed as a percentage of that in the original sugar solution.

The uptake of this D-glucose analogue was measured at one, two and five minutes in the presence and absence of 25mU/ml bovine insulin (Table 5).

TABLE 5

The Uptake of 0.5mM 2-deoxy-D-glucose into Fat Tissue Pieces at 37°C ± Insulin.

Time in minutes	*Uptake no insulin	*Uptake + insulin	Stimulation by insulin (approx.)
1	8.5	32.5	3.8-fold
2	29.5	60.5	2.1-fold
5	49.5	88.0	1.8-fold

Table 5: Insulin was at a concentration of 25mU/ml. *Uptake was measured in μmoles sugar taken up per mg wet tissue. Values given are to the nearest 0.5μmole. Data is from one experiment; each uptake was measured in duplicate.

The extent of insulin stimulation was low, and poorly reproducible on a day-to-day basis. Since viable, insulin-sensitive tissue was essential to the isolation of cells and preparation of "ghosts", some considerable attention was devoted to the details of the extraction of the tissue and also to the subsequent cell isolation and production of ghosts, with the aim of optimising cell lifetime and hormone-sensitivity.

3.1.2. Method Improvements for Adipocyte Preparation.

The preparation of adipocytes, described in Section 2.2.2, Method (b), had been modified, during attempts to measure insulin-sensitive methylglucose uptake, in several respects: the collagenase concentration was reduced to 0.5mg/ml and the digestion was terminated by inspection of the incubations (usually 30 to 40 minutes), 1mM D-glucose was added during the collagenase digestion and the first wash, a hypertonic (150mM NaCl) Krebs Ringer bicarbonate buffer was used, all glass- and plastic-ware was soaked in 6M HCl overnight and thoroughly rinsed in hot tap water, singly

distilled and then doubly distilled water. The first two modifications were suggested by Belsham et al. (1980). The hypertonic buffer was used to prevent swelling, and thereby lysis, by creating a tendency for cells to shrink. However, the preparation remained insufficiently sensitive to insulin, so a more detailed investigation into other possible modifications was undertaken.

Regarding collagenase digestion, screening of the commercially available crude collagenase preparations for any reduction in hormonal activity has been recommended (G. Belsham, personal communication). In one analysis, this was assessed by monitoring glucose oxidation (Section 2.2.6) immediately after the collagenase digestion, which was at 0.5mg/ml for forty minutes. Table 6 shows increases of four- to sixteen-fold.

TABLE 6

Insulin Sensitivity of Fat Cells immediately after Collagenase Digestion.

Type of collagenase	Basal Uptake of [^{14}C]-D-glucose (nmoles/mg cells/hr)	Uptake of [^{14}C]-D-glucose in insulin-treated tissue (nmoles/mg cell/hr)	Increase in insulin-treated uptake over basal.
PL, Lot 20	55.8	217.9	4-fold
Sigma, Lot 37C	34.6	312.0	9-fold
Sigma, Lot 49C	28.5	454.1	16-fold

Table 6: Data is from one experiment. In this and subsequent experiments, except where stated otherwise, each value represents a single or duplicate experimental determination.

However, the response was lost after the cells were washed and collected by centrifugation at 1000xg (Table 7).

TABLE 7

Insulin-sensitivity of Fat Cells after Collagenase Treatment and Washing.

Type of Collagenase	Basal Uptake of [^{14}C]-D-glucose (nmoles/mg cell/hr)	Uptake in [^{14}C]-D-glucose in insulin-treated cells (nmoles/mg cell/hr)	Comments
PL, Lot 20	28.3	37.7	Slight insulin stimulation
Sigma, Lot 37C	16.3	12.5	Slight decrease with insulin
Sigma, Lot 49C	18.5	11.6	Slight decrease with insulin

Table 7: Data is from one experiment.

This loss could be a direct effect of the washing procedure, or the time of, and collagenase concentration in, the digestion could affect the long-term stability of the cells. This latter possibility was investigated further.

In one such experiment, a range of collagenase concentrations from 0.1mg/ml to 2.0mg/ml was tested, at incubation times of between 25 and 60 minutes. A preparation of defatted albumin as used (Section 2.2.7.(a)), at 1% (w/v). However, a significant degree of lysis was noted for each condition and no improvement in sensitivity to insulin was noted (data not shown). A development that gave a small (2-fold) insulin stimulation was the use of the McIlwain tissue chopper to produce uniformly cut ($1mm^3$) tissue pieces for digestion. The conditions of digestion were as follows: a collagenase concentration of 0.5mg/ml and digestion for 25 minutes with PL Chemicals' collagenase, Lot 20. Defatted albumin at 1% (w/v), and 1mM D-glucose was used in all the buffer. The basal rate of 1mM[U-^{14}C]-D-glucose uptake, measured at 30 minutes was 5.55nmoles glucose taken up per mg of packed cells per minute. With insulin present, the rate was 10.0nmoles/mg/minute. Each value was measured in duplicate. The whole

experiment was performed in a room maintained at 37°C.

This slight improvemnt was followed up by measuring the response to 1pM to 10mM insulin (porcine, monocomponent) of a preparation prepared by a similar collagenase digestion step (Table 8). In an attempt to improve cell stability during the wash period, the cells were collected after each wash

TABLE 8

The Sensitivity of Fat Cells to a Range of Insulin Concentrations.

Insulin concentration	Uptake (nmoles/10^5 cells/hr)
0	29.6
1pM	28.3
10pM	41.1
100pM	43.3
1nM	92.6
10nM	84.9

by allowing them to float to the surface of the solution under gravity for one minute, without centrifugation. The number of washes was increased to four, with approximately 10mls of buffer for each. 1mM [U-^{14}C]-D-glucose uptake was measured as previously. A three-fold increase in uptake was observed in cells treated with 1nM porcine insulin, and lysis was minimal during the experiment.

When siliconized glass vessels were used to contain the fat cell suspensions, there appeared to be a further increase in the insulin-sensitivity, and a reduction in cell damage with time (assessed visibly). Table 9 records a four-fold insulin response, in an experiment with this modification. This suggests that even the stringent acid-treatment of the plastic vessels was inadequate.

TABLE 9

The Sensitivity of Fat Cells in Siliconised Glass Vessels to Insulin.

Insulin Concentration	Uptake of [U-^{14}C]-D-glucose (nmoles/10^5 cells/hr)
0	24.9
1pM	28.2
10pM	33.3
100pM	41.0
1nM	98.0
10nM	103.6

A more comprehensive measurement of the amount of [^{14}C]-D-glucose incorporation into adipocytes was subsequently used. The [^{14}C]-glucose uptake was measured as previously, but in addition, the [^{14}C]-carbon dioxide evolved was collected by adsorption to hyamine hydroxide, and this was also estimated by liquid scintillation counting. The extent of lysis was assessed by the release of lactate dehydrogenase into the medium during the preparation.

An unsuccessful modification was the digestion of uncut fat pads in 0.5mg/ml collagenase. Even after one hour's incubation at 37°C, the tissue was not wholely digested and cell damage occurred during the mesh-filtration stage. A 1.8-fold increase in [^{14}C]-carbon dioxide evolution and [^{14}C]-D-glucose uptake was observed (Table 10).

TABLE 10

The Sensitivity of Fat Cells to Insulin: Uncut Pads, and Low Collagenase.

Insulin Concentration	[^{14}C]-incorporation into cells (nmoles/10^5 cells/hr)	[^{14}C]-released as carbon dioxide (nmoles/10^5 cells/hr)
0	6.14	2.78
1pM	6.74	2.89
10pM	7.69	3.06
100pM	8.90	5.02
1nM	11.35	4.69
10nM	11.64	4.50
100nM	11.54	4.50
1μM	11.46	-

Table 10: Data is from one experiment.

Several points had emerged from the previous experiments. One critical point was that gentle treatment of the cells was essential. Thus, in the next experiment, the collagenase-digested tissue was allowed to pass through the mesh during filtration under very little pressure. The washing buffer was removed by aspiration through a flamed and siliconised Pasteur pipette to avoid disturbance of the floating cell layer. Complete digestion in a low concentration of collagenase was used and 2% (w/v) dialysed albumin (see Section 2.2.7.(b)) was used during the digestion, although 1% (w/v) was present in other suspending buffers. A thirty minute period of "stabilisation" after cell preparation was used as this appeared to increase the sensitivity of the cells (Kono et al., 1977).

These changes in method were apparently small, but they led to a seventeen-fold insulin-stimulation of [^{14}C]-D-glucose incorporation into the adipocytes (Table 11).

TABLE 11

Improved Sensitivity of Fat Cells to Insulin

Type of collagenase	Insulin Concentration	[^{14}C]-incorporation into cells (nmoles/10^5 cells/hr	[^{14}C] released as carbon dioxide (nmoles/10^5 cells/hr
Worthington Enzymes, Lot 1	0	0.207	0.355
	10nM	3.42	5.185
PL Chemicals Lot 23	0	0.115	0.159
	10nM	1.497	2.460

The sensitivity of the preparation had been improved. This method, with very small modifications was used for adipocyte preparation in the kinetic experiments: Hepes-saline was chosen, instead of Kreb's Ringer bicarbonate buffer, for its pH-stability; continuous gassing was found to be unnecessary; the hypertonicity of the buffer was not necessary for the insulin-sensitivity.

In the experiment reported in Table11Ø, neither of the two collagenases appeared significantly better than the other. In a later experiment (data not shown) a new batch of Worthington Enzyme's collagenase showed a twenty two-fold stimulation of [^{14}C]-D-glucose incorporation into the "lipid" phase with the "Dole" method (Section 2.2.7.(b)), by comparison to a seventeen-fold stimulation given by the same cell population by Worthington, Lot 1. Since there is this small variation between batches of collagenases, for subsequent kinetic experiments, one collagenase (PL Chemicals, Lot 40B011P) was used throughout. The practice of chopping the tissue into a 0.5mm cube with a tissue chopper was compared to scissor cutting: an eighteen-fold stimulation of [^{14}C]-D-glucose incorporation was shown with insulin in the former case, and nineteen-fold in the latter.

Throughout this series of experiments, the number of cells per sample was estimated by using a cytometer grid slide in a phase contrast microscope. The apparent discrepancies in the apparent rates of D-glucose uptake between experiments may, in part, be due to inaccuracies in this method, when applied to fat cells: an even distribution of cells was difficult to obtain, and the cells became fragile in the heat of the microscope lamp. The capillary method (Section 2.2.3) was therefore adopted during the kinetic experiments, since this was more reliable, provided that the cells were evenly distributed throughout the container before capillary sampling.

3.2. Transport Kinetics in Adipocytes Treated with Insulin.

3.2.1. The Insulin Concentration in Kinetic Experiments.

The sensitivity of the cell preparation (Section 2.2.2.(a)) to insulin over the range 10^{-3} to 10nM was measured. The histogram (Figure 1) shows that 0.1nM insulin maximally stimulates the cells, and that the concentration (10nM) used for the kinetic analyses shows that same rate of uptake.

3.2.2. The Stability of Cells, and their Response to Insulin during Experiments.

In order to assess whether the rates of sugar transport were constant over the maximum time of any assay, the entry of 1mM 3-O-methyl-D-glucose was measured at intervals up to three hours after cell preparation. Two conclusions may be drawn from the resultant ~~histogram~~ graph (Figure 2). Firstly, the basal rate of uptake is stable for the duration of the experiment: none of the values is significantly different from the mean uptake at 30 mins (Student's *t* test). This shows that a negligible number of cells die during the experiment. Secondly, the cells remain equally sensitive to insulin over this time: again, no individual value is significantly different from

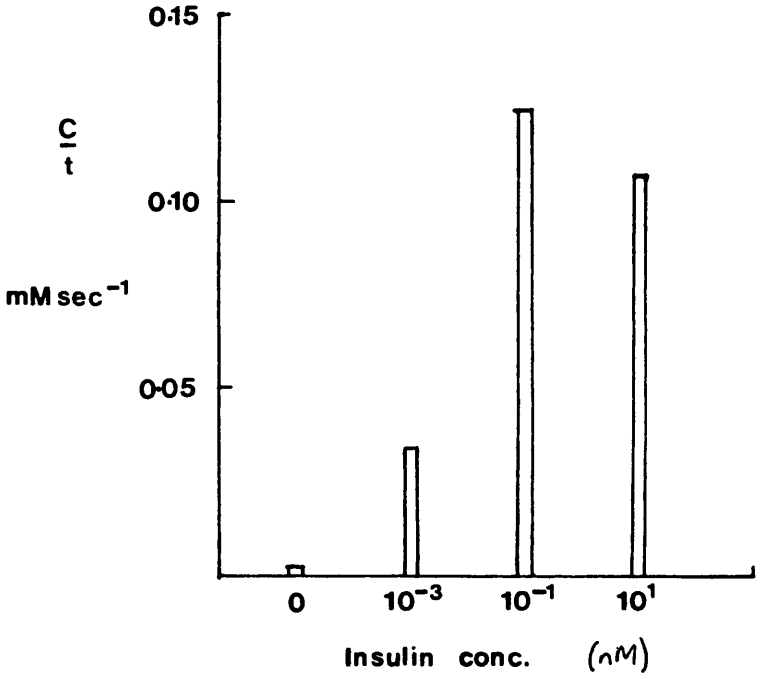


FIGURE 1

The Response of Isolated Adipocytes to Insulin.

Fig. 1: The cell suspension were treated with porcine insulin for $\geq$ 20 minutes, and the uptake of 1mM 3-O-methyl-D-glucose measured over one second, as described in Section 2.3.2. The extent of uptake per second is plotted against the insulin concentration in nM. At each concentration, uptake was measured in quadruplicate. The uptakes at 10^{-1}nM and 10^{1}nM insulin are insignificantly different (Student's t test).

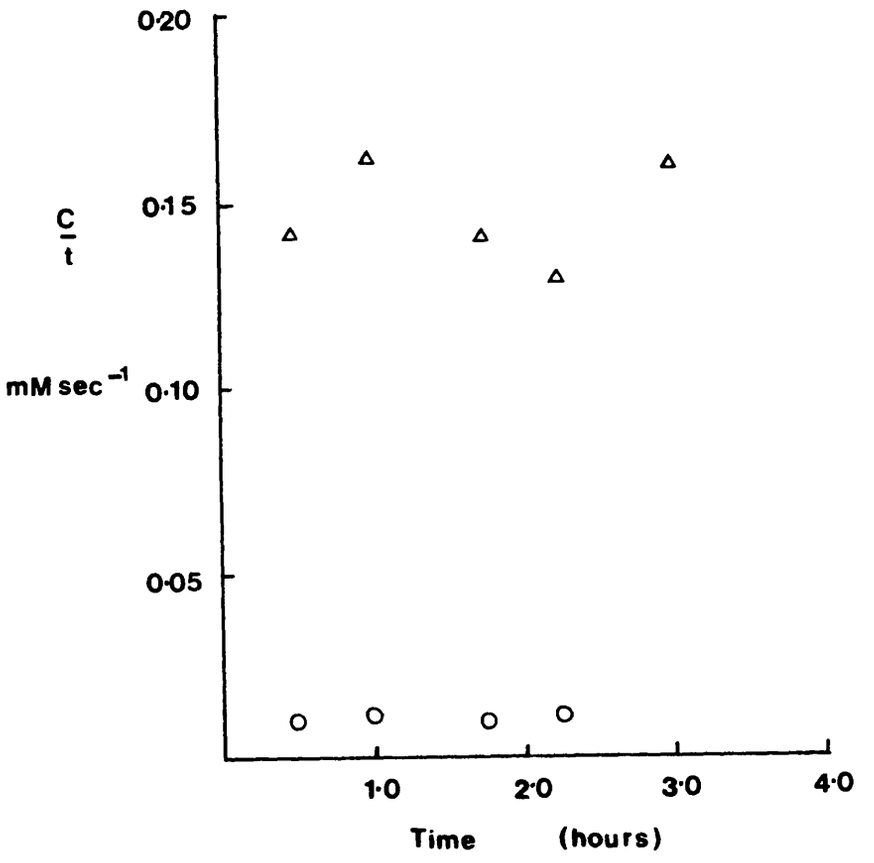


FIGURE 2

The Stability of Transport Rates.

Fig. 2: The uptake of 1mM 3-O-methyl-D-glucose was measured under zero trans conditions, from basal and insulin-stimulated adipocytes at 15 seconds and 1 second respectively. Transport assays were performed at intervals up to 3 hours after cell preparation. Points are means of quadruplicate values. (Δ) refers to insulin-treated cells and (O) to basal cells.

the mean uptake at 30 mins (Student's *t* test).

During prolonged transport assays, cell suspensions were used for up to four hours after preparation. The suspending buffer was free of D-glucose as, in physiological concentrations, this would interfere with some of the 3-O-methyl-D-glucose transport assays. Sodium pyruvate at 0.5mM was therefore added to prevent possible cell starvation, and associated hormonal-response changes (Kono *et al.*, 1977). In experiments where the transport assay was rapid (as was the case for insulin-stimulated cells, and singe time-point assays) sodium pyruvate was omitted from the suspending buffer, but the results obtained did not differ significantly from other experiments of the same type where pyruvate was included.

In order to confirm that the addition of sodium pyruvate did not affect the rate of sugar uptake, the exit of 1mM 3-O-methyl-D-glucose under zero *trans* conditions was measured on the samples from the same cell preparation in the presence and absence of 0.5mM sodium pyruvate. The $V_{initial}$ values, measured over ten seconds, were 0.027 +.0016mMs^{-1} (n=4) for pyruvate containing suspensions, and 0.0298 +.0055mMs^{-1} (n=3) for pyruvate-free suspensions, where "n" was the number of individual observations and the rates were measured 2 1/2 hours after cell preparation. Initial rates measured over ten seconds did not differ significantly when assayed two and a half hours after cell preparation.

3.2.3. The Measurement of Initial Rates.

For the determination of the zero *trans* kinetic parameters for the transport system it is necessary to know the longest single point time interval that could be used to directly measure the true initial rate of uptake. This may be evaluated by using an integrated rate equation to calculate the true initial rate. A time course for net entry at 1mM

methylglucose was measured at 1, 2, 3 and 4 seconds for cells which had been treated with insulin (Figure 3). The low methylglucose concentration would show the maximum deviation from linearity of the amount of sugar taken up with time. To calculate the initial rates, equation 3 devised by Ginsberg & Stein (1975) is used:

$$(A + BS_o)\ \frac{\ln(1-C/S_o) + C/S_o}{C}\ -\ \frac{A}{S_o} = -\frac{t}{C}$$

Equation 3

where $A = KR_{oo} + R_{12}S_o$,

and $B = KR_{oo}/\Pi + R_{12} + (R_{12}/\Pi + R_{21}/\Pi + R_{ee}/K)S_o + R_{ee}S_o^2/K\Pi$,

where Π is the concentration of osmotically active but impermeant material, S_o is the external concentration of substrate, C is the internal concentration, R_{ee} is the reciprocal of the maximum velocity in the equilibrium exchange experiment, R_{12} is the reciprocal of the maximum velocity in the zero <u>trans</u> efflux experiment and R_{21} is the corresponding value for the influx direction.

$$R_{oo} = R_{12} + R_{21} - R_{ee}.$$

A plot of t/C, that is, the reciprocal of the net rate of entry, V, vs. $-[\ln(1-C/S_o) + C/S_o]/C$ will give the reciprocal of the initial influx rate ($1/v_o$) on the ordinate (Eilam & Stein, 1974; Holman, 1979). From Figure 4 this calculation gives an initial rate for 1mM 3-O-methyl-D-glucose entry into insulin-stimulated cells of $0.21 mMs^{-1}$. Since the apparent initial rate calculated as C/t when uptake was directly measured at one second is $0.16 mMs^{-1}$, the true initial rate is 24% underestimated even at one second. At 2 seconds the initial rate is underestimated by 43%.

Measurement of time intervals of less than one second were not

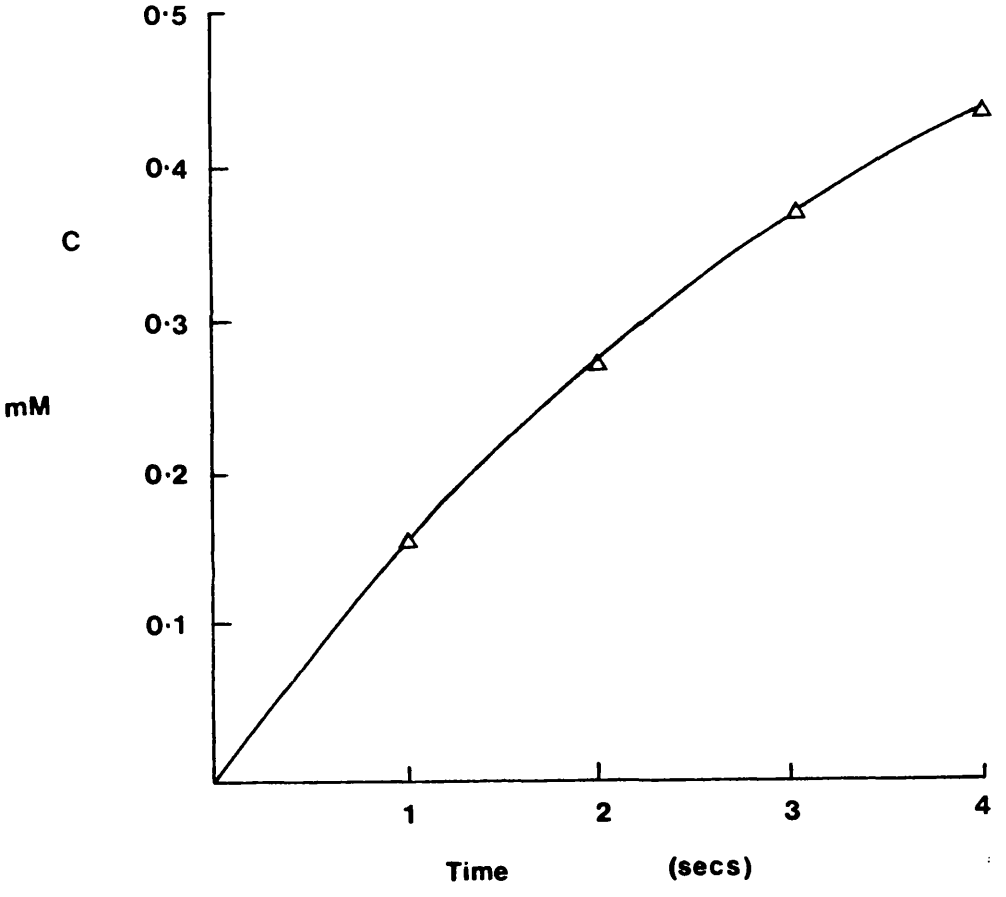


FIGURE 3

Entry of Methylglucose in Insulin-treated Adipocytes.

Fig. 3: A time course for the net entry of 1mM 3-O-methyl-D-glucose, in cells pre-treated with 10nM porcine insulin, at 37°C. 34 observations, from 2 populations of cells.

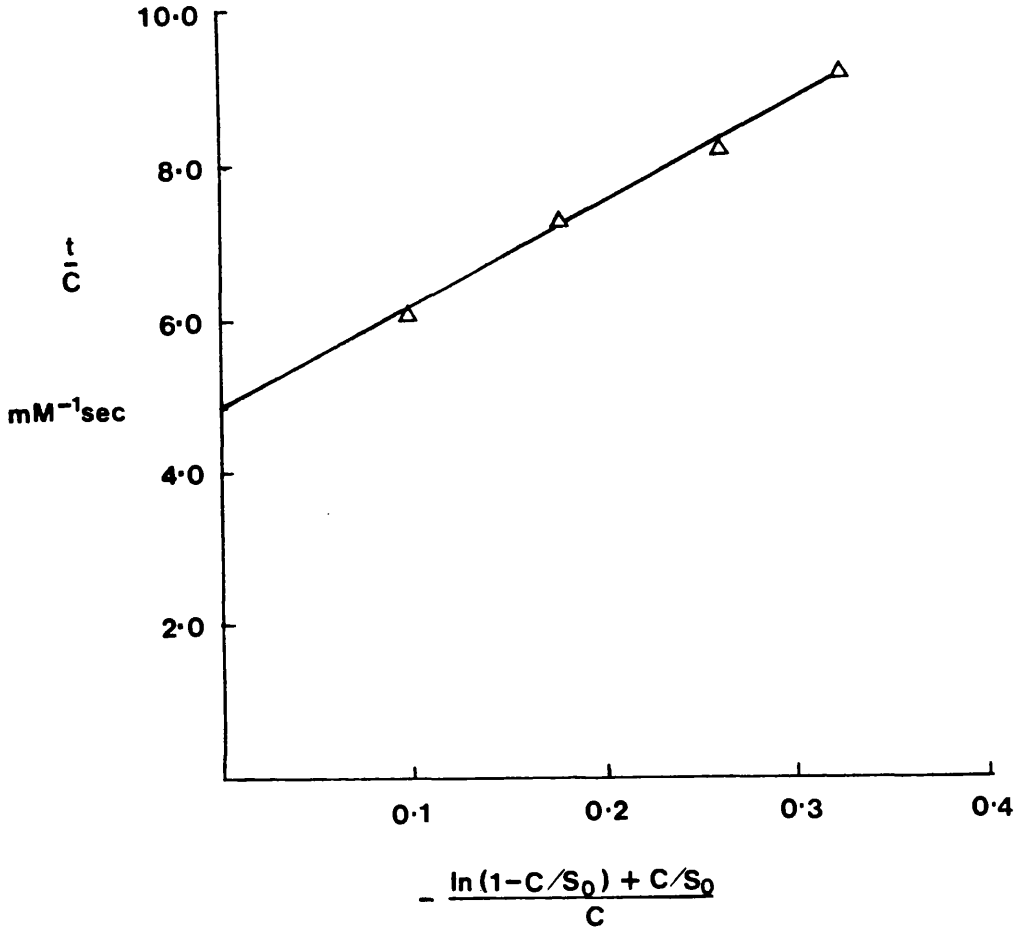


FIGURE 4

Entry of Methylglucose in Insulin-treated Adipocytes.

Fig. 4: An integrated rate equation replot of 1mM 3-O-methyl-D-glucose net entry shown in Fig. 3. The initial rate, V_o = 0.210mMs^{-1}.

attempted for these assays because my response time for manually stopping a stop-watch (using equivalent actions to those used for ejecting liquid from automatic pipettes as in the transport assays) was inaccurate at time intervals of less than one second.

3.2.4. Zero trans Entry Experiments in Adipocytes.

The zero *trans* entry parameters were determined for insulin-treated cells when rates were calculated as C/t where the uptake was measured directly, over a specified time interval, t, of one second. The mean data of 34 observations is shown in Figure 5(Δ). The zero *trans* influx parameters are K_{zt}^{oi} = 6.11 ± 1.66mM, V_{zt}^{oi} = 1.21 ± 0.19mMs^{-1}.

In view of the apparent underestimation of initial rates of uptake of 1mM 3-O-methyl-D-glucose shown in Figure 4, these zero *trans* influx parameters were compared with values estimated from initial rates calculated from integration of time courses at five concentrations, using Equation 3. The time courses and the integrated rate plots derived from them are shown in Figures 6 to 10 in (a) and (b) respectively. The apparent initial rates of uptake (V_{app}) calculated from the integrated rate plots were used to plot a graph of S/V vs. S (Figure 5, (▲)); the kinetic parameters were K_{zt}^{oi} = 7.49 ± 0.59mM, V_{zt}^{oi} = 1.79 ± 0.08mMs^{-1}. The comparison of these two calculations of the zero *trans* influx parameters indicates that the maximum velocity, V_{zt}^{oi}, is apparently low when the initial rates are underestimated, but the affinity constant, K_{zt}^{oi}, is not significantly affected. It must, however, be noted that the number of observations (n) at each time point for the integrated time courses was only three, and uptakes were measured on cells from the same pool, so the graphically calculated values of V_{app} are statistically less significant than the values directly measured.

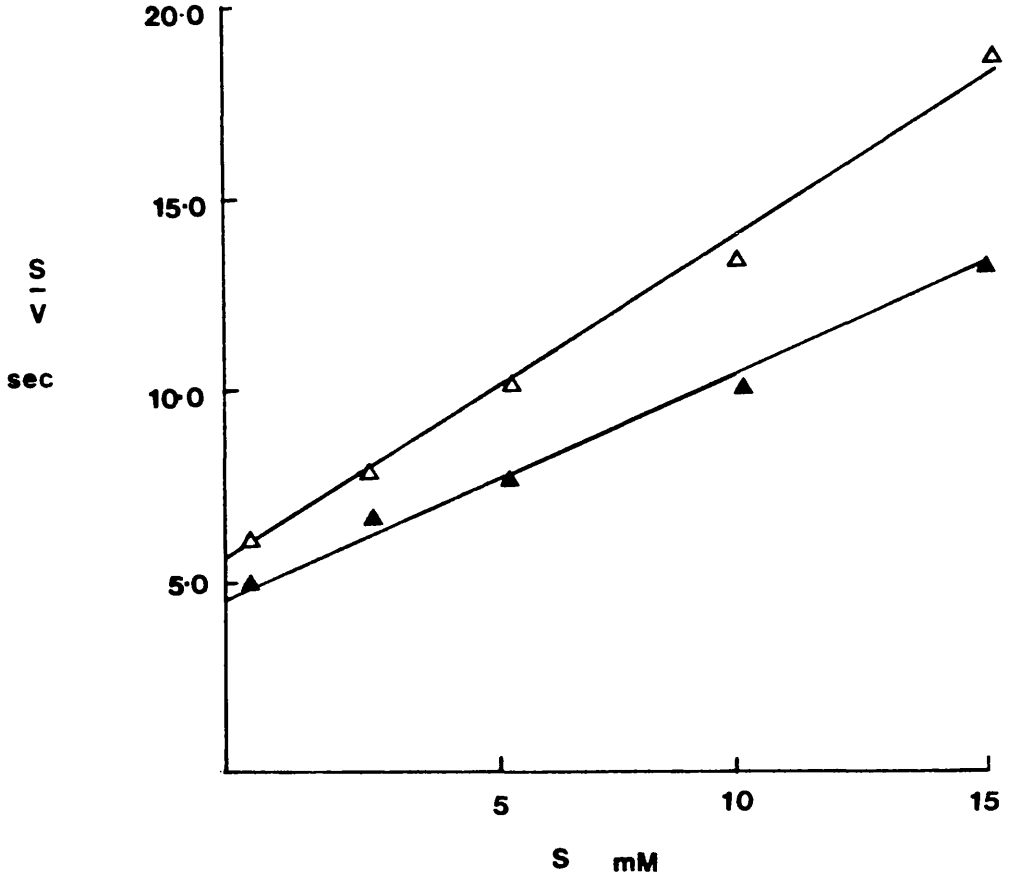


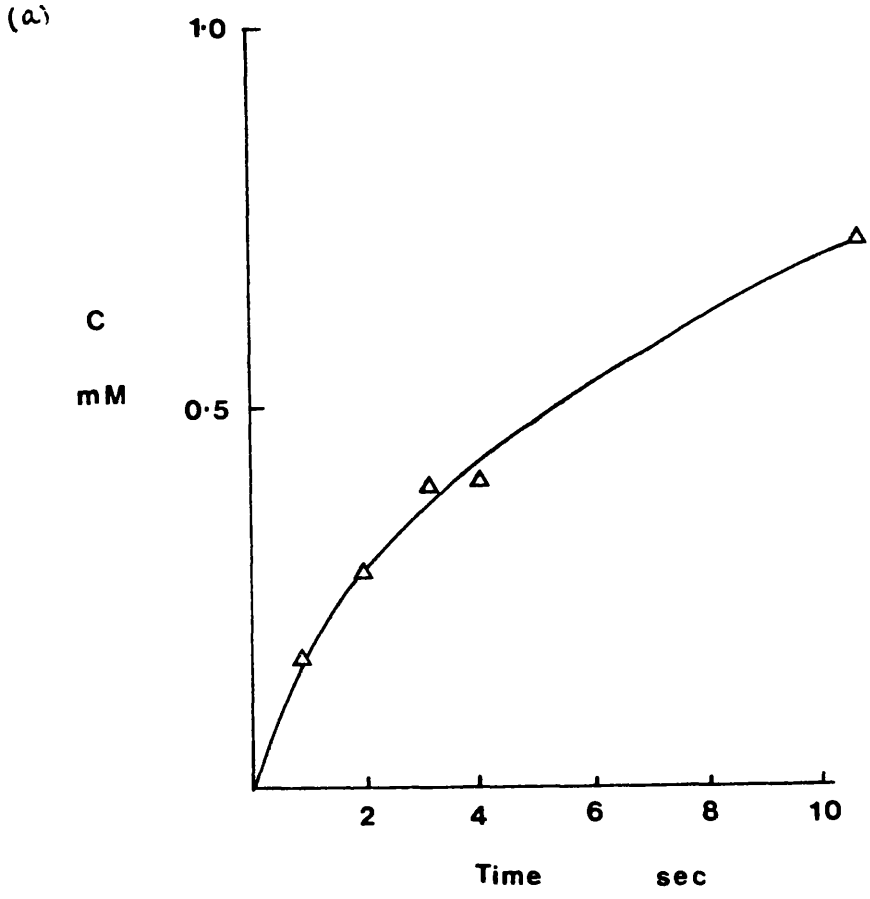
FIGURE 5

Zero trans Entry of Methylglucose in Insulin-treated Adipocytes.

Fig. 5: (Δ) Zero trans influx of 3-O-methyl-D-glucose in cells pre-treated with 10nM insulin. Uptakes were measured at 1 second, and at 37°C. 45 observations, experiments) K_{zt}^{oi} = 6.10 ± 1.65mM and V_{zt}^{oi} = 1.20 ± 1.19mMs^{-1}. (▲) Zero trans influx of 3-O-methyl-D-glucose at 37°C in cells pre-treated with 10nM insulin. Initial rates were calculated from 5 time courses, where each point represents mean values of triplicate measurements. (See Figures 6 to 10).

FIGURES 6 to 10

Time Courses and Integrated Rate Analyses of Methylglucose Uptake in Insulin-stimulated Adipocytes.



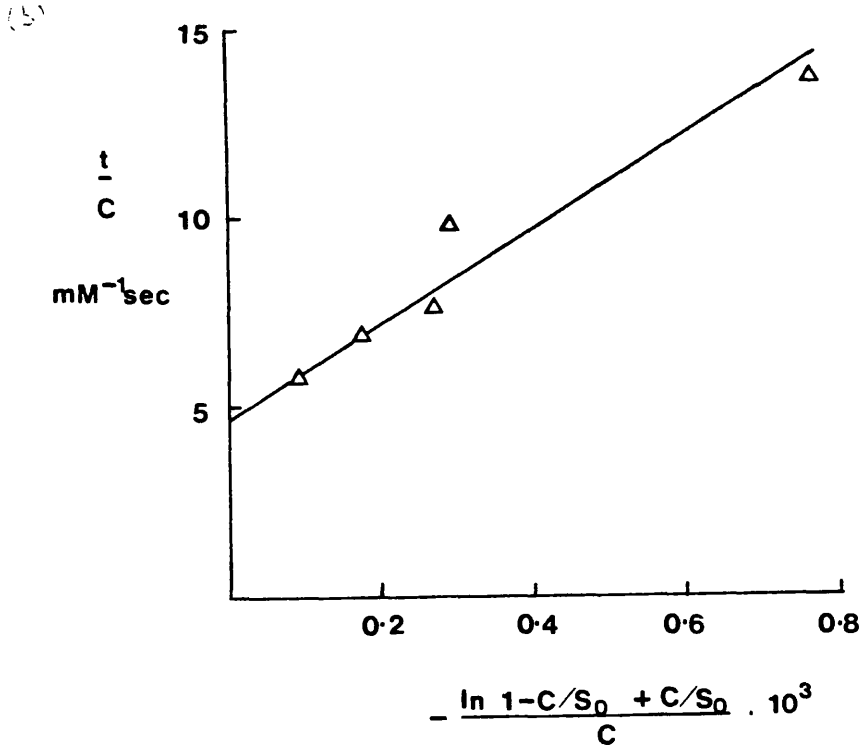
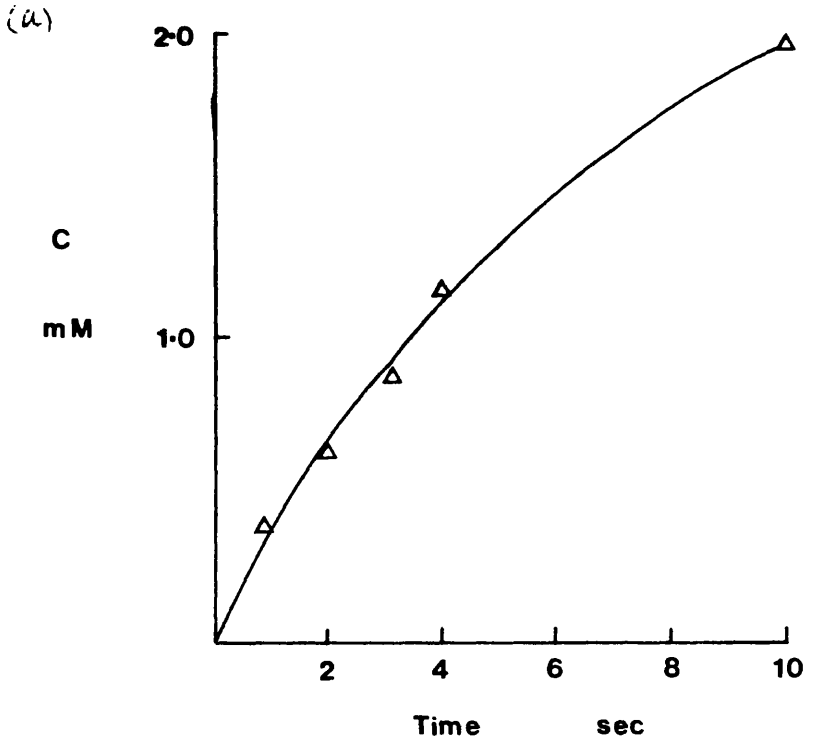


Fig. 6 (a): The net uptake of 1mM 3-O-methyl-D-glucose into insulin-treated adipocytes. Points are mean values of triplicate measurements from one pool of cells, with two experimenters.

(b): An integrated rate replot of the data shown in (a). The line was drawn by regression eye. The apparent initial rate, $V_{app} = 0.21 mMs^{-1}$.



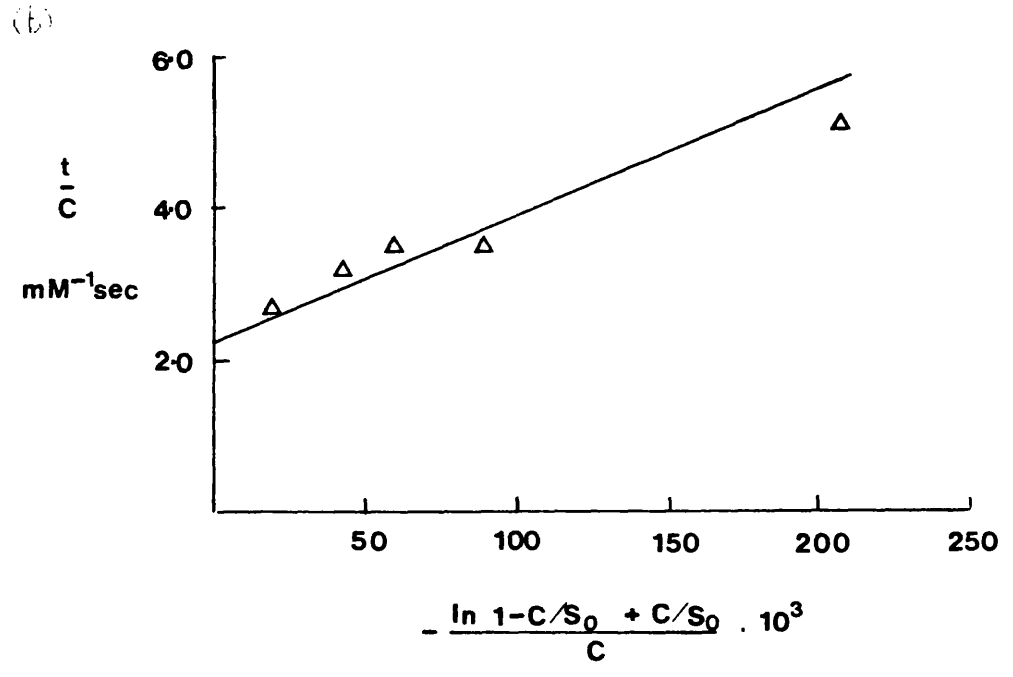
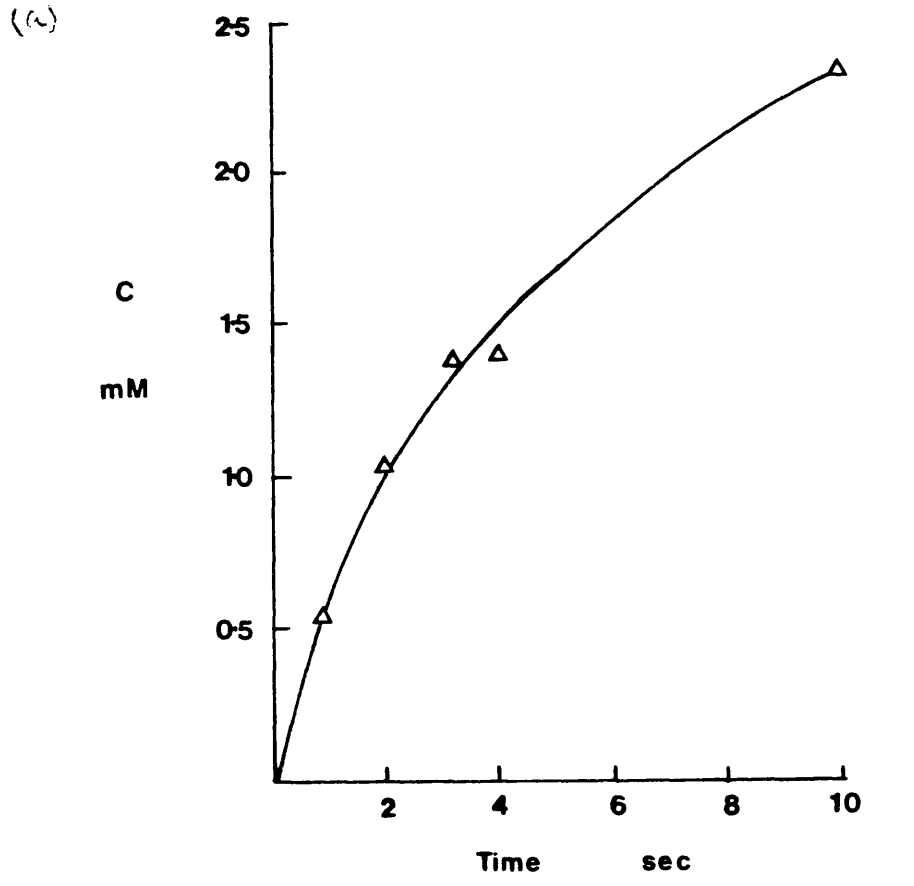


Fig. 7 (a): The net uptake of 3mM 3-O-methyl-D-glucose into insulin-treated adipocytes. Points are mean values of triplicates.
(b): An integrated rate replot of the data shown in (a). V_{app} = 0.46mMs^{-1}.



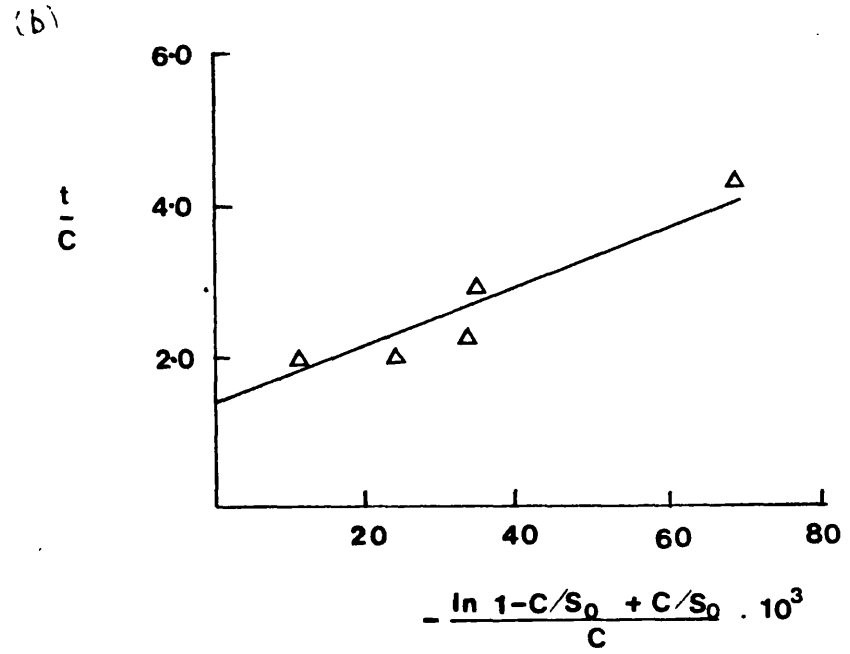
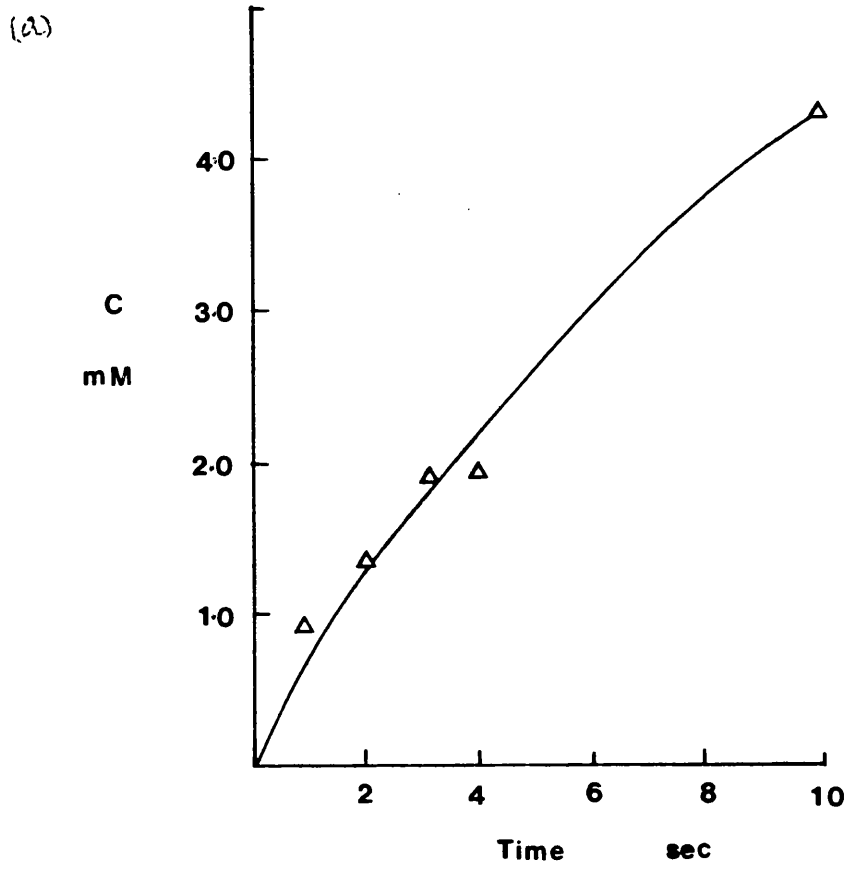


Fig. 8 (a): The net uptake of 5mM 3-O-methyl-D-glucose into adipocytes treated with insulin. Points are means of triplicates.
(b): The integrated rate replot of the data shown in (a) V_{app} = $0.71 mMs^{-1}$.



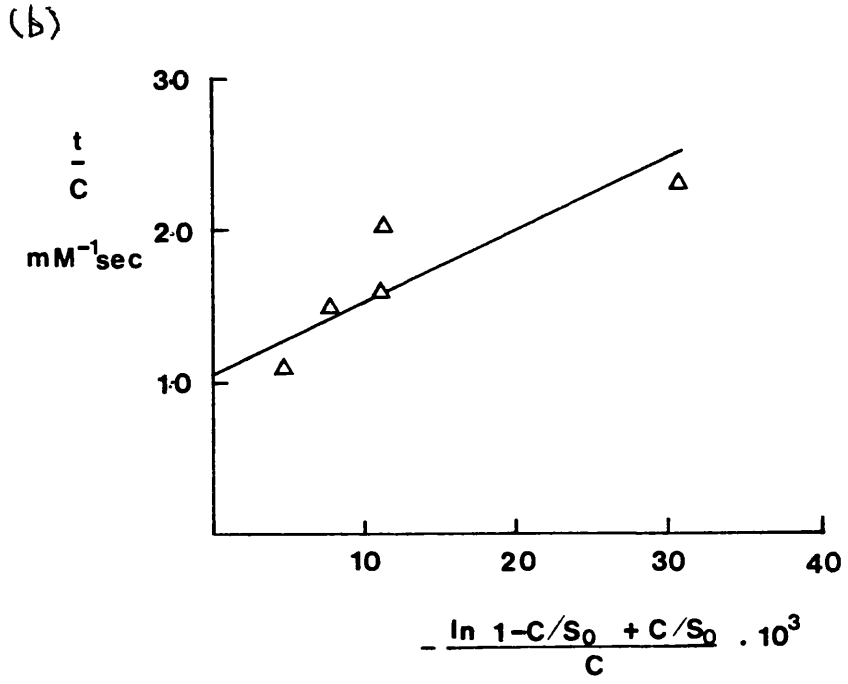
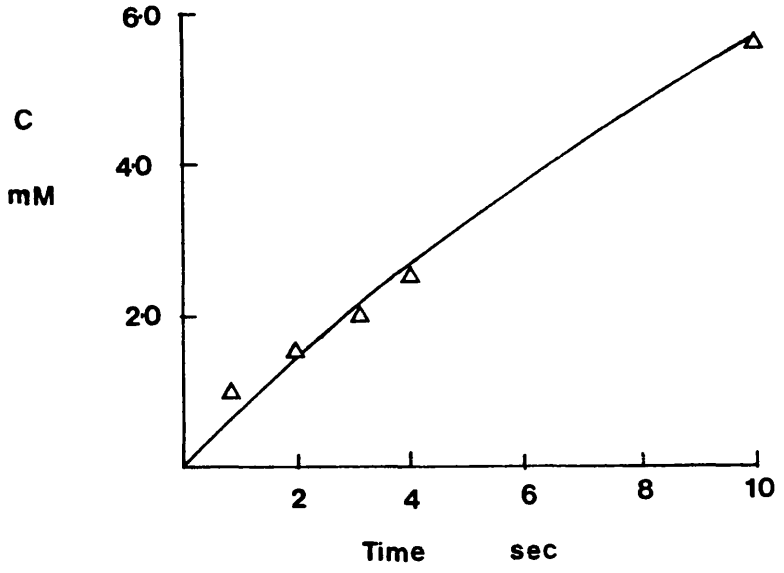


Fig. 9 (a): The net uptake of 10mM 3-0-methyl-D-glucose into insulin-treated adipocytes. Points are mean values of duplicates or triplicates.

(b): An integrated rate replot of the data shown in (a). V_{app} = $1/1.06 mMs^{-1}$.

(a)



(b)

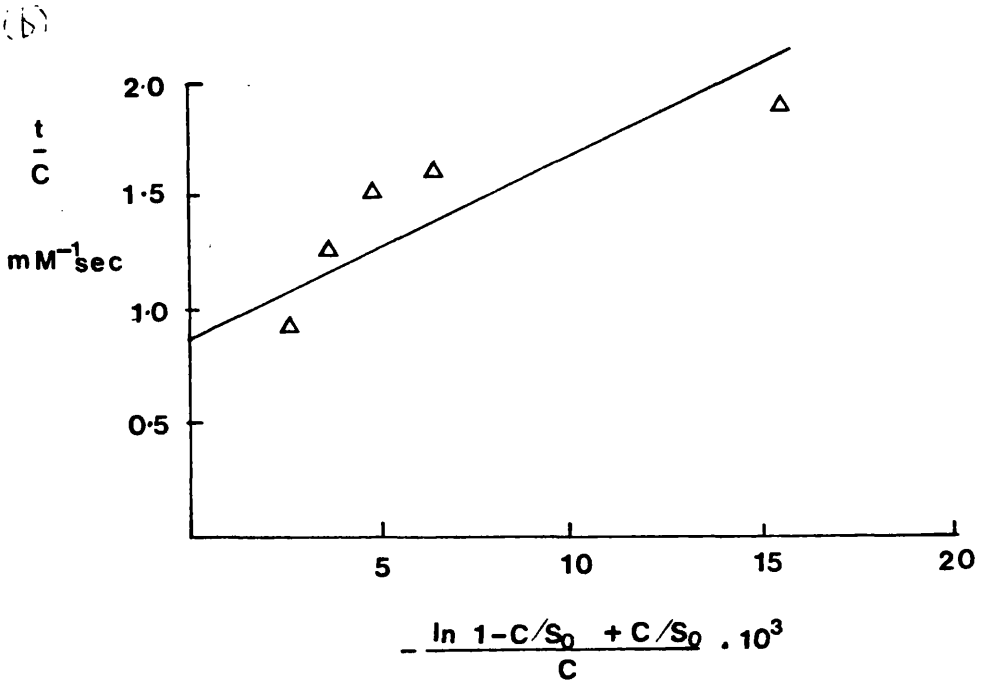


Fig. 10 (a): The net uptake of 15mM 3-O-methyl-D-glucose into insulin-treated adipocytes. Points are mean values of triplicates (except where the number of observations is one, at 10 seconds).

(b): An integrated rate replot of the data shown in (a). V_{app} = $1.16 mM s^{-1}$.

3.2.5. Equilibrium Exchange (Entry) Experiments in Adipocytes.

Measurement of exchange flux of 3-O-methyl-D-glucose in adipocytes has previously been documented (Vinten et al., 1976; Whitesell & Gliemann, 1979). This type of experiment was repeated here as a comparison of the results with those of other groups, and further, to enable a direct comparison of the zero trans entry and equilibrium exchange parameters to be made. The equilibrium exchange results for insulin-treated adipocytes are shown in Figure 11: $K_{ee}^{oi} = 4.45 \pm 0.26$mM, $V_{ee}^{io} = 0.84 \pm 0.002 mMs^{-1}$. The value for V_{ee}^{oi} appeared to be slightly lower than would be expected from the zero trans entry parameters V_{zt}^{oi} ($V_{zt}^{oi} = 1.20 \pm 0.19 mMs^{-1}$). In order to show whether this represented a real difference or simply day-to-day variation between cell populations, time courses at 15mM 3-O-methyl-D-glucose were measured under both zero trans and exchange conditions, in the same cell population. Two such experiments are shown (Figures 12 and 13). Although the experiments shown in both figures indicated that there was no discrepency between the uptakes under the two conditions, the rate of uptake shown in Figure 12, appeared low. This was shown by a calculation of the V_{app} from the integrated rate analysis (using Equation 3), and a comparison of this value, $V_{app} = 0.581 mMs^{-1}$, with that shown for previous zero trans uptake data in Figure 10, where $V_{app} = 1.16 mMs^{-1}$. The experiment was repeated with fresh stock solutions of insulin (Figure 13). V_{app} was significantly increased, $V_{app} = 1.02\ mMs^{-1}$. These two experiments also indicate, therefore, that insulin stock solutions were liable to loose their potency after storage at -20°C for two months.

3.2.6. Zero trans Exit Experiments in Adipocytes.

The reliable measurement of the zero trans efflux of 3-O-methyl-D-glucose from adipocytes proved difficult. In order to achieve zero trans

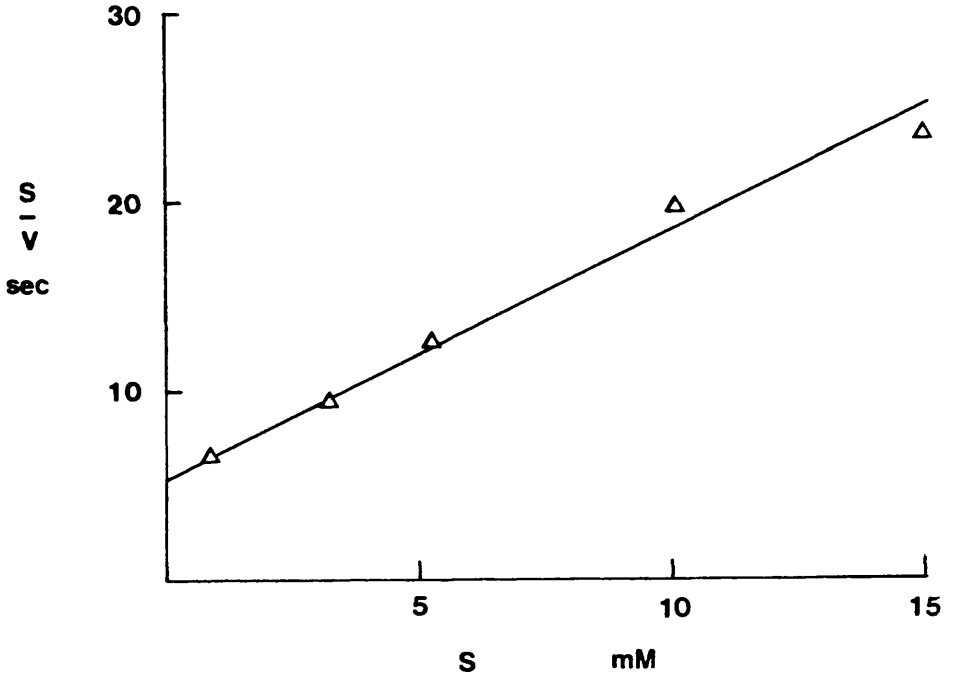


FIGURE 11

Equilibrium Exchange of Methylglucose in Insulin-treated Adipocytes.

Fig. 11: Equilibrium exchange, measured by entry of isotope, of 3-O-methyl-D-glucose in insulin-stimulated adipocytes, at 37°C. K_{ee}^{oi} = 4.45 ± 0.26mM, V_{ee}^{oi} = 0.84 ± 0.002mMs^{-1} (42 observations from 3 experiments).

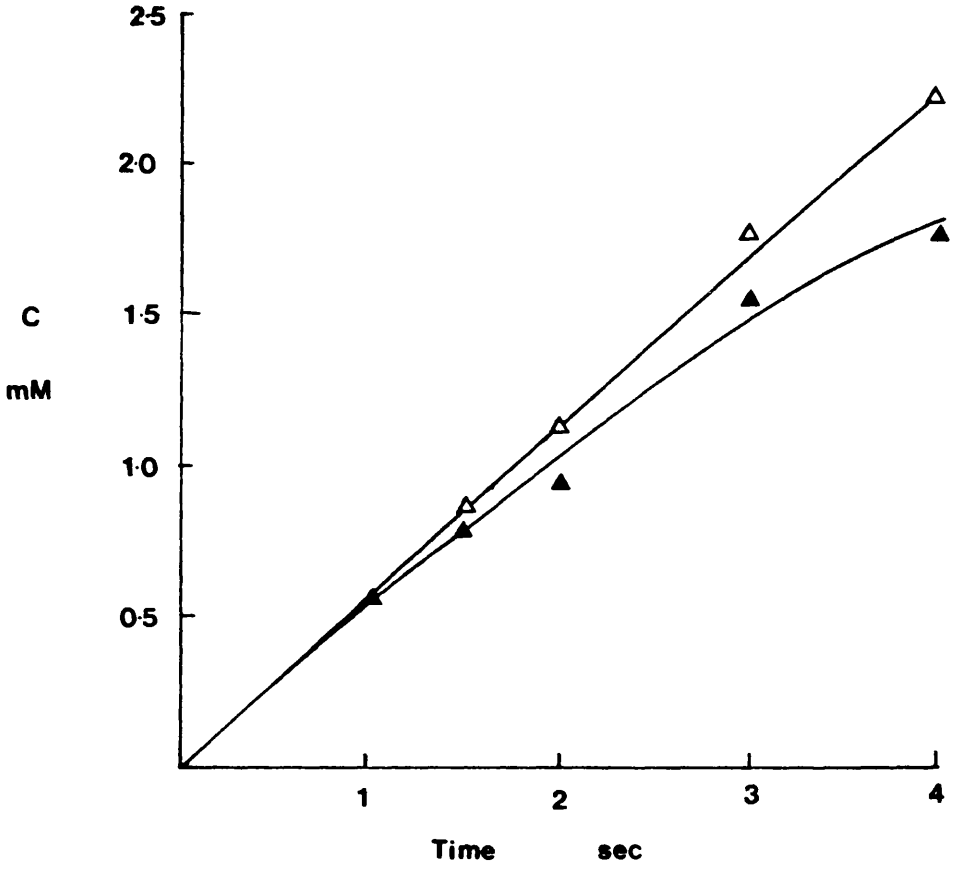


FIGURE 12

A Comparison of Equilibrium Exchange and Zero trans Time Courses.

Fig. 12: Zero trans (▲) and equilbrium exchange (△) time courses of 15mM 3-O-methyl-D-glucose entry at 37°C. Cells had been pre-treated with insulin. The insulin stock solution was 2 months old. Each point is the mean of 2 or 3 observations. There were a total of 12 observations for the exchange, and 14 for the zero trans experiment. The "background" and "infinity" values were measured in triplicate.

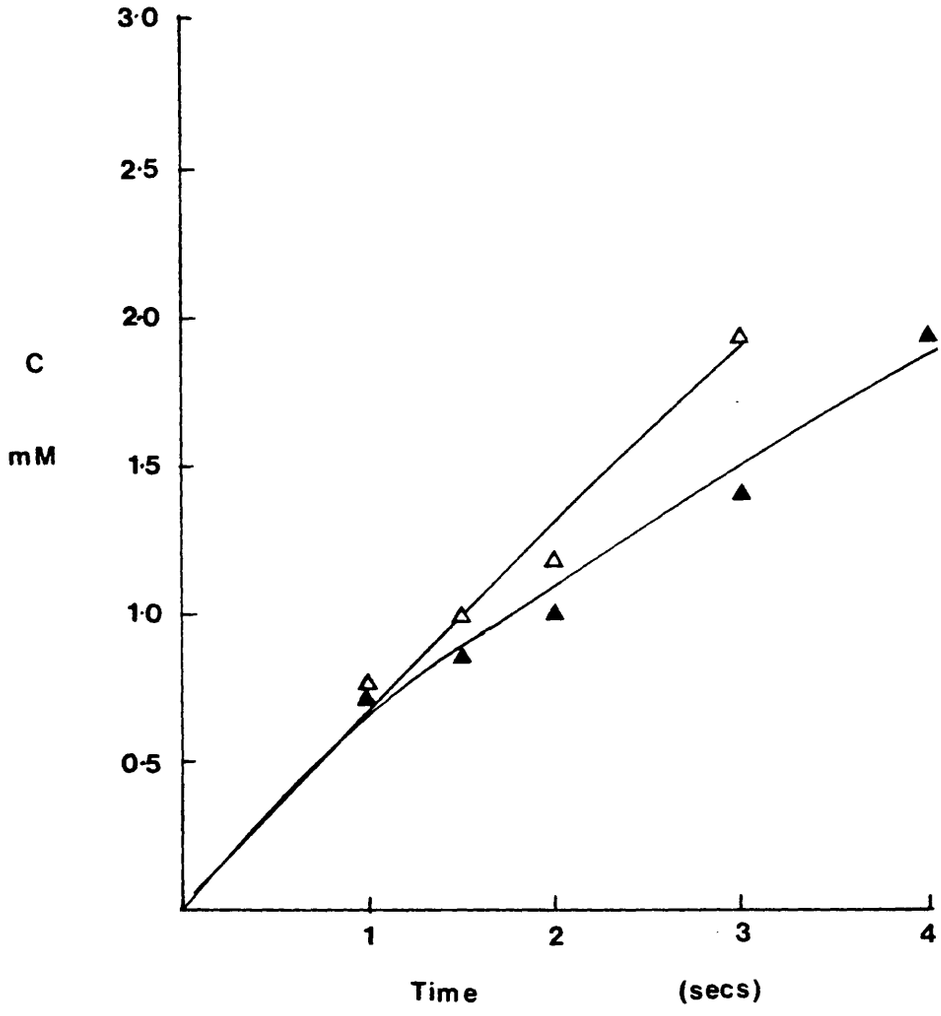


FIGURE 13

A Comparison of Equilibrium Exchange and Zero trans Time Course: New Insulin.

Fig. 13: A comparison of short time courses for zero trans (▲) and equilibrium exchange (△) uptake with cells pre-treated with 10nM insulin; from a stock solution which was prepared on the same day as the uptake experiments were carried out. The number of observations was 3 to 5 for each time point. The total number of observations was 18 for the exchange, and 20 for the zero trans experiment.

conditions, it was necessary to dilute cell suspensions into a large excess of buffer to prevent any significant backflux of extracellular label. Extracellular label can be minimized by using a densely packed cell suspension. However, the maximum practical cytocrit for fat cells was found to be 50% (v/v). Suspensions greater than 50% were unstable when kept for the necessary length of time. In the first zero _trans_ exit experiments a one in 400 dilution of the extracellular label was obtained by the addition of 50 µl of a 50% (v/v) suspension of cells, to 10mls of the efflux buffer. Effective mixing of the solutions in this experimental design is likely to be awkward, particularly when the extent of efflux is low, and errors in the estimation of the uptake at zero time will appear large. Under such conditions, the amount of additional unstopped efflux would be a significant percentage of the efflux over the initial phase. Therfore the early experiments were not designed to measure initial rates but time points were used in the region where 15% to 60% of the substrate was expected to have been released on the basis of the V_{max} predicted from the infinite _cis_ exit experiment ($V^{io}_{ic} = 1.76 \pm 0.63 mMs^{-1}$) and a K_m predicted from the infinite _cis_ entry K_m ($K^{oi}_{ic} = 6.51 \pm 0.83 mM$). The estimation of the initial rates necessitates use of an integrated rate analysis, since the fall in the internal concentration of substrate rapidly becomes non-linear.

The equation used is:

$$-\frac{\ln S_t/S_o}{S_o - S_t} = \frac{V_{max} \cdot t}{K_m(S_o - S_t)} - \frac{1}{K_m}$$

Equation 4

where S_o and S_t are the internal cell concentrations at times zero

and t. $t/(S_o-S_t)$ is plotted against $-\ln(S_t/S_o)/(S_o-S_t)$ and $1/K_m$ is estimated from the intercept on the ordinate (Baker & Naftalin, 1979).

The results of a combination of six zero *trans* exit experiments are shown in Figure 14. In order to minimize the problems of mixing described above the smallest extent of exit measured was 10% of the total exit. At the longer time intervals, errors due to the extent of backflux of extracellular label would be greatest, thus exits greater than 60% of the total were not used in the calculations. Figure 14 shows the time courses measured over this range of exit which were used in the integrated rate equation replot shown in Figure 15. The zero *trans* exit parameters were $K_{zt}^{io} = 2.66 \pm 0.26$mM, $V_{zt}^{io} = 1.19 \pm 0.07$mMs^{-1}.

Another method which may be used to calculate the zero *trans* exit parameters is to calculate the inital rates from integrated rate plots at a range of concentrations. When $-\ln(S_t/S_o)/(S_o-S_t) = 1/S_o$, then $t/(S_o-S_t) = 1/V_o$ (Cornish-Bowden, 1976). Initial rates have been calculated in this manner, and the resultant data is shown in Figure 16. The estimated parameters are $K_{zt}^{io} = 2.83\pm0.34$ mM, $V_{zt}^{io} = 1.21\pm0.13$ mMs^{-1}. These values confirm the values calculated from the integrated rate plot (Figure 15).

The zero *trans* entry and exit V_{zt} parameters are approximately equal (Table 12), although there is a slight difference between K_{zt} for entry and exit. This may reflect the technical difficulties of measuring efflux.

3.2.7. Infinite cis Entry Experiments in Adipocytes.

Infinite *cis* experiments may be used as an additional way of measuring the influx and efflux kinetic parameters. In this study, they were used to confirm the parameters measured under zero *trans* procedures.

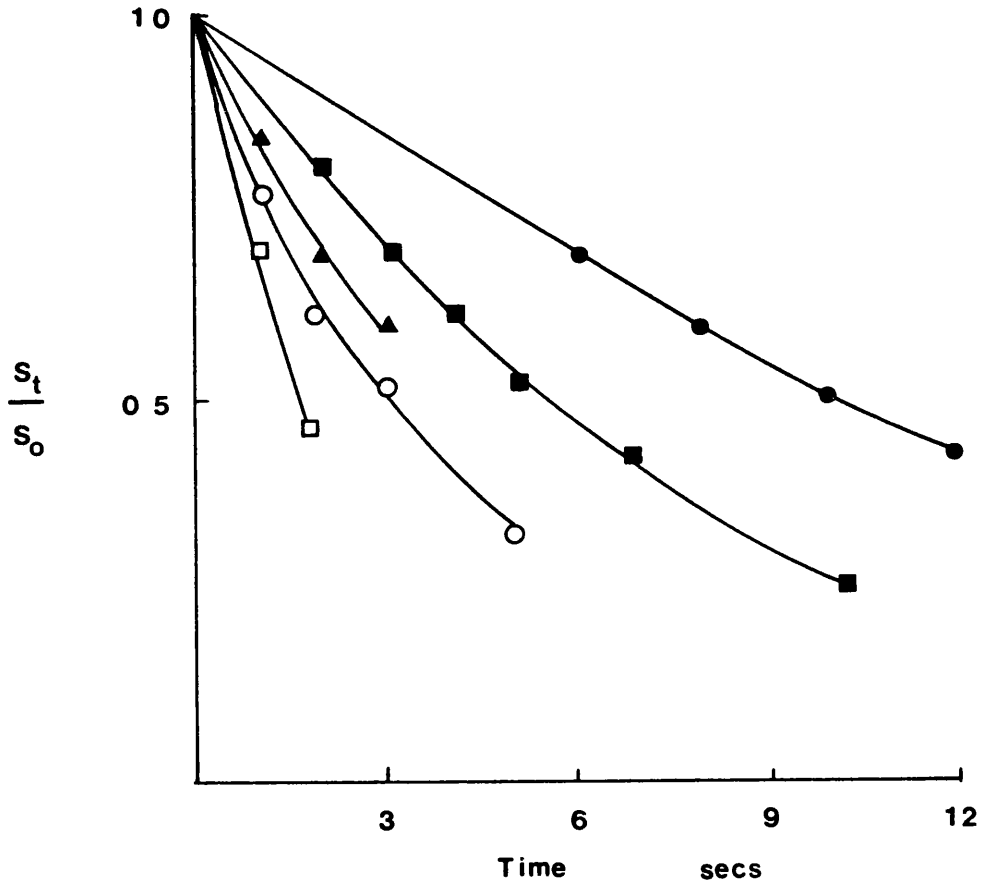


FIGURE 14

Zero trans Efflux of Methylglucose from Insulin-treated Adipocytes.

Fig. 14: Time courses for exit of 3-O-methyl-D-glucose over a range of sugar concentrations into sugar-free solutions. Exit was monitored at 37°C in cells pre-treated with 10nM insulin. There were 130 observations from 6 experiments. Concentrations of sugar: (□) 1mM, (○) 3mM, (▲) 5mM, (■) 10mM, (●) 20mM.

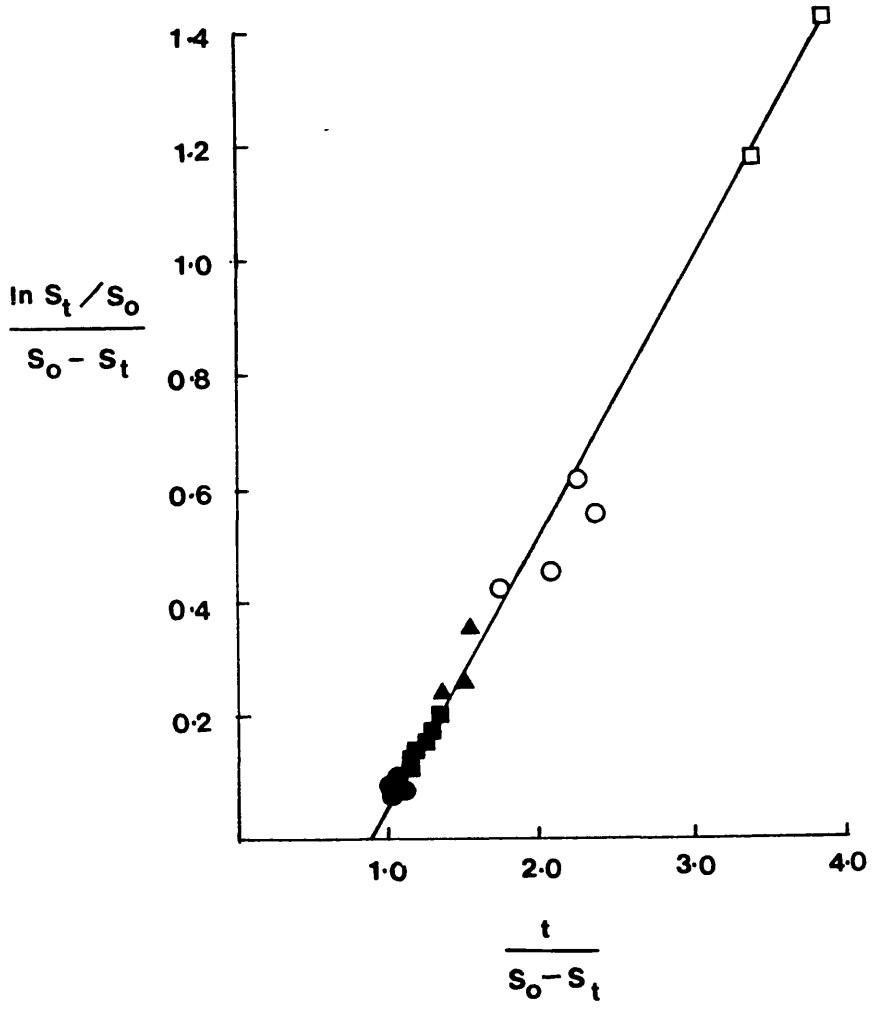


FIGURE 15

An Integrated Rate Analysis of Zero trans Exit (Figure 14).

Fig. 15: An integrated rate equation replot of the zero trans efflux of 3-O-methyl-D-glucose in insulin-treated cells. K_{zt}^{io} = 2.66 ± 0.26mM, V_{zt}^{io} = 1.19 ± 0.07mMs^{-1}.

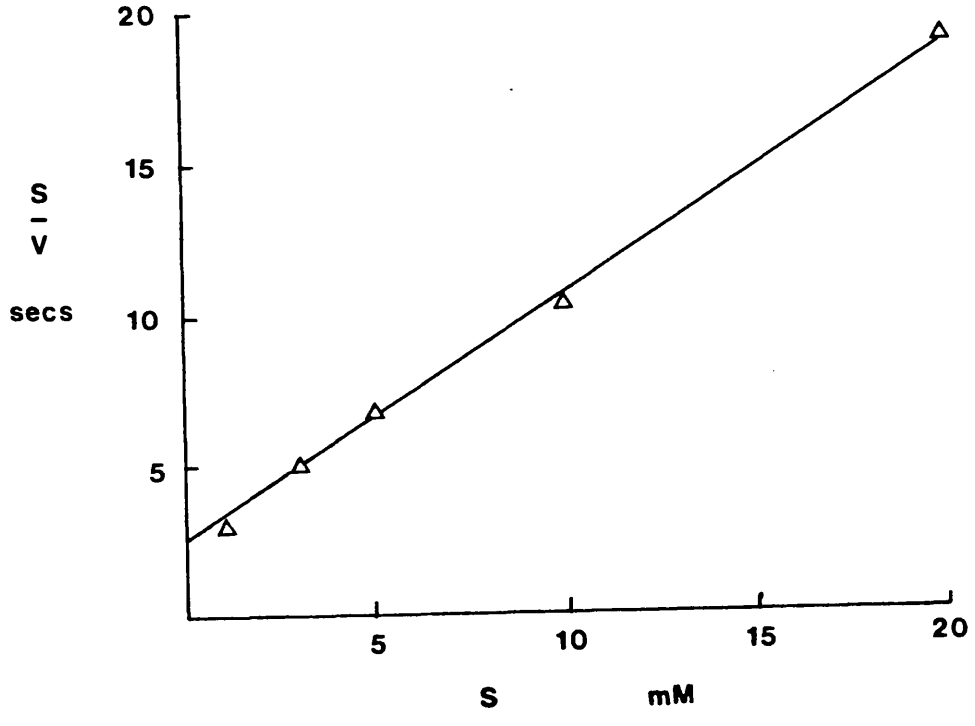


FIGURE 16

Zero trans exit of Methylglucose from Insulin-treated Adipocytes.

Fig. 16: Zero trans exit parameters calculated from the integrated plot (Equation 4) as described in the text. K_{zt}^{io} = 2.83 ± 0.34 mM, V_{zt}^{io} = 1.21 ± 0.13 mMs^{-1}.

The infinite cis entry was measured by following the time course of entry of a high substrate concentration (Figure 17), and the integrated rate equation (3) was applied to the data. When the cis (external) concentration is much greater than the K_m, the intercept on the x-axis of the "infinite cis plot" (Figure 18) gives the infinite cis K_m (Holman, 1979), whilst the intercept on the ordinate gives the infinite cis V_{max}. For insulin-treated cells, $K_{ic}^{oi} = 6.51 \pm 0.83$mM; $V_{ic}^{oi} = 0.98 \pm 0.09$mMs^{-1}. These values confirm those for the external V_{max} and the internal K_m found in zero trans entry, and zero trans exit experiments, respectively.

3.2.8. Infinite cis Exit Experiments in Adipocytes.

The infinite cis exit parameters were determined from the use of a single high internal concentration of 3-O-methyl-D-glucose, when the apparent permeability was low. The efflux was linear with respect to time until the internal concentration fell below the saturating concentration for the internal site. The efflux rate from insulin-stimulated cells was measured at 20 seconds in the presence of varying external concentrations of 3-O-methyl-D-glucose (Figure 19). I/V was plotted against the external concentration, and the infinite cis K_m was given by the intercept on the abscissa. The kinetic parameters were calculated as $K_{ic}^{io} = 3.60 \pm 1.33$mM, $V_{ic}^{io} = 1.76 \pm 0.63$mMs^{-1}. As with the infinite cis entry experiments, zero trans experimental determinations were confirmed: the infinite cis exit K_m confirms the external K_m found in the zero trans entry experiment, and the infinite cis exit V_{max} agrees with the zero trans exit, or internal, V_{max}.

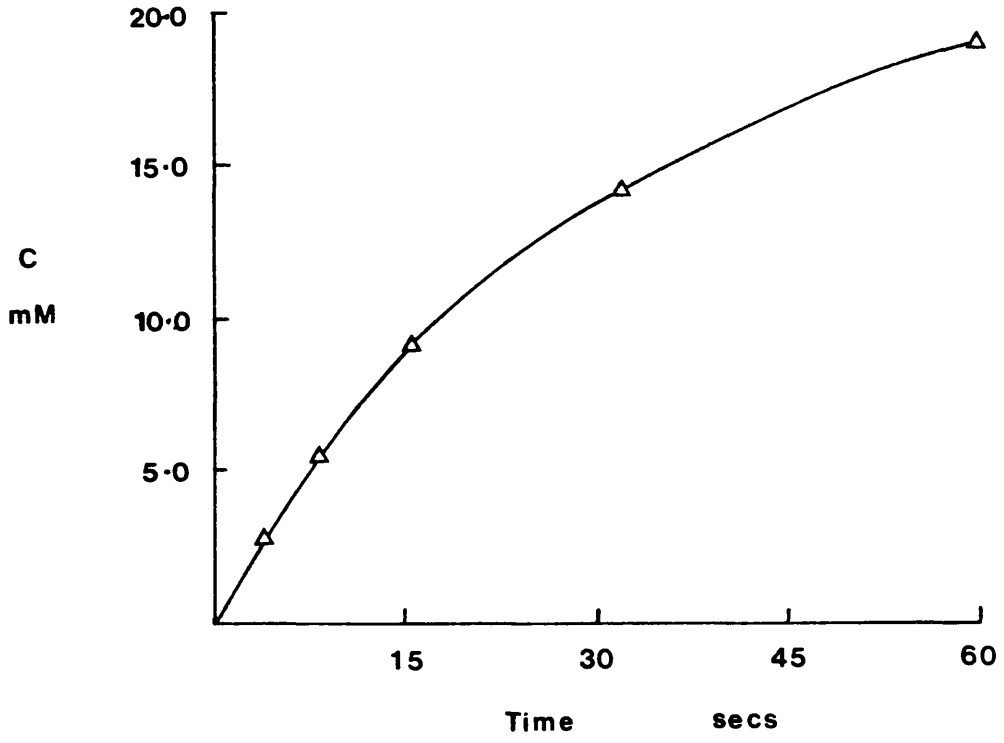


FIGURE 17

Infinite cis Entry of Methylglucose into Insulin-treated Adipocytes.

Fig. 17: A time course for net influx of 40mM 3-O-methyl-D-glucose at 37°C, in cells pre-treated with 10nM insulin. There were 51 observations from 2 experiments.

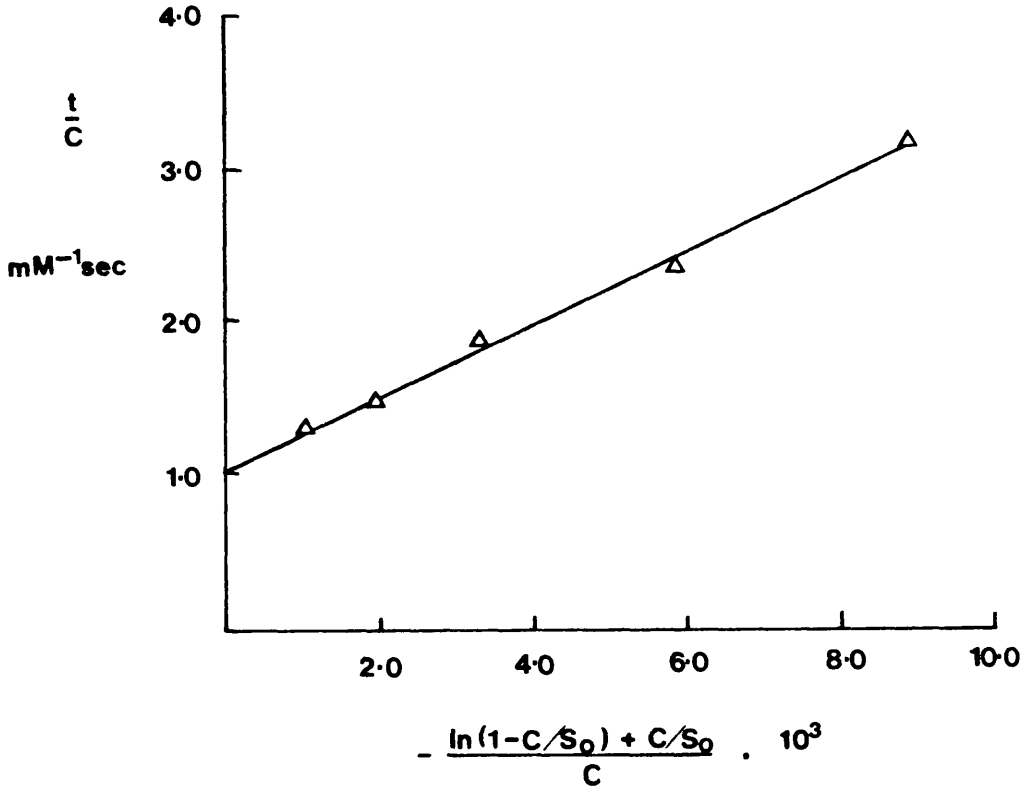


FIGURE 18

An Integrated Rate Analysis.

Fig. 18: An integrated rate analysis of the infinite cis entry data shown in Fig. 17. K^{oi}_{ic} = 6.51 ± 0.83mM, V^{oi}_{ic} = 0.98 ± 0.09mMs^{-1}.

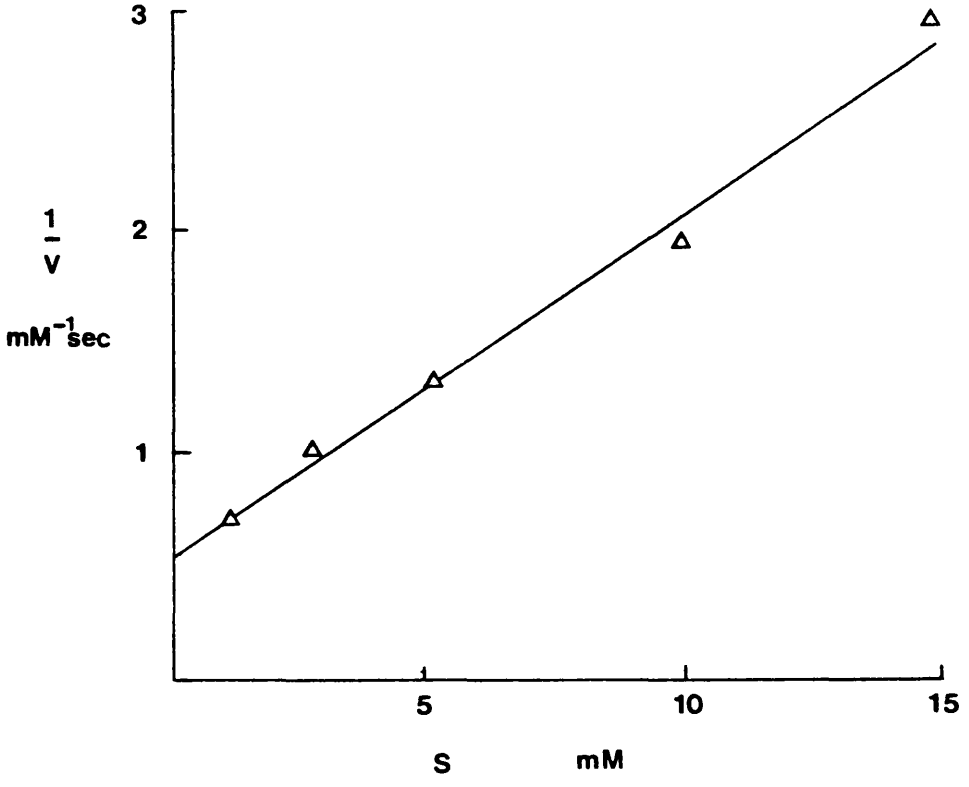


FIGURE 19

Infinite cis Exit of Methyl Glucose from Insulin-treated Adipocytes.

Fig. 19: Infinite cis efflux of 40mM 3-O-methyl-D-glucose into 1mM, 3mM, 5mM, 10mM and 15mM 3-O-methyl-D-glucose, from cells pre-treated with 10nM insulin. K_{ic}^{io} = 3.60 ± 1.33mM, V_{ic}^{io} = 1.76 ± 0.63mMs^{-1}.

3.3. Transport Kinetics in Adipocytes under Basal Conditions.

3.3.1. Zero trans Entry Experiments in Adipocytes.

The kinetic parameters of sugar transport in isolated adipocytes were determined when they were not hormonally stimulated. Previous work (Whitesell & Gliemann, 1979) had shown an influx K_m of 2.5 to 5mM at 22°C. A time course to show the rate of sugar uptake at 1mM 3-O-methyl-D-glucose was therefore measured (Figure 20). This showed that the uptake was sufficiently slow for a 15-second time interval to be used to measure initial rates over a 1 to 15mM range of substrate concentrations. These initial rates were used to estimate the zero trans entry parameters (Figure 21), $K_{zt}^{oi} = 5.41 \pm 0.98$mM, $V_{zt}^{oi} = 0.034 \pm 0.014$mMs^{-1}.

3.3.2. Zero trans Exit Experiments in Adipocytes.

In the first zero trans exit experiments, the samples were occasionally shaken by hand during the efflux period in order to minimize possible cell damage caused by mechanical stirring (as seen in early experiments on insulin-treated cells). This type of stirring proved to be inefficient, especially over the longer time intervals where a significant backflux of extracellular label had apparently occurred. Direct measurement of initial rates for a range of sugar concentrations between 1mM and 15mM was therefore adopted and the Hanes analysis in the form of S/V vs. S plots was used. Figure 22 shows $K_{zt}^{io} = 4.09 \pm 1.05$mM and $V_{zt}^{io} = 0.153 \pm 0.023$mMs^{-1}. In this experiment, cells were incubated with labeled sugar for sixty minutes, the cell stabilisation period being included within this time. Figure 23 shows data obtained from an experiment where cells were loaded with sugar for ninety minutes after the stabilisation period. These cell populations show a higher value for V_{zt}^{io} ($K_{zt}^{io} = 3.89 \pm 0.68$ mM, $V_{zt}^{io} = 0.255 \pm 0.44$ mMs^{-1}). The integrated rate treatment was necessary.

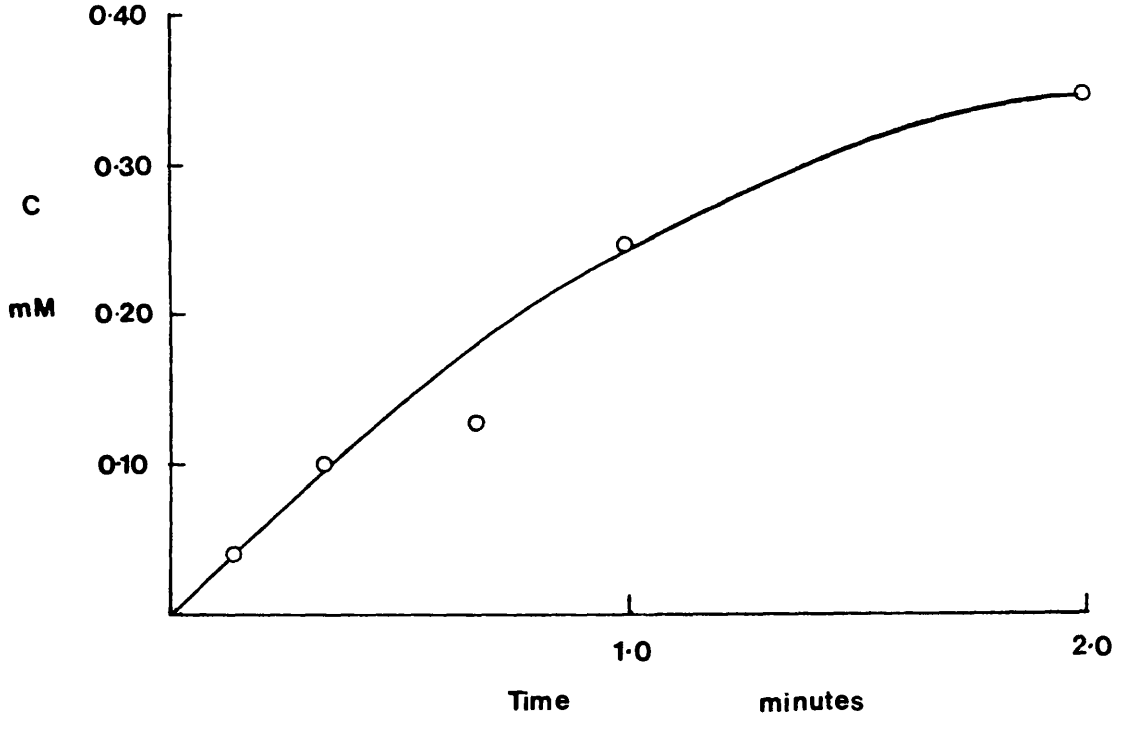


FIGURE 20

The Entry of Methylglucose into Non-stimulated Adipocytes.

Fig. 20: A single experiment showing a time course for the uptake of 0.9mM 3-O-methyl-D-glucose under zero trans conditions, into basal cells at 37°C. The points are means of duplicate measurements. (At 40s, one of the values gave a very low uptake, whilst the other value was 0.172mmoles sugar taken up/l cell water).

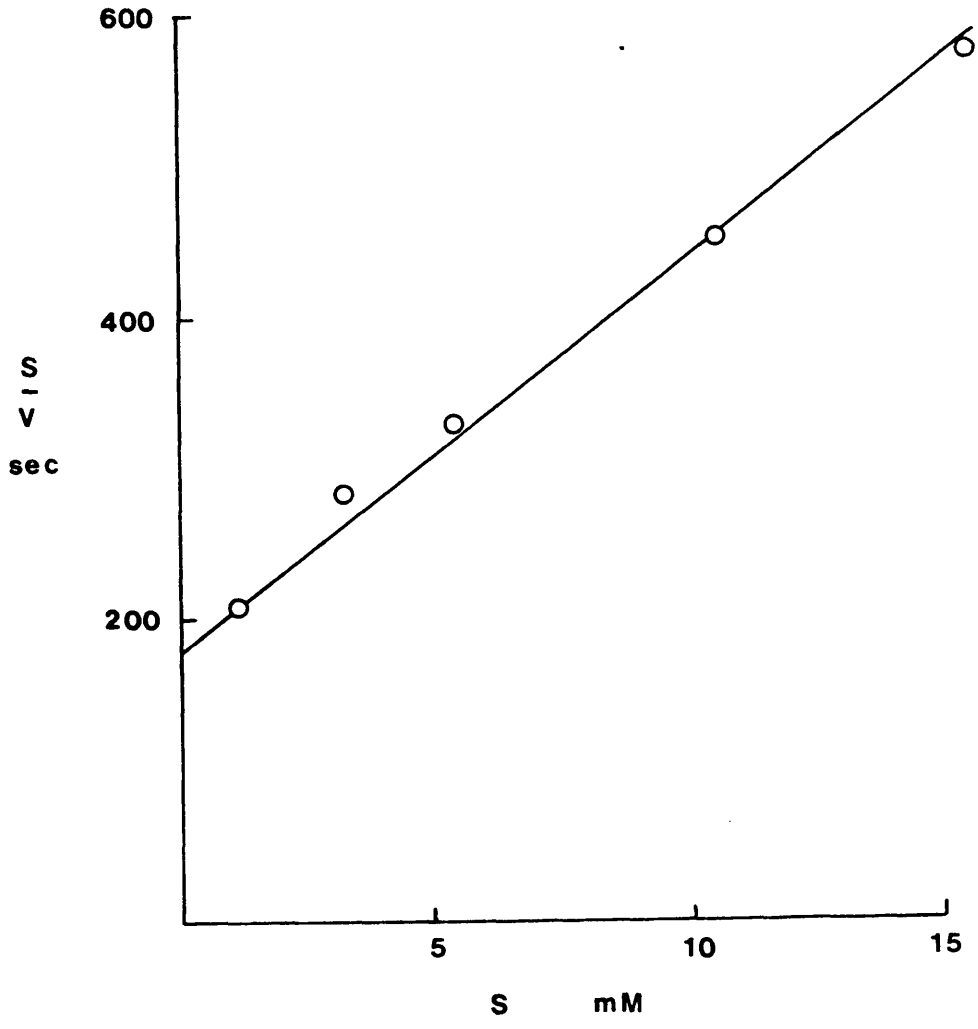


FIGURE 21

Zero trans Entry of Methylglucose into Basal Adipocytes.

Fig. 21: Zero trans entry of 3-O-methyl-D-glucose into basal cells at 37°C. K_{zt}^{oi} = 5.41 ± 0.98mM, V_{zt}^{oi} = 0.34 ± 0.014mMs^{-1}. There were 24 observations from 1 experiment.

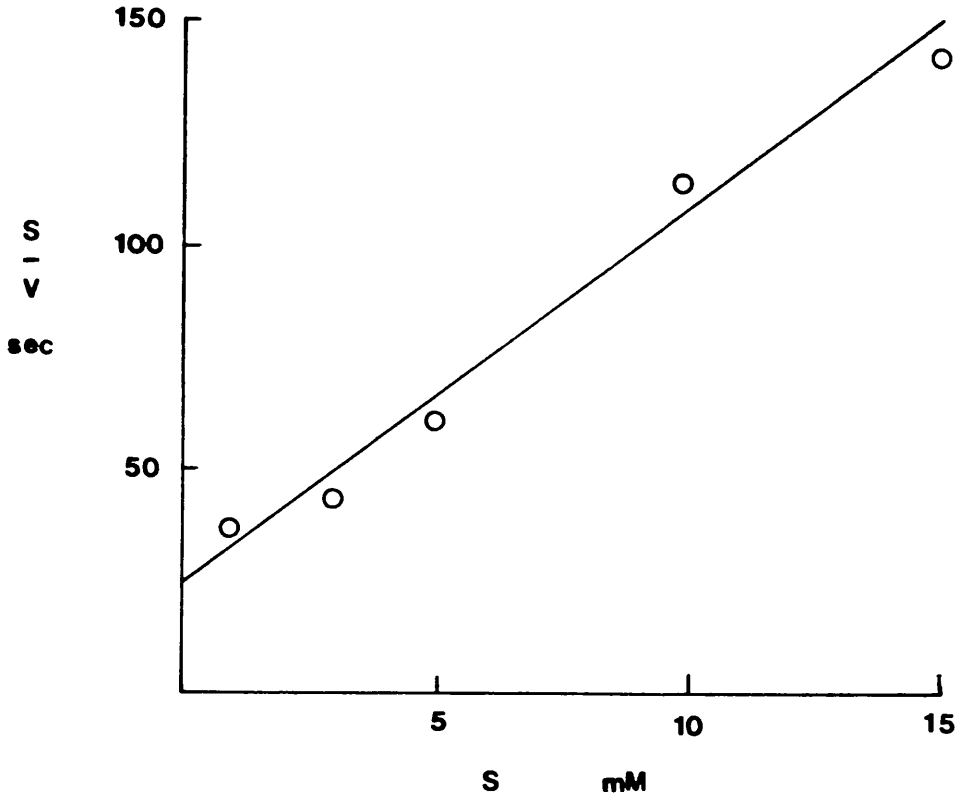


FIGURE 22

Zero trans Exit Kinetics for Basal Cells.

Fig. 22: Zero trans exit of 3-O-methyl-D-glucose from basal adipocytes, at 37°C. (22 observations from one experiment). K_{zt}^{io} = 4.09 ± 1.05mM, V_{zt}^{io} = 0.153 ± 0.023mMs^{-1}.

FIGURE 23

Zero trans Exit for Older Adipocytes.

Concentration (mM)	1	3	5	10	15
Time (seconds)	10	15	15	25	30
Extent of Efflux (S_t) in mM	0.576	1.62	3.24	5.88	8.01

The table shows the extent of exit at a range of 3-O-methyl-D-glucose concentrations at the various times. Data from one experiment, 15 observations.

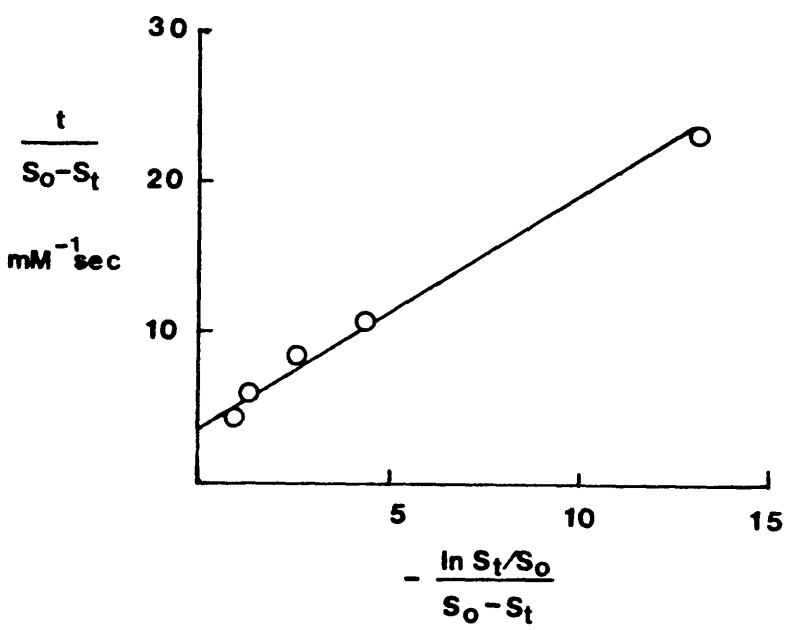


Figure 23: An integrated rate treatment of the data shown in the Table. K_{zt}^{io} = 3.89 ± 0.68 mM, V_{zt}^{io} = 0.255 ± 0.44 mMs^{-1}.

A comparison of the results from Figures 22 and 23 indicated that prolonged periods of incubation caused the internal V_{max} to increase.

3.3.3. Equilibrium Exchange Experiments in Adipocytes.

Entry of 3-O-methyl-D-glucose was also measured under exchange conditions. This was to verify the present work against that done in other laboratories, and to compare the zero *trans* and exchange parameters. The equilibrium exchange parameters for basal cells are shown in Figure 24. $K_{ee}^{oi} = 4.22 \pm 1.24$mM and $V_{ee}^{oi} = 0.058 \pm 0.001 mMs^{-1}$.

3.3.4. Infinite cis Entry Experiments in Adipocytes.

Infinite *cis* parameters were estimated for adipocytes in the basal condition. In order to achieve this for the entry of 3-O-methyl-D-glucose, a time course of uptake of a high substrate concentration (40mM) was followed, and the integrated rate equation (3) analysis applied. Figure 25 shows the results of three such experiments, and Figure 26, the integrated rate replot.

3.3.5. Infinite cis Exit Experiments in Adipocytes.

For a determination of the infinite *cis* exit kinetics for basal cells, a time point of four minutes was chosen to measure the efflux rate in the presence of varying external concentrations of 3-O-methyl-D-glucose (Figure 27). The kinetic parameters were calculated from this plot of I/V vs. the external concentration. The infinite *cis* (external) K_m is given by the intercept on the abcissa, $K_{ic}^{io} = 4.54 \pm 1.32$mM, and the internal V_{max}, V_{ic}^{io} from the ordinate intercept, is $0.106 \pm 0.026 mMs^{-1}$.

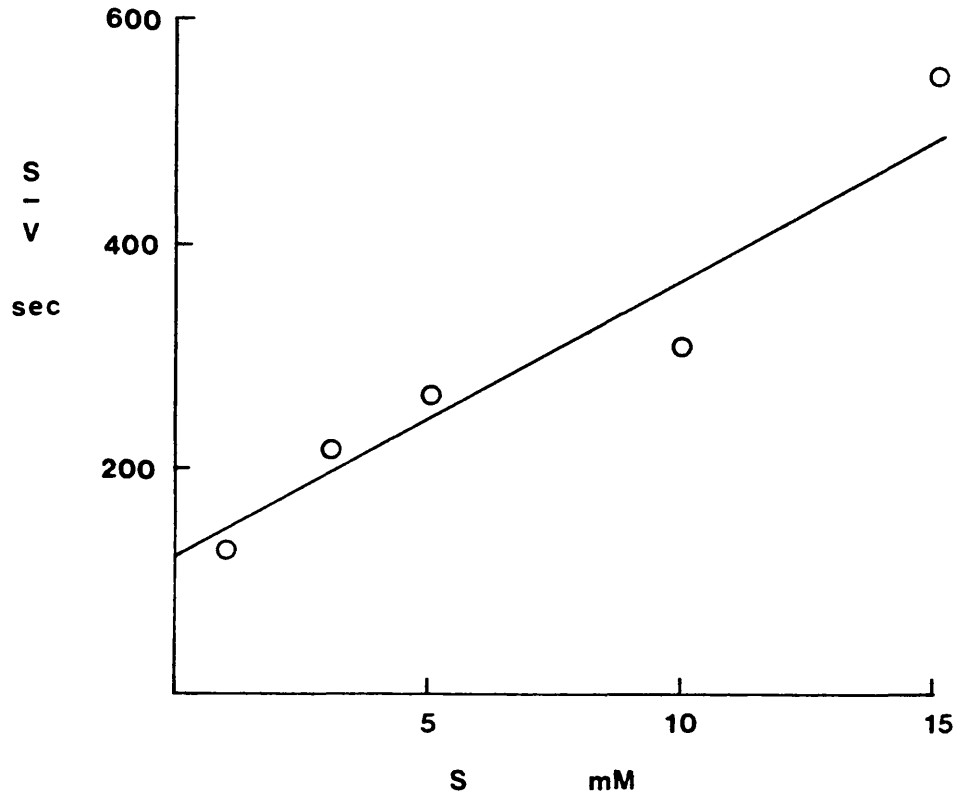


FIGURE 24

Equilibrium Exchange Kinetics for Basal Adipocytes.

Fig. 24: The kinetics under exchange conditions of 3-O-methyl-D-glucose uptake in unstimulated adipocytes measured at 15 and 30 seconds, at 37°C. $K_{ee}^{oi} = 4.22 \pm 1.24$mM, $V_{ee}^{oi} = 0.058 \pm 0.0001 mMs^{-1}$. Data was from 3 experiments, 78 observations (n), n = 17 or 18 at all concentrations except 15mM, where n = 7, data from 1 experiment was not included where the apparent rate was very high.

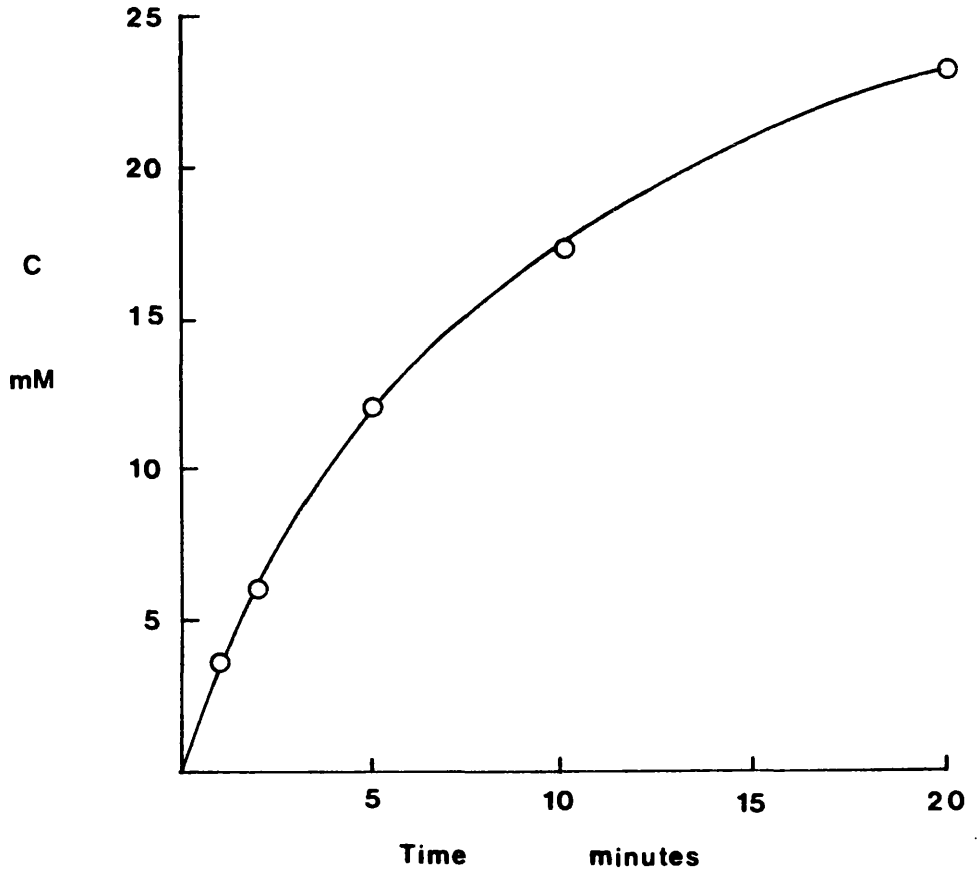


FIGURE 25

Infinite cis Entry Kinetics for Basal Adipocytes.

Fig. 25: A time course for net influx of 40mM 3-O-methyl-D-glucose at 37°C, in the absence of insulin. 49 observations, from 3 experiments.

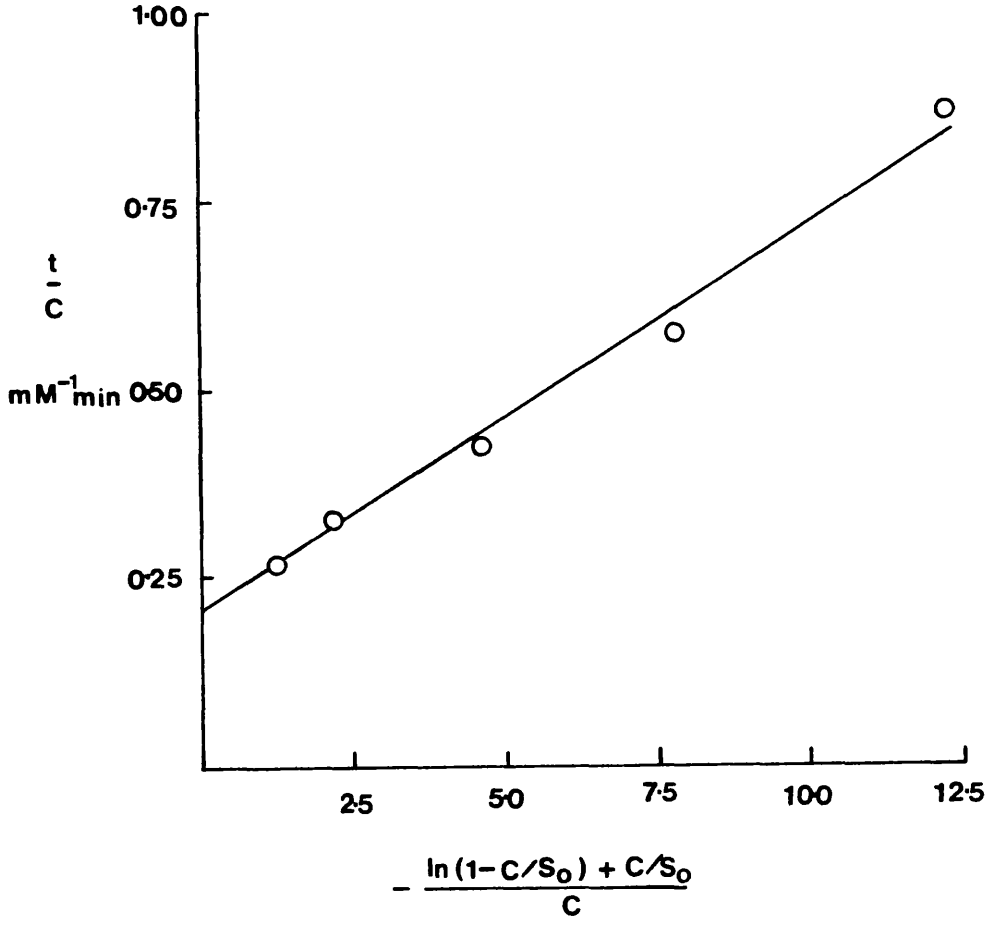


FIGURE 26

An Integrated Rate Analysis of Infinite cis Data (Figure 25).

Fig. 26: An integrated rate equation analysis of the infinite cis entry data of Figure 25. $K_{ic}^{oi} = 9.03 \pm 3.28$mM, $V_{ic}^{oi} = 0.066 \pm 0.13$mMs^{-1}.

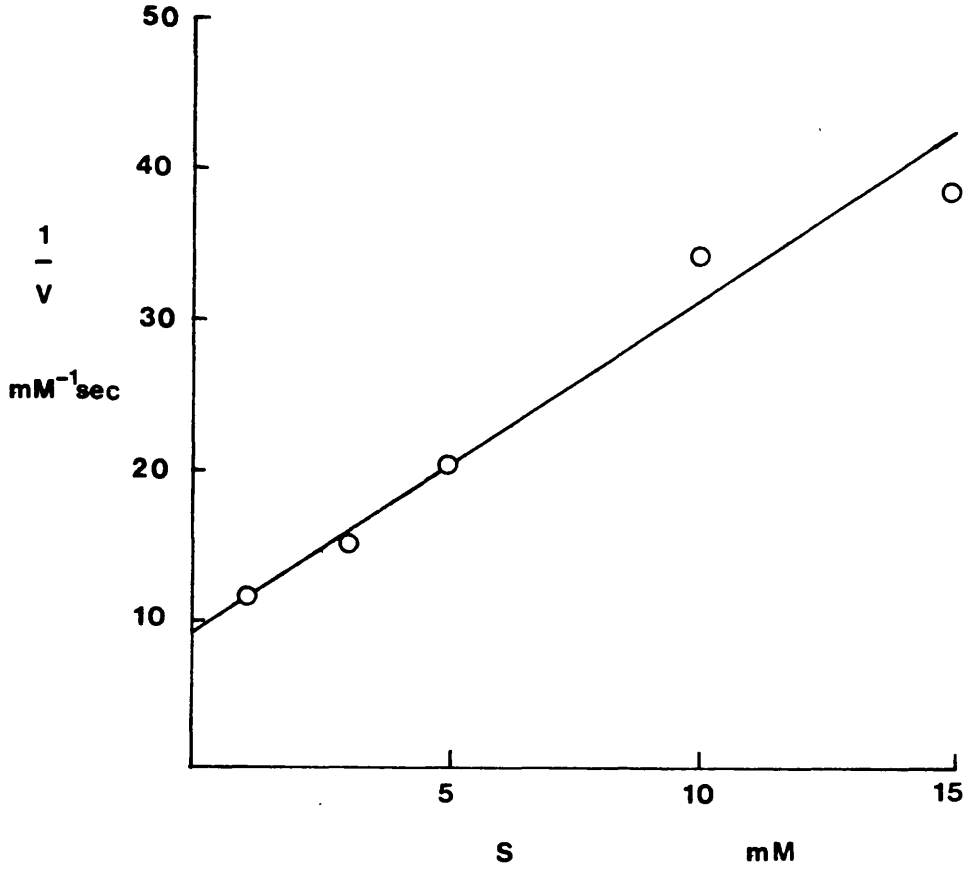


FIGURE 27

Infinite cis Efflux Kinetics for Basal Adipocytes.

Fig. 27: The infinite cis efflux of 40mM 3-O-methyl-D-glucose at 37°C. K_{ic}^{io} = 4.54 ± 1.32mM, V_{ic}^{io} = 0.106 ± 0.026mMs^{-1}. There were 60 observations, from 3 experiments.

3.3.6. Transport Kinetics in Adipocytes: A Summary.

All the parameters described in the previous sections are summarized in Table 12 (Taylor & Holman, 1981). Parameters for both basal and insulin-stimulated cells are shown.

3.4. The Testing of Potential Effectors of 3-O-methyl-D-glucose Uptake in Adipocytes.

The role of protein synthesis in the mechanism of insulin stimulation was investigated. 3-O-methyl-D-glucose uptake activity of the adipocytes was used as an assay for the effect of protein synthesis inhibitors on the extent of insulin-stimulation in the cell. Figure 28 shows the results of treatment with the protein synthesis inhibitors cordecypin and puromycin. The conditions of treatment are shown in Table 13. Neither compound appears to have a statistically significant effect on the insulin response.

The effect of a specific inhibitor of protein glycosylation, tunicamycin (Takatsuki et al., 1975) on the insulin stimulated and basal 3-O-methyl-D-glucose transport was also tested. The recommended method for dissolving tunicamycin is to use a solution of 25mM sodium hydroxide (Howe et al., 1980); the effect of 25mM NaOH alone was also tested.

In the first instance, Table 14, tunicamycin was added to the unstimulated cell preparation, to insulin-treated cells, and to basal cells with subsequent insulin treatment. Tunicamycin treatment was for one hour and at a final concentration of 1.25µg/ml as recommended by Howe et al. (1980). From Table 14 it may be seen that tunicamycin does not significantly affect the basal uptake. The treatment of the cells with tunicamycin prior to insulin addition showed a slight inhibition of the insulin response. However, tunicamycin treatment of previously insulin-stimulated cells appeared to produce a stimulation of sugar uptake that was greater than that observed with insulin alone. In order to check

TABLE 12

Kinetic Parameters for 3-O-Methyl-D-glucose Transport in Rat Adipocytes at 37°C.

Experiment	+Insulin(10nM)		Basal	
	K_m(mM)	V(mM/s)	K_m(mM)	V(mM/s)
Zero *trans* entry	6.10 ± 1.65 (n = 45)	1.20 ± 0.19	5.41 ± 0.98 (n = 24)	0.034 ± 0.014
Zero *trans* exit	2.66 ± 0.26 (n = 130)	1.19 ± 0.07	4.09 ± 1.05 (n = 22)	0.153 ± 0.023
Infinite *cis* entry	6.51 ± 0.83 (n = 51)	0.98 ± 0.09	9.03 ± 3.28 (n = 49)	0.066 ± 0.013
Infinite *cis* exit	3.60 ± 1.33 (n = 47)	1.76 ± 0.63	4.54 ± 1.32 (n = 60)	0.106 ± 0.026
Equilibrium Exchange	4.45 ± 0.26 (n = 42)	0.84 ± 0.002	4.22 ± 1.24 (n = 63)	0.058 ± 0.0001

Table 12: Results are the mean ± S.E. (from regression).

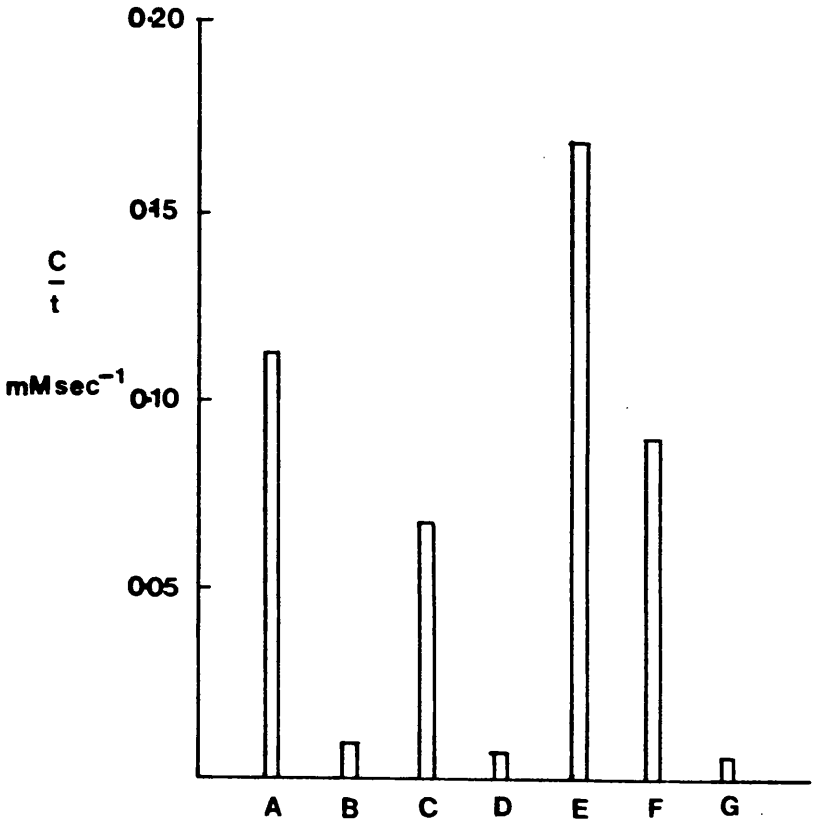


FIGURE 28

The Effect of Protein Synthesis Inhibitors on Methylglucose Uptake in Adipocytes.

Fig. 28: The figure shows the effect of 0.1mM cordecypin and 46μM puromycin on the zero trans entry of 1mM 3-O-methyl-D-glucose at 37°C. The conditions A to G are described in Table 13. Each histogram comprises mean values of 4 observations.

TABLE 13

Condition	Time of Incubation in minutes	*Addition	Time over which uptake was measured in seconds
A	0-30	10nM insulin	1
B	0-30	None	15
C	0-30	None	1
	30-90	0.1mM cordecypin	
	90-110	10nM insulin	
D	0-30	None	15
	30-90	0.1mM cordecypin	
	90-110	None	
E	0-30	10nM insulin	1
	30-90	0.1mM cordecypin	
	90-110	None	
F	0-30	None	1
	30-90	46μM puromycin	
	90-110	10nM insulin	
G	0-30	None	15
	30-90	46μM puromycin	
	90-110	None	

*Additions in the experiment shown in Figure 28, immediately after cell preparation. All concentrations shown are the final concentrations in the cell suspensions. Additions were sequential.

TABLE 14

Condition	*Time of incubation (minutes)	**Addition (sequential)	#Uptake per second ($mMsec^{-1}$)
A	0-30	None	0.0853
	30-90	tunicamycin	
	90-110	10nM insulin	
B	0-30	None	0.00610
	30-90	tunicamycin	
	90-110	None	
C	0-30	10nM insulin	0.171
	30-90	tunicamycin	
D	0-30	10nM insulin	0.112
E	0-30	None	0.00811

* Zero-time for the additions was taken immediately after cell preparation.

** The insulin concentration was the final concentration in the cell suspension. Tunicamycin was at a final concentration of 1·25μg/ml.

n = 2 to 4.

that this apparent stimulation was not an experimental artefact, the insulin response over the maximum time necessary to complete the assays was assessed. The results, shown in Figure 2, Section 3.2.2, show that the stimulation was constant over this period. The maximum variation in the stimulated uptake was 12.9% of the mean of the five uptakes ($0.147 mMsec^{-1}$) at varying times. This variation was 6.5% of the uptake of the basal condition ($0.0123 mMs^{-1}$), measured at four different times. Tunicamycin was tested on this cell preparation as in the previous experiment by adding it to the basal cells, to the insulin pre-treated cells, and to basal cells subsequently treated with insulin. The results, shown in Table 15, confirm those shown in Table 14.

In two further experiments, the large tunicamycin-stimulated insulin responses were not observed (Tables 16 and 17), although a slight stimulation was shown both during the one hour's treatment and when tunicamycin was present for one to 2.5 minutes (Table 17). Tunicamycin had no effect on adipocytes treated with a sub-maximal insulin concentration.

The results from the control experiment with 0.25mM sodium hydroxide were somewhat ambiguous. In the experiment reported in Table 15, sodium hydroxide appears to show an insignificant stimulation of the basal rate of 3-O-methyl-D-glucose uptake ($0.0166 \pm .0023 mMs^{-1}$ as compared to the basal rate of $0.0123 \pm 0.0006\ mMs^{-1}$). A similar slight stimulation occurs in the experiment shown in Table 16, and could account for the apparent slight tunicamycin stimulation of the insulin response. However, the insulin stimulation is low compared to that expected from other experiments (see Table 13, 15, and Figure 28). Experimental error in the measurement of the insulin-stimulated transport value (Table 16) could nullify the difference between values for condition B, compared with A or C.

Although 0.25mM NaOH appears to slightly increase the uptake of

TABLE 15

Condition	*Time of incubation (minutes)	**Addition (sequential)	#Uptake per second ($mMsec^{-1}$)
A	0-30	None	0.0952
	30-90	tunicamycin	
	0-110	10nM insulin	
B	0-30	None	0.0151
	30-90	tunicamycin	
	90-110	None	
C	0-30	10nM insulin	0.357
	30-90	tunicamycin	
D	0-30	10nM insulin	0.147
E	0-30	None	0.0123
F	0-30	25mM NaOH	0.0166

* see Table 14.

** see Table 14.

\# n = 4 for conditions A, B, C, & F.
n = 20 for condition B
n = 16 for condition C } see text

TABLE 16

Condition	*Time of incubation (minutes)	**Addition (sequential)	#Uptake per second ($mMsec^{-1}$)
A	0-20 20-80	10nM insulin tunicamycin	0.104
B	0-20	10nM insulin	0.0701
C	0-20 20-80	10nM insulin 25mM NaOH	0.107
D	0-180	None	0.00935

*, **, # see Table 14.

TABLE 17

Condition	*Time of incubation (minutes)	**Addition (sequential)	#Uptake per second ($mMsec^{-1}$)
A	0-20 20-80	10nM insulin tunicamycin	0.131
B	0-20 20-(21 to 23.5)	10nM insulin tunicamycin	0.142
C	0-20 20-80	0.001nM insulin tunicamycin	0.0371
D	0-20 20-80	0.001nM insulin None	0.0339
E	0-20	10nM insulin	0.106
F	0-180	10nM insulin	0.106
G	0-20	None	0.00302
H	0-180	None	0.0111
J	0-20 20-80	10nM insulin 25mM NaOH	0.197

* see Table 14.

** see Table 14.

n = 4.

insulin-stimulated cells shown in another experiment (Table 17), it must be noted that in this experiment, the basal rate of uptake increased 3.5-fold during the course of the experiment. This suggested that this particular cell preparation was unstable, and therefore the apparent stimulation of the insulin response by tunicamycin should be viewed with considerable doubt.

3.5. Electron Microscopy of the Adipocyte Ghost Preparation.

In order to assess the efficacy of the resealing process for fat cell ghosts, to determine the approximate size of the ghosts, and to examine the extent to which material other than cell membranes "contaminated" the preparation, ghosts were examined by electron microscopy.

Of prime importance for using the ghosts for transport studies was the question whether the plasma membrane had resealed adequately for the vesicle to contain the sugar that had been taken up by facilitated diffusion, without leakage. At the resolution of electron micrographs, sac-like structures and not membrane sheets should be visible. Figure 29 shows quite clearly that intact membrane sacs were formed (features A and B). These sacs are about 10μM in diameter. Numerous smaller vesicles were also visible (region C). Also, in Figure 30, intact round structures (≅2μM wide) may be seen, bounded by a single membrane. It is unclear whether these structures represent individual vesicles, or are projections from a larger sac.

It is notable, that in Figure 30, the vesicles all appear to be empty of cell debris, although this material is found outside of the vesicular structures. Although many empty structures were seen in the sections, there were examples of membrane sacs containing variable amounts of cell matter. Figure 31 illustrates a typical vesicle of this type (feature A). This one is of the order of 10μM wide; part of a similar sac structure is seen in

FIGURES 29 TO 33

Electron Micrographs of the Fat Cell Ghost Preparations.

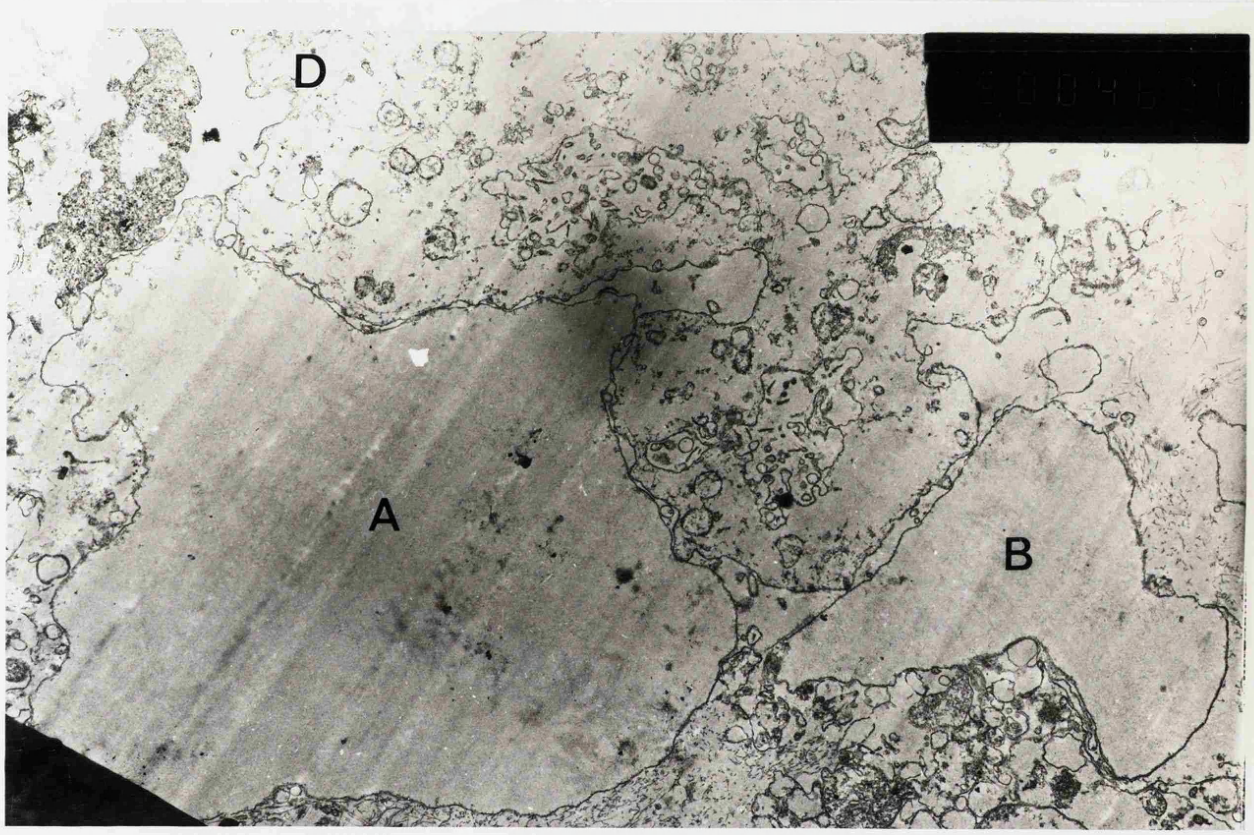


Fig. 29: Magnification = 5.0 x 10^3. Features A, B and D are vesicles.

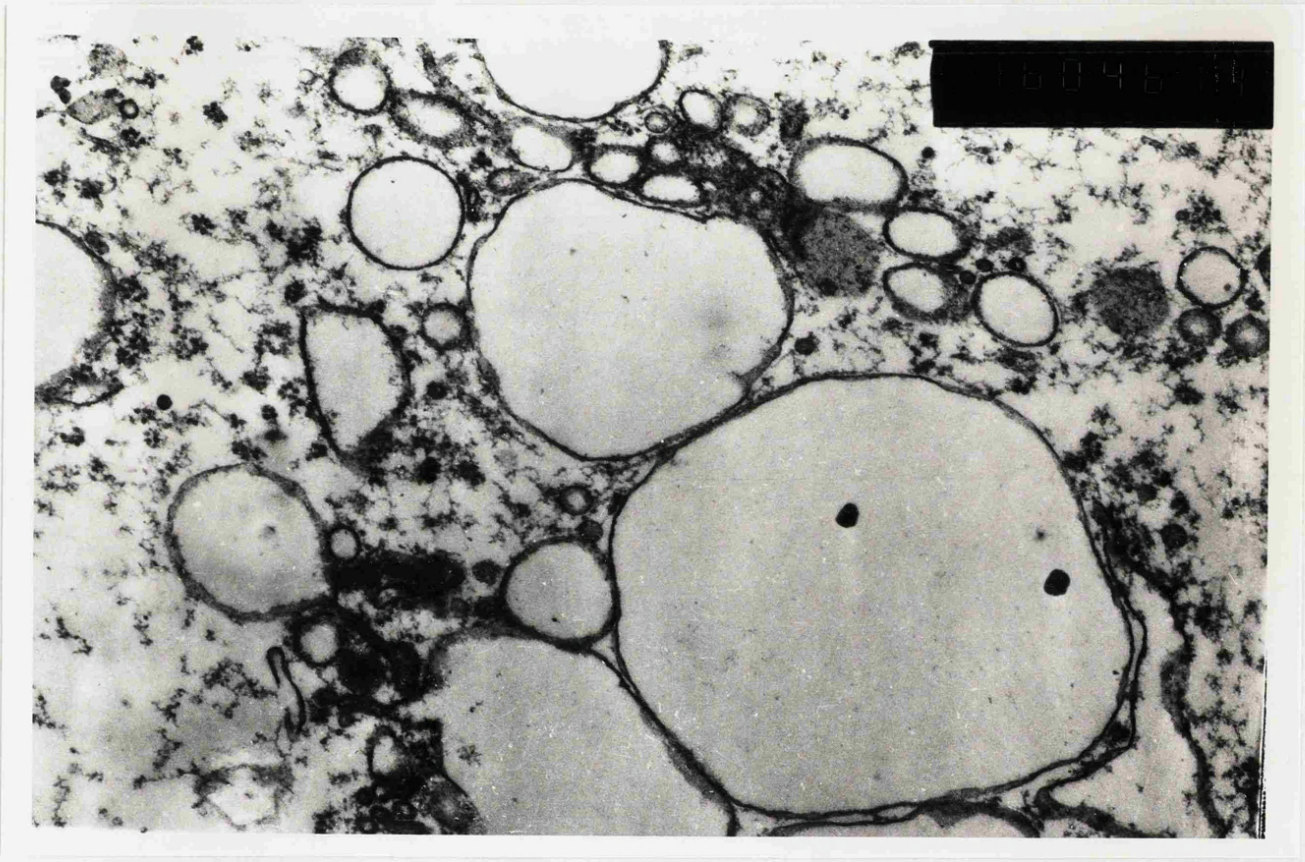


Fig. 30: Magnification = 16 x 10^3.

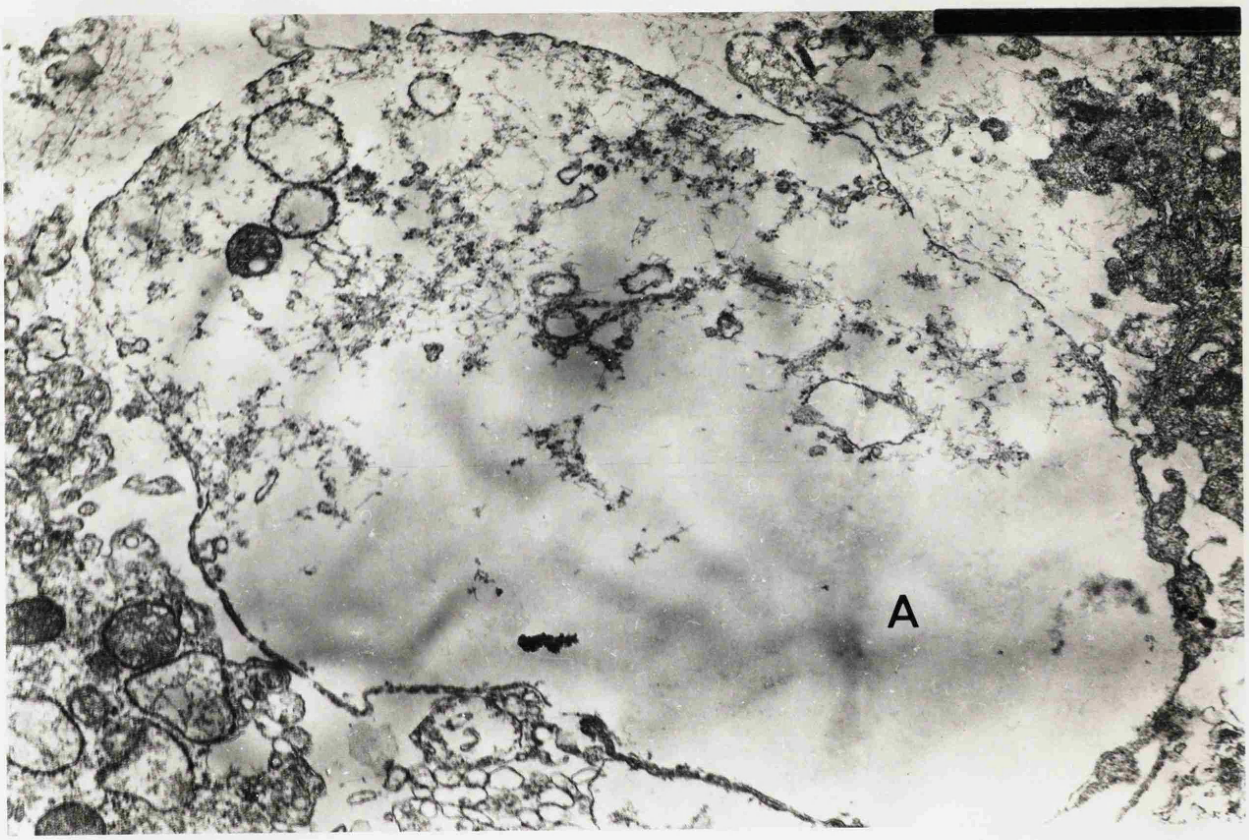


Fig. 31: Magnification = 8.3 x 10^3. Feature A is a vesicle.

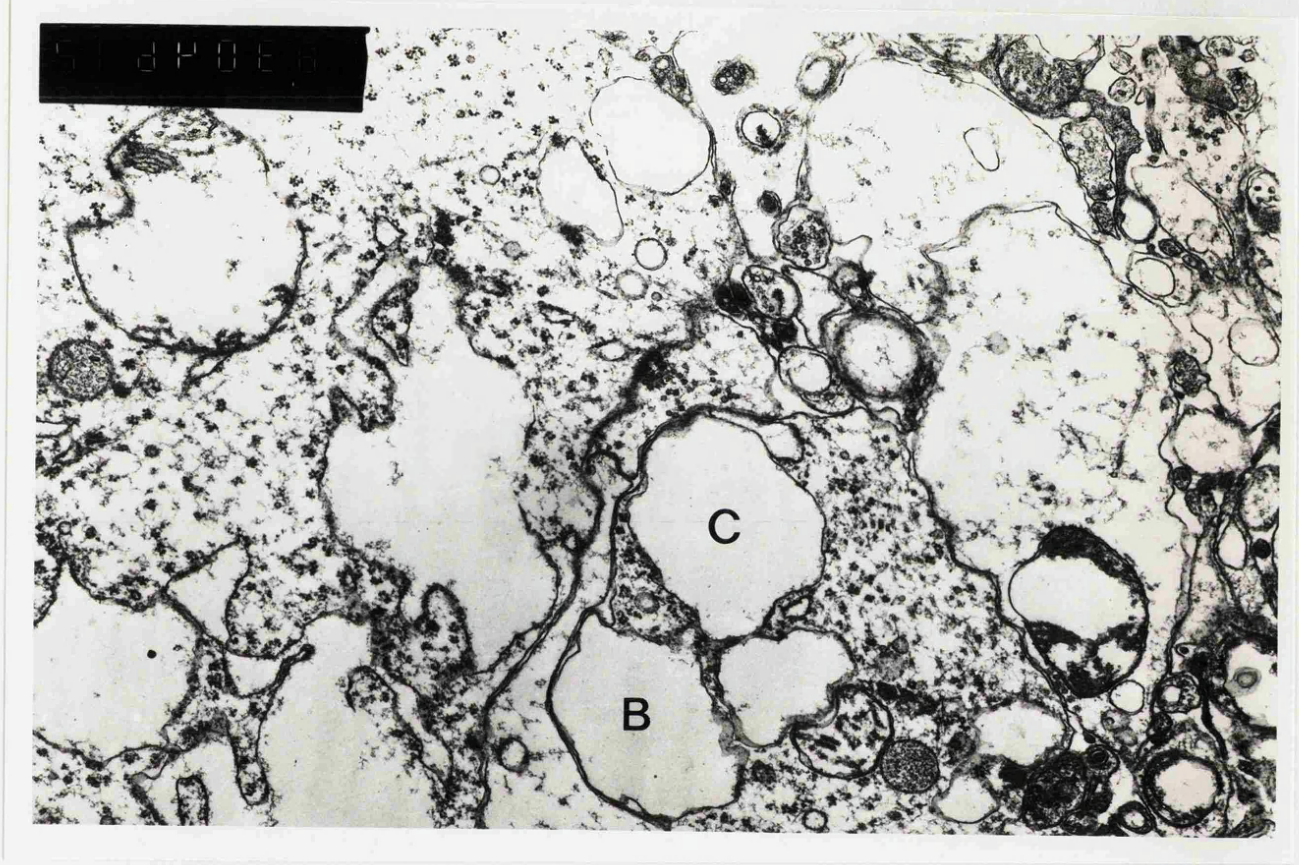


Fig. 32: Magnification = 8.3 x 10^3. Features B and C are small vesicles within a larger sac.

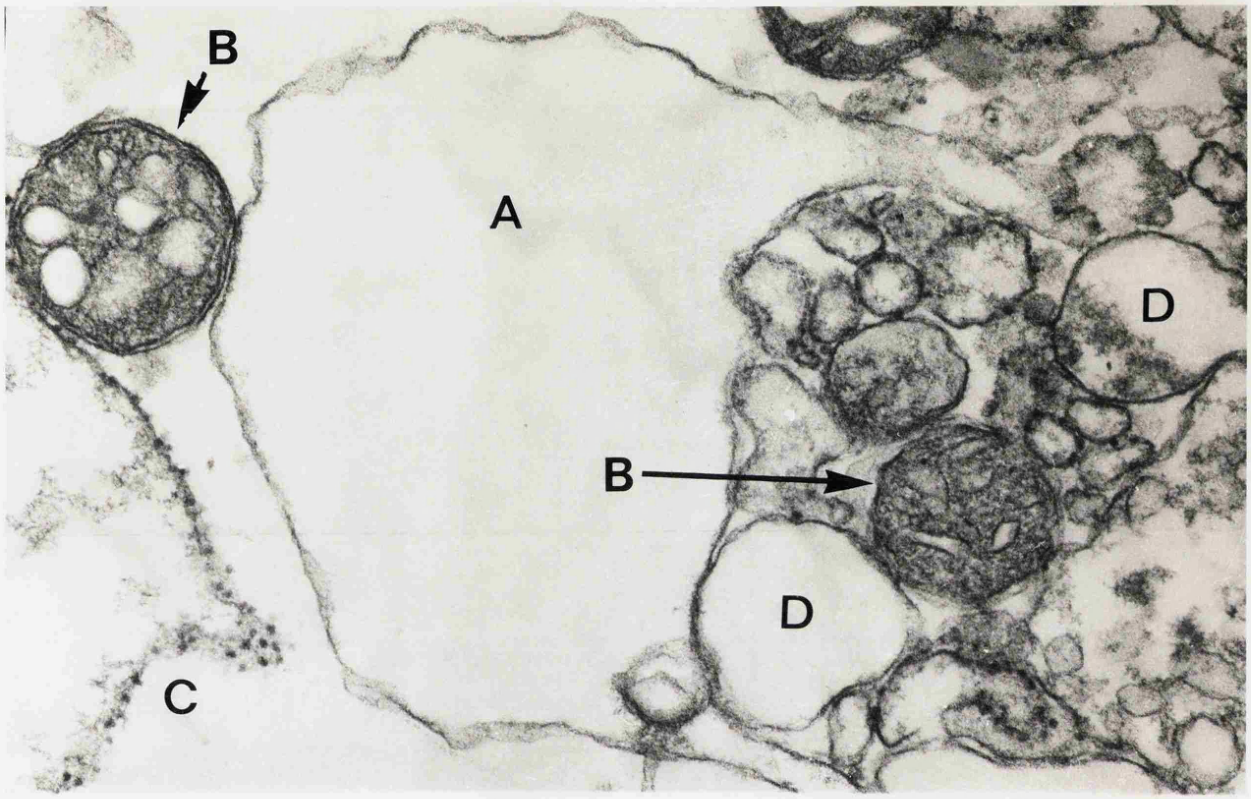


Fig. 33: Magnification = 20 x 10^3. Feature A is an empty vesicle, B are mitochondria, C is endoplasmic reticulum and D are small vesicles.

Figure 29 (feature D). In this particular sac (≅ 5μm wide) it is quite clear that many smaller vesicles are present within the larger sac. This is illustrated even more graphically in Figure 32: feature A contains several vesicles (eg. B and C) which, in this case, contain a large proportion of the interval volume visible. This phenomenon of vesicles within sacs could give rise to internal sequestration of the sugar, thus complicating the transport kinetics.

The type of organelle found amongst the membrane vesicles is shown in Figure 33. In this figure, the vesicle (A) is about 2μm wide. Outside it, rather distorted mitochondria (B) may be observed, as may a structure which is probably endoplasmic reticulum (C), dotted with ribosomes. In addition, smaller membrane vesicles (D) occur. Nuclear material was not detected, probably because if present, it was very disrupted by the hypotonic lysis.

In Rodbell's preparation of ghosts (Rodbell, 1967a), phase contrast light microscopy clearly showed remnants of nuclear and mitochondrial material within the ghosts. The modified preparation produces different, empty membrane structures, although some structures have similar features to those shown made by Rodbell.

There was no detectable difference between basal, insulin-treated, ATP, or IBMX-treated ghosts.

The apparently intact, vesicular nature of the membranes, makes them suitable for transport studies, although the variability in formation of internal vesicles may hinder an accurate analysis of the transport.

3.6. 3-O-Methyl-D-glucose Transport by Adipocyte Ghosts.

The early experiments on D-sugar uptake in adipocyte ghosts were designed to show several basic features. Firstly, that the ghosts took up the sugar, 3-O-methyl-D-glucose and that this uptake was by facilitated

diffusion. Using filtration methods, which had previously been applied successfully to the measurement of sugar transport in red blood cell ghosts (see Section 3.7) this first point was demonstrated in the experiment shown in Figure 34. Here, the uptake of 3mM 3-O-methyl-D-glucose into sugar-free cells was followed. "Infinity" was measured at thirty minutes. Several points deviate from the curve; this probably reflects the scatter of the data points since only two observations were taken at each time shown. In this particular experiment, uptake was measurable. In other experiments, no sugar was apparently taken up, or the extent of it was variable, demonstrating the variability of the ghost preparation.

Similar problems were encountered when the second question was considered: namely, whether the transport process could be affected by insulin or not, and if so, to what extent? Insulin-stimulation of transport was observed in some experiments. Figures 35, 36, and 37 document the effect of 8mU/ml bovine insulin on the uptake of 0.5, 3 and 10mM 3-O-methyl-D-glucose at 20°C or 25°C. In all these cases, the stimulation was in the region of a two-fold increase. (The "early peak" shown in Figure 37 was probably artefactual, points are only means of duplicate measurements and other experiments did not mimic this pattern). However, in other experiments, the ghosts were unresponsive to insulin.

A third purpose of this series of experiments, was to determine whether initial rates could be measured directly, and if so, what would be a suitable time interval to use for direct measurement. From previous experience with the filtration method, the lowest accurate and reproducible time point appeared to be at two seconds. Figure 35 showed that it would be necessary to use this time interval, since even with this low 3-O-methyl-D-glucose concentration, the rate was not initial at four seconds. The data was not sufficiently accurate to allow the use of integrated rates to

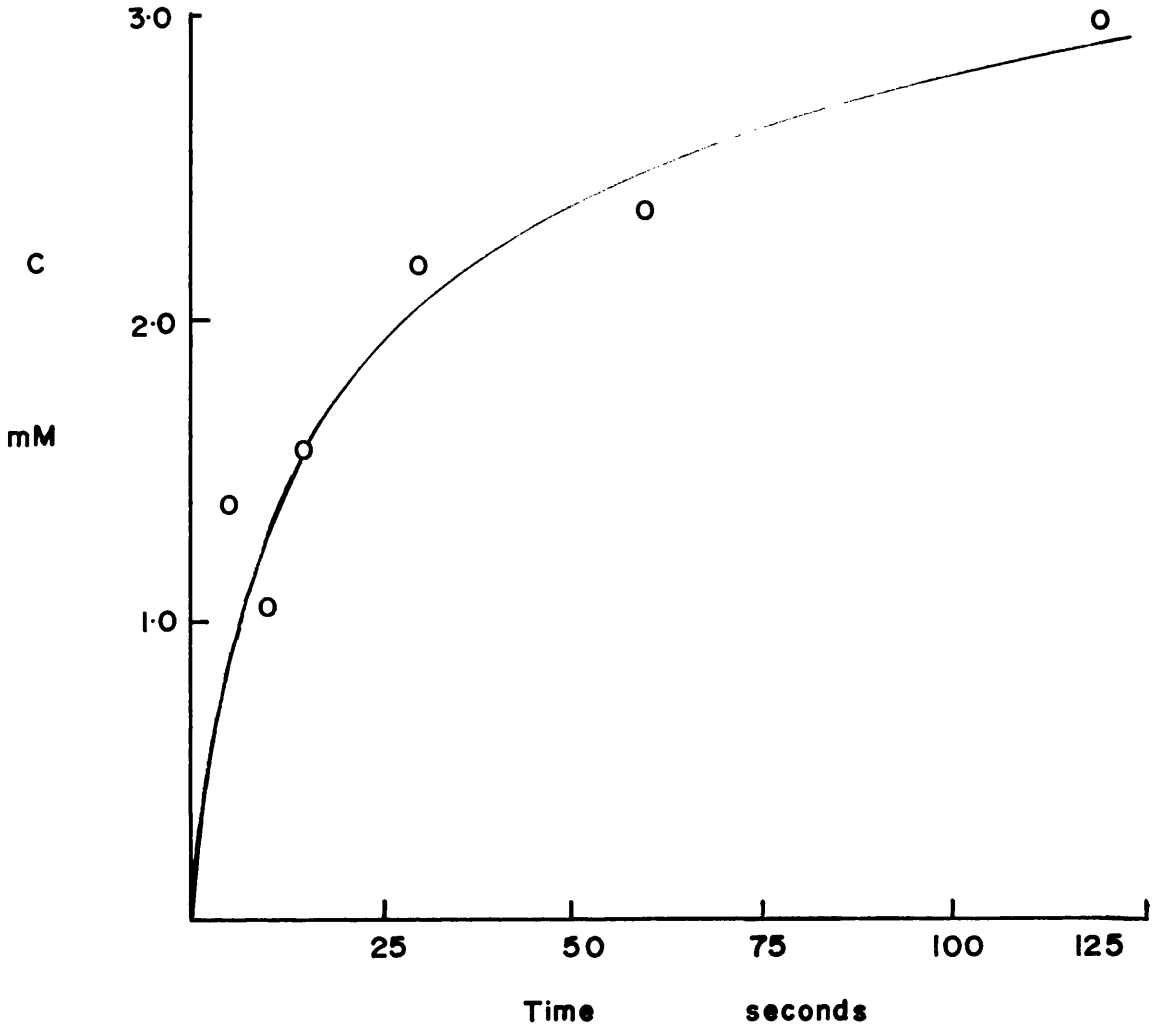


FIGURE 34

The Uptake of Sugar by Adipocyte Ghosts.

Fig. 34: The curve shows the uptake of 3mM 3-O-methyl-D-glucose under basal conditions in adipocyte ghosts, at 20°C. Data ~~are~~ from only one experiment, where points represent mean values of duplicates.

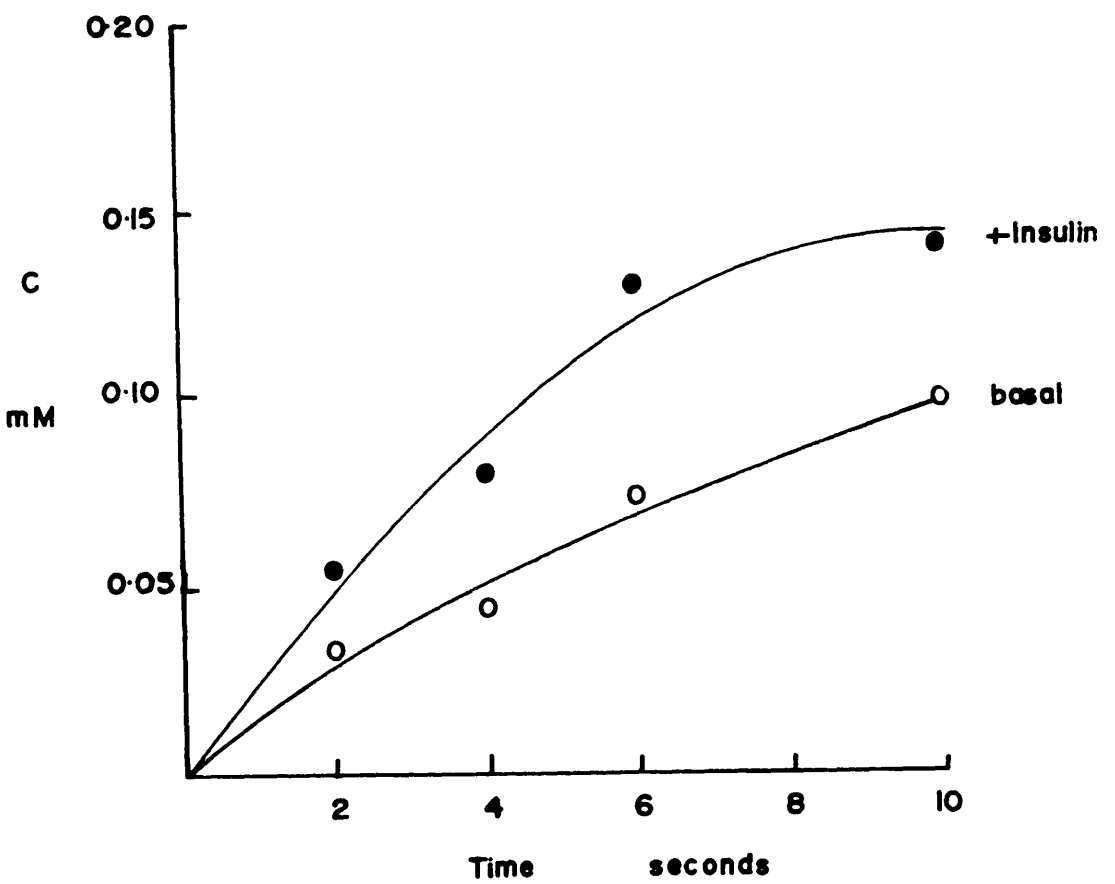


FIGURE 35

The Transport of a Low Concentration of Methylglucose into Adipocyte Ghosts.

Fig. 35: The graph shows the time courses for entry of 0.5mM 3-O-methyl-D-glucose, into adipocyte ghosts at 20°C, both in the presence (●) and absence (O) of 8 mU/ml bovine insulin. Data are from one experiment, each value is the mean of duplicates.

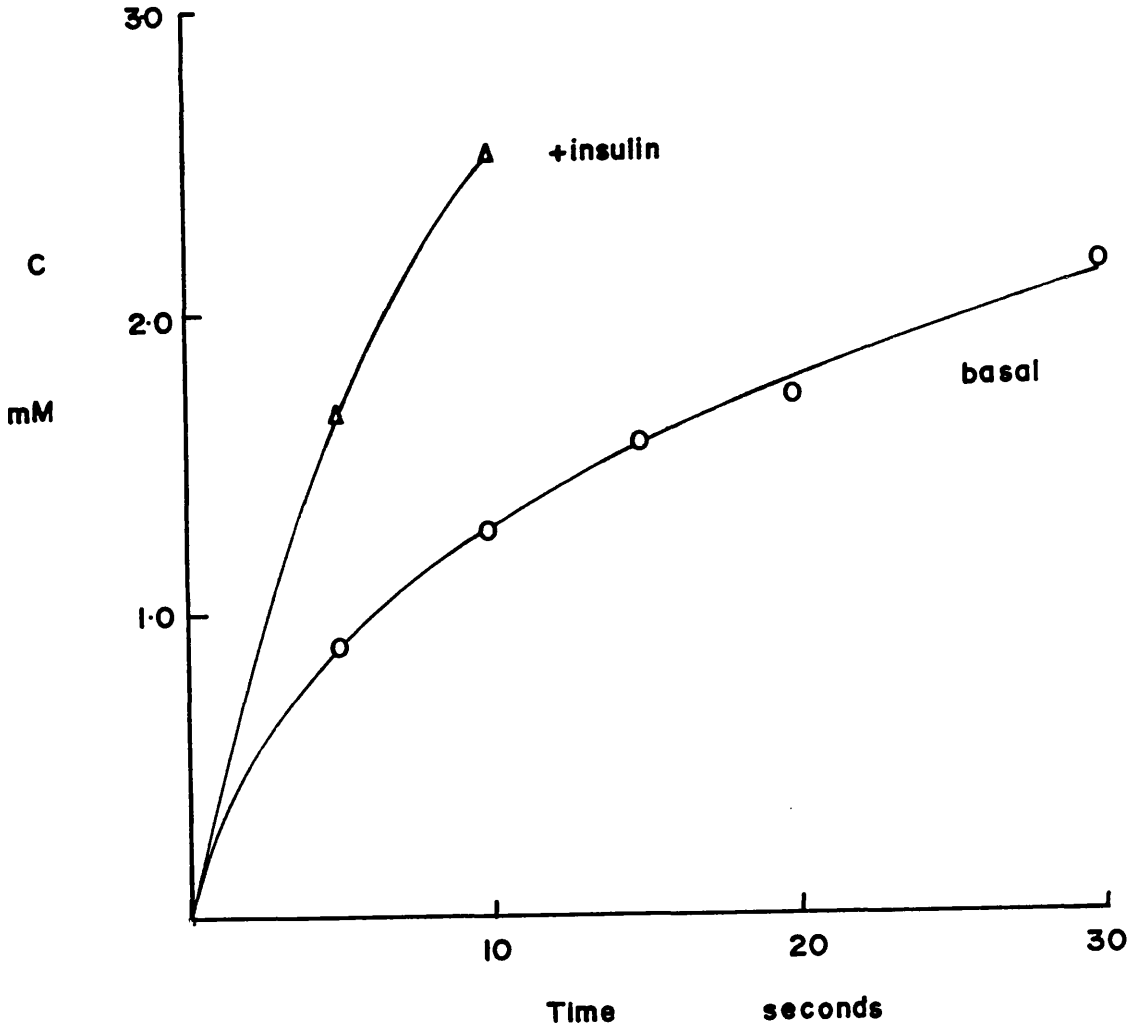


FIGURE 36

The Transport of Methylglucose into Adipocyte Ghosts.

Fig. 36: The curve (O) shows the entry of 3mM 3-O-methyl-D-glucose into rat adipocyte ghosts at 20°C. The data is from 3 experiments, where the total number of observations was 24. Points represent mean values of experimental means. In one of these cell populations, an insulin-stimulation was observed (Δ) when n = 2 for each point, which is a mean value.

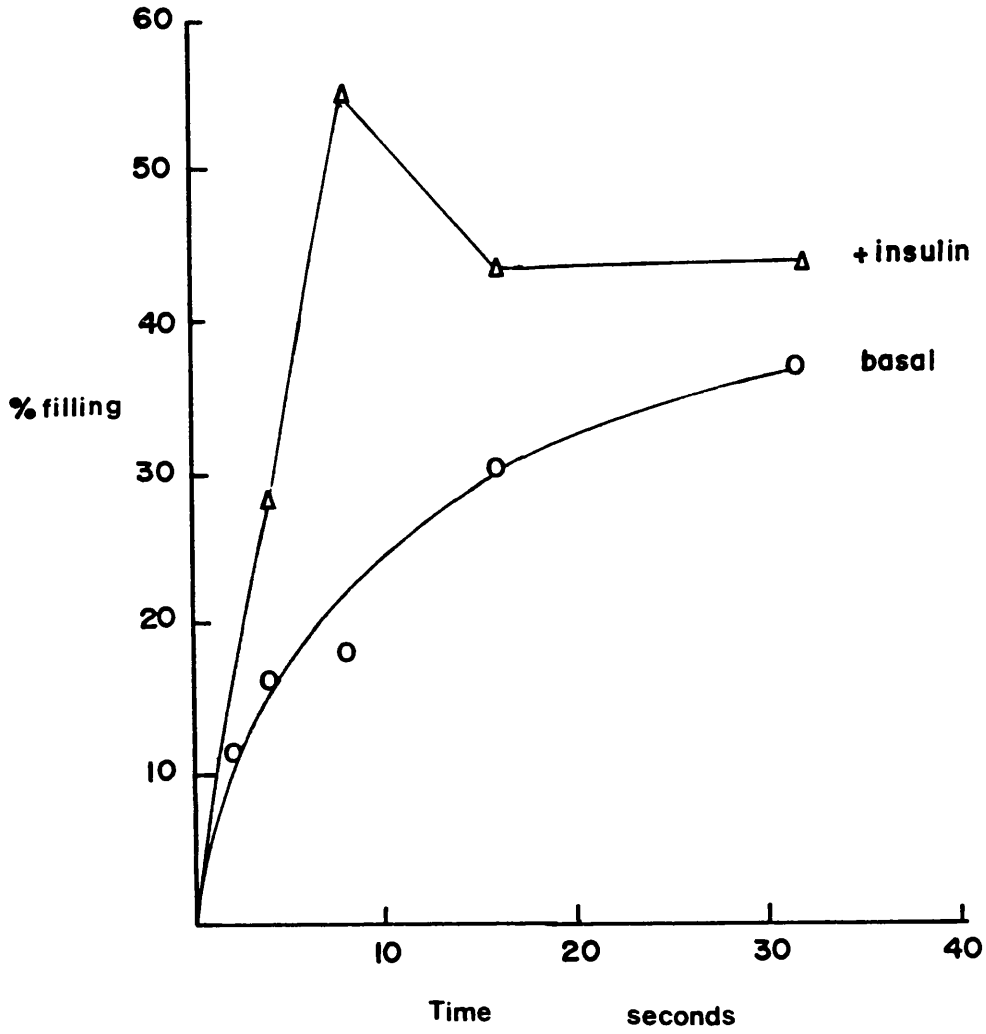


FIGURE 37

The Transport of Methylglucose into Adipocyte Ghosts.

Fig. 37: The transport of 10mM 3-O-methyl-D-glucose into adipocyte ghosts at 25°C, in the presence (Δ) and absence (O) of 8mU/ml bovine insulin. Each point is the mean of duplicate measurements from a single experiment.

confirm the initial rate of about 0.015mMs^{-1}, determined graphically. It should be noted that the basal uptake in Figure 37 appears rather high when compared to that expected from the 0.5mM and 3mM uptake curves (Figures 35 and 36), and also the later experiments where the zero *trans* entry of 10mM 3-O-methyl-D-glucose was measured over two seconds (Figure 38). This again demonstrates the variable nature of the fat cell ghost sugar transport.

3.6.1. Zero trans entry Kinetics in Adipocyte Ghosts.

Although the data shown in Figure 38 shows zero *trans* entry kinetics for 3-O-methyl-D-glucose transport under both basal and insulin-stimulated conditions, an equal number of experiments showed very erratic uptake - basal levels as high as those for insulin-stimulated ghosts, and *vice versa*. In view of these discrepancies the parameters K_{zt}^{oi} and V_{zt}^{oi} have not been analysed by rigorous statistical methods. In Figure 38, an indication of the values has been given from lines drawn by eye through points which are mean values from several experiments. From these rough estimates, it appears that insulin increases the V_{max} by about 4-fold, without affecting the K_m for zero *trans* entry. The value for the parameter K_{zt}^{oi} appears similar to that found in intact adipocytes (see Section 3.3.6). Although the V_{zt}^{oi} value for insulin-treated ghosts is of the same order as that found in whole cells, the basal value is considerably higher.

3.6.2. Equilibrium Exchange Kinetics in Adipocyte Ghosts.

Equilibrium exchange experiments are shown in Figure 39. Only two experiments contribute to the mean values shown - the scatter of data in these experiments was not so great as that seen in other experiments (Figure 40). These other experiments gave variable parameters, initial rates at 30mM 3-O-methyl-D-glucose being as high as 2.79mMs^{-1}, and 0.045mMs^{-1} at

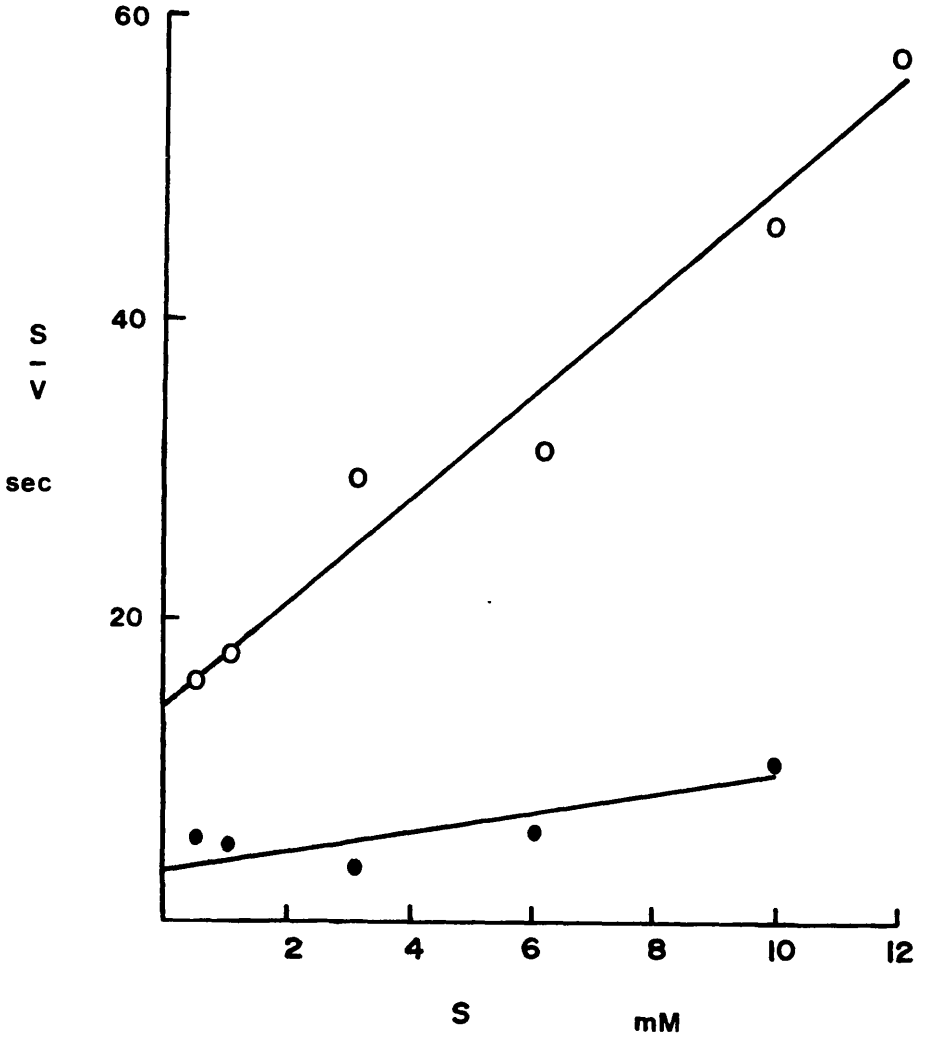


FIGURE 38

Zero trans Entry Kinetics in Adipocyte Ghosts.

Fig. 38: The zero trans entry of 3-O-methyl-D-glucose into basal (o) and insulin-stimulated (●) adipocyte ghosts is shown. Transport was measured at 20°C and over two seconds. For the basal transport, K_{zt}^{oi} = about 5mM, V_{zt}^{oi} = about 0.30mMs^{-1} (32 observations from up to 4 experiments). For insulin-stimulated transport, K_{zt}^{oi} = approximately 6mM and V_{zt}^{oi} = approximately 1.3mMs^{-1} (30 observations from 3 experiments).

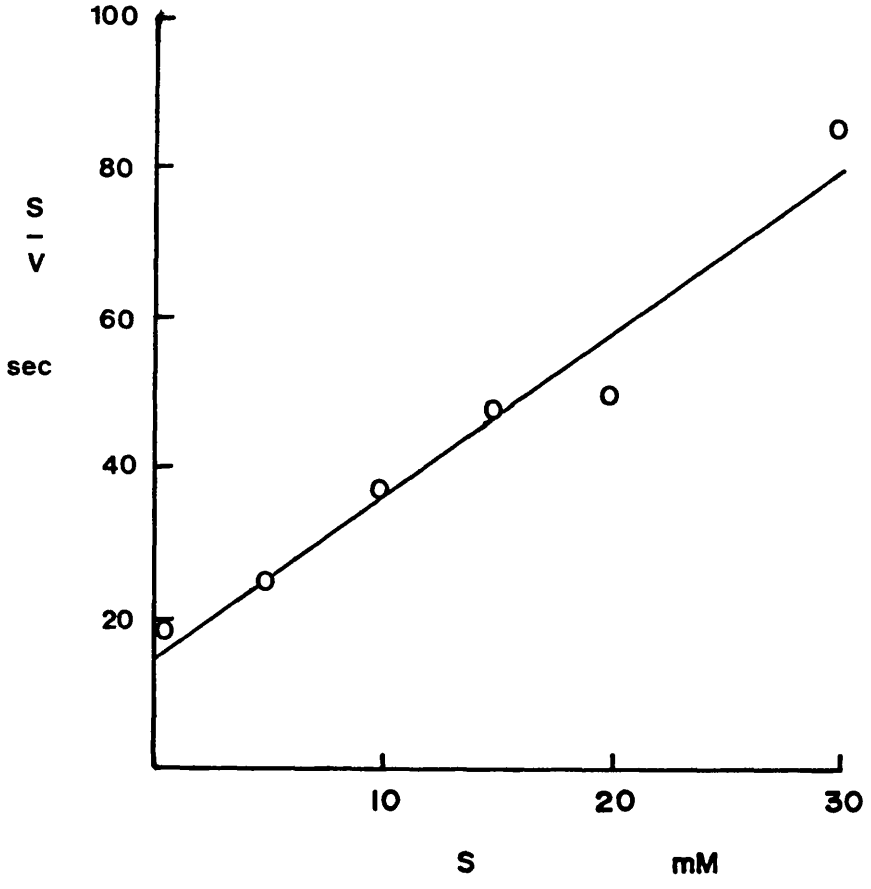


FIGURE 39

Equilibrium Exchange Kinetics in Adipocyte Ghosts.

Fig. 39: The equilibrium exchange entry of 3-O-methyl-D-glucose into basal (O) adipocyte ghosts. Transport was measured at 25°C and over two seconds. K_{ee}^{oi} = 6.06±2.59 mM, and V_{ee}^{oi} = 0.445±0.245 mMs^{-1} (18 observations from 2 experiments).

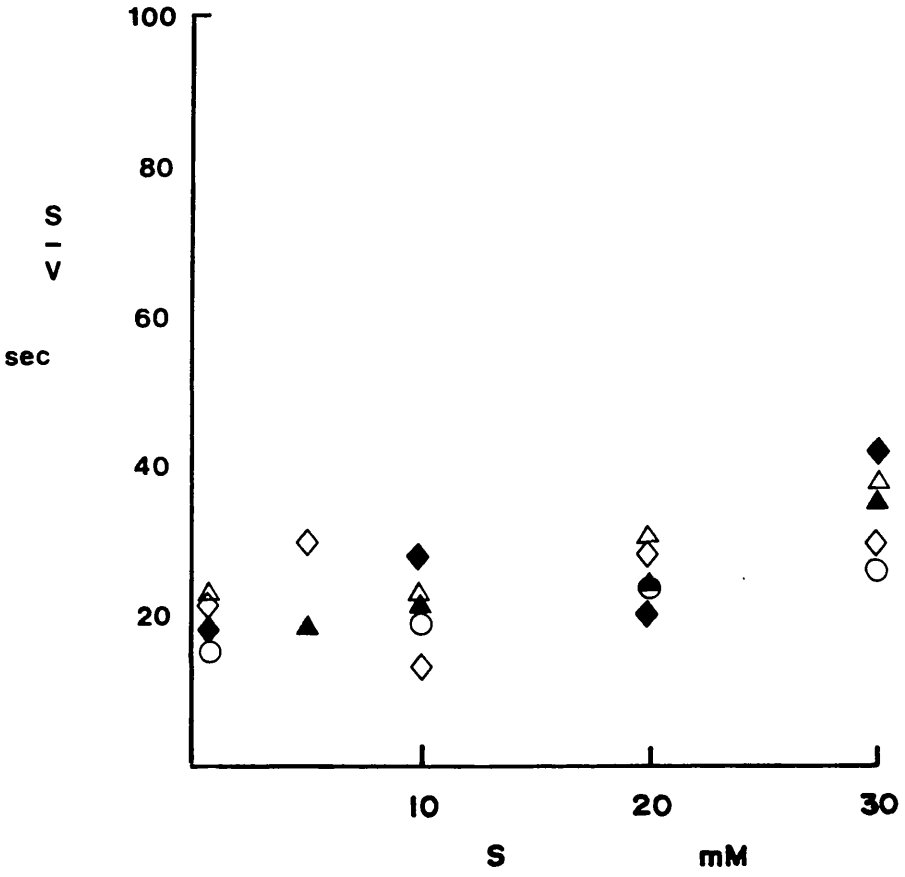


FIGURE 40

Further Equilibrium Exchange Experiments on Adipocyte Ghosts.

Fig. 40: Equilibrium exchange entry of 3-O-methyl-D-glucose into basal adipocyte ghosts. Each point is from a single experiment, where points are mean values measured in duplicate.

0.5mM 3-O-methyl-D-glucose, in one instance. Although both V^{oi}_{ee} and K^{oi}_{ee} were apparently higher in these experiments (Figure 40) the permeability (K/V) was about the same, indicating that sugar leakage did not account for the differences.

None of the equilibrium exchange experiments in fat cell ghosts appeared to show insulin stimulation. In the two experiments on basal cells reported in Figure 39, insulin treatment was not assessed in one case, and in the other, no insulin effect was seen.

These results indicated that the ghost preparation was not suitable for rigorous equilibrium exchange analyses to obtain accurate kinetic parameters from initial rates. The data was very scattered, possibly due to the rather non-homogenous nature of the ghost suspensions: clumps of material were frequently found, which were very difficult to disperse, whilst treating the ghosts gently so as not to rupture the sacs.

3.6.3. Infinite cis Entry Kinetics in Adipocytes Ghosts.

One further type of kinetic experiment was attempted: the infinite *cis* entry experiment. In this experiment, the design is such that transport is measured over a longer time course, so that the percentage errors which are incurred by assaying uptake over short time intervals are of less importance.

The transport of 20mM 3-O-methyl-D-glucose is shown in Figure 41. Further examination, by application of the integrated rate equation (see above) to the data (Figure 42) showed that the inifinite *cis* affinity and velocity parameters changed when cells were treated with insulin. Values of the parameters for basal ghosts were, K^{io}_{ic} = about 5.20mM and V^{oi}_{ic} = $0.370 \pm 0.055 mMs^{-1}$ whereas with insulin, the K^{oi}_{ic} = 1.52mM and V^{oi}_{ic} = $1.89 \pm 0.40 mMs^{-1}$.

In some infinite *cis* experiments, 1mM ATP was added to the resealing

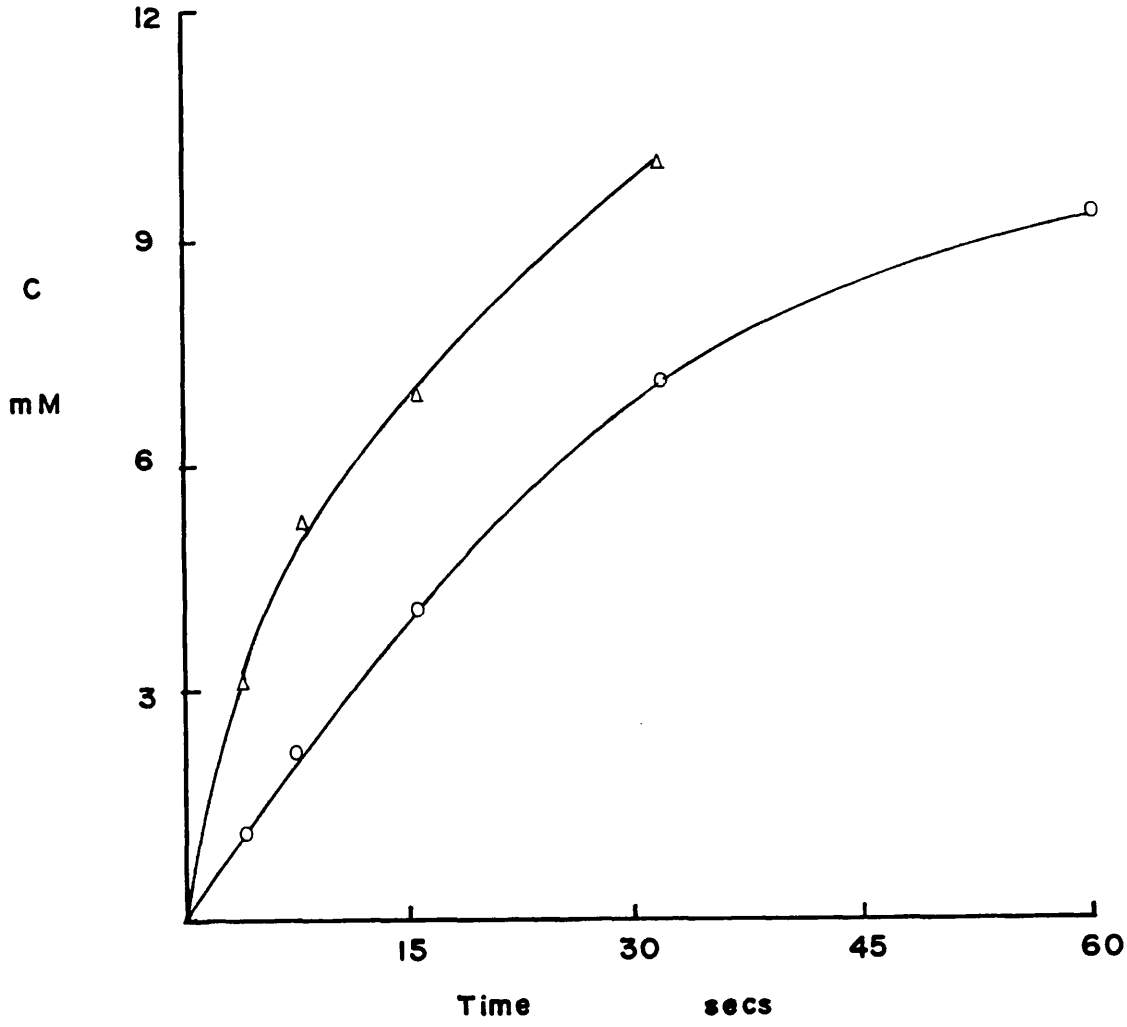


FIGURE 41

Infinite cis Entry Experiments in Adipocyte Ghosts.

Fig. 41: This graph shows two time courses for the net entry of 20mM 3-O-methyl-D-glucose into adipocyte ghosts at 25°C. One curve represents uptake in basal ghosts (O) and the other, uptake in ghosts treated with 25mU/ml bovine insulin (Δ).

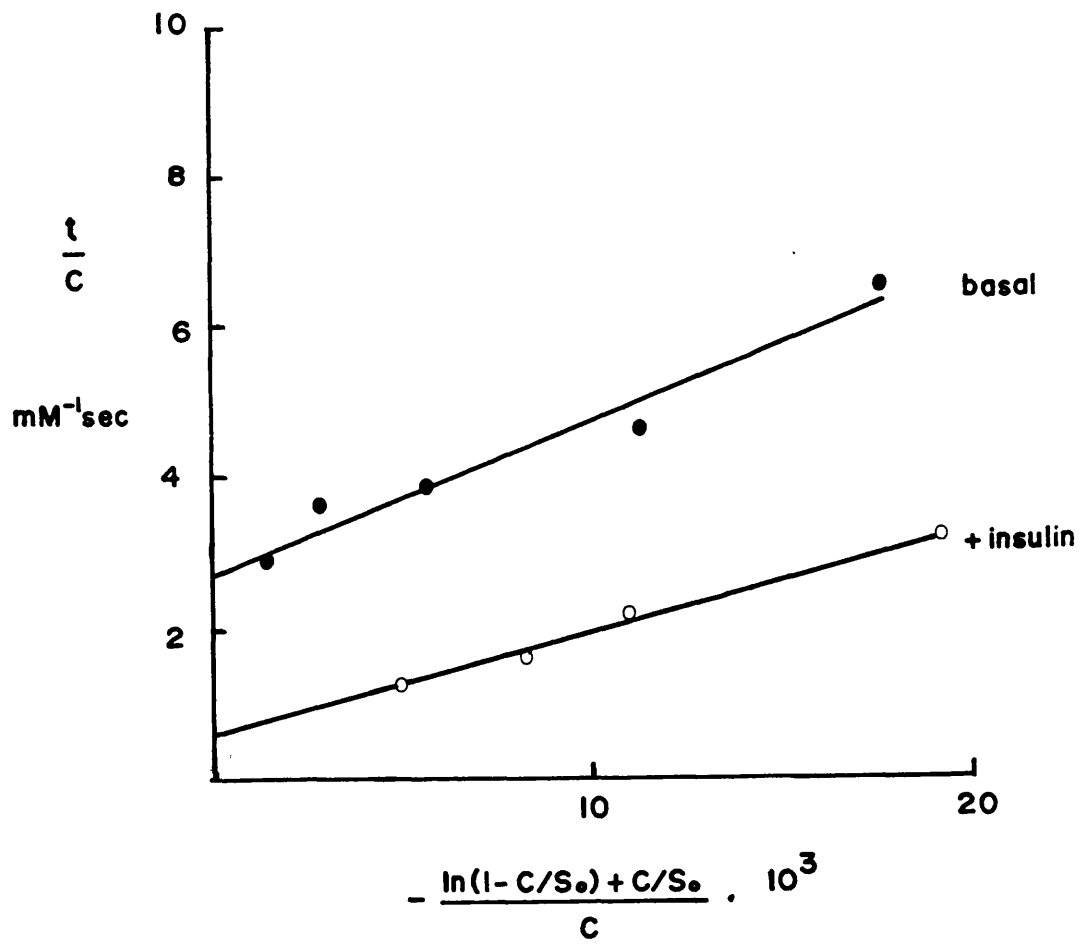


FIGURE 42

An Analysis of the Infinite cis Entry Data.

Fig. 42: The data shown in Fig. 41 is replotted here, following analysis with the integrated rate equation (3). (○) represents insulin-treated ghosts, (●) basal ghosts. The infinite cis parameters were, for basal ghosts, $K_{ic}^{oi} = 5.20$mM, $V_{ic}^{oi} = 0.37 \pm 0.055$mMs^{-1}; for insulin-stimulated ghosts, $K_{ic}^{oi} = 1.52$mM, $V_{ic}^{oi} = 1.89 \pm 0.40$mMs^{-1}.

buffer. In red blood cell ghosts, this compound appears to allow the plasma membrane to behave more like that of an intact cell (Hoffman, 1962), and it was of interest to see if it produced a more stable and hormone-responsive fat cell ghost suspension, and thereby enable data scatter to be reduced. The results from three experiments are shown in Figure 43, and the data, analysed and replotted, is shown in Figure 44. In this graph, the line represents mean values for the infinite *cis* entry shown in Figure 42. Although data is still very scattered, it appears that insulin does not stimulate the basal 3-O-methyl-D-glucose transport in ghosts resealed in the presence of 1mM ATP. In view of the previously demonstrated phenomenon, that ghosts respond variably to insulin, this result should be treated with caution, but it is notable that three experiments illustrate this trend.

Such a result could suggest that a depletion of ATP by insulin is one prerequisite for a stimulation of D-glucose transport. Several mechanisms may be conceived of for this, but will be discussed later in the context of other results.

3.6.4. The Effect of Temperature on 3-O-methyl-D-glucose Transport in Adipocyte Ghosts.

In order to study the effect of temperature on the sugar transporter, the zero *trans* entry of 3mM 3-O-methyl-D-glucose was assayed at several temperatures, ranging from 4°C to 25°C. Initial rates were measured over two seconds for both basal, and insulin-stimulated ghosts. Figure 45 shows the results from six experiments. It can be seen from this figure that transport occurs even at temperatures as low as 4°C. The basal rate of 3-O-methyl-D-glucose entry appears to increase gradually with increasing temperature, the rate at 25°C being about seventeen times greater than

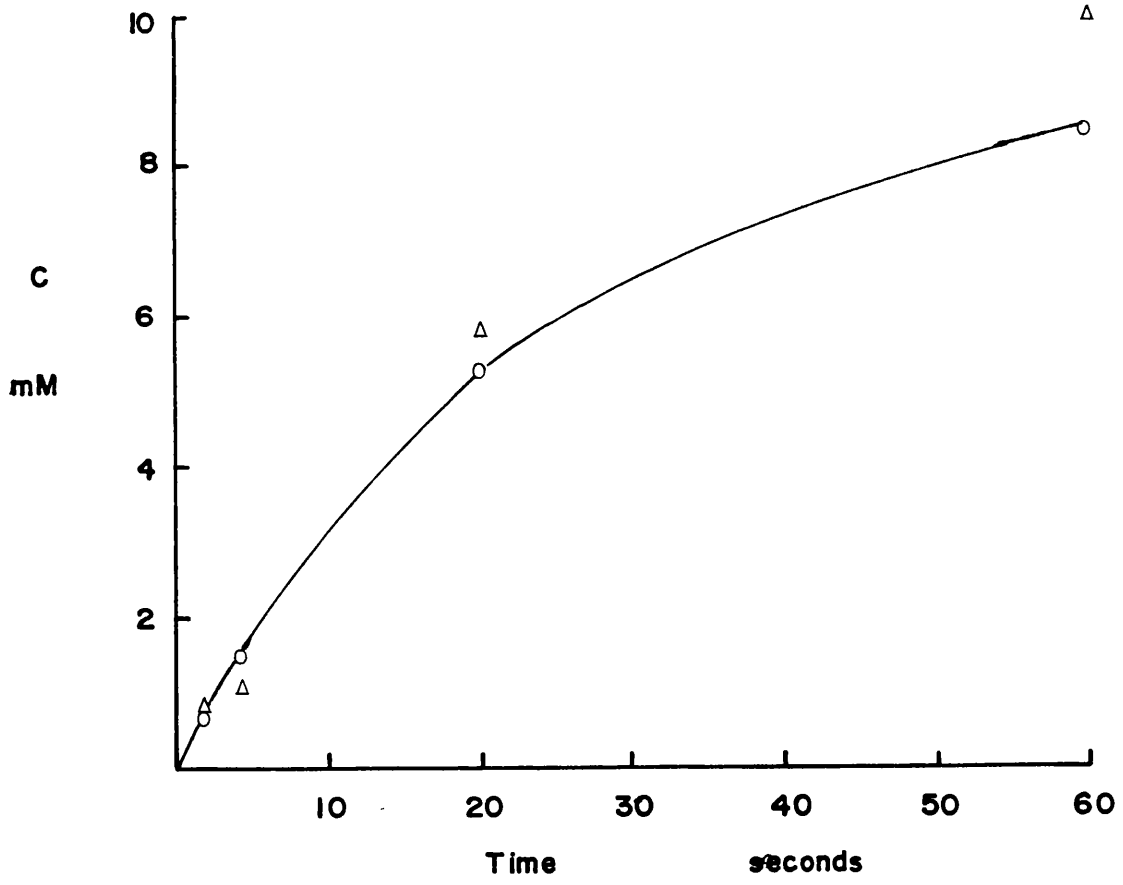


FIGURE 43

Resealing Ghosts in the Presence of ATP.

Fig. 43: The uptake of 20mM 3-O-methyl-D-glucose into adipocyte ghosts at 25°C is shown for basal (O) and insulin-treated (Δ) ghosts. Ghosts were resealed in the presence of 1mM ATP. Data is from three experiments where uptakes were measured in duplicate.

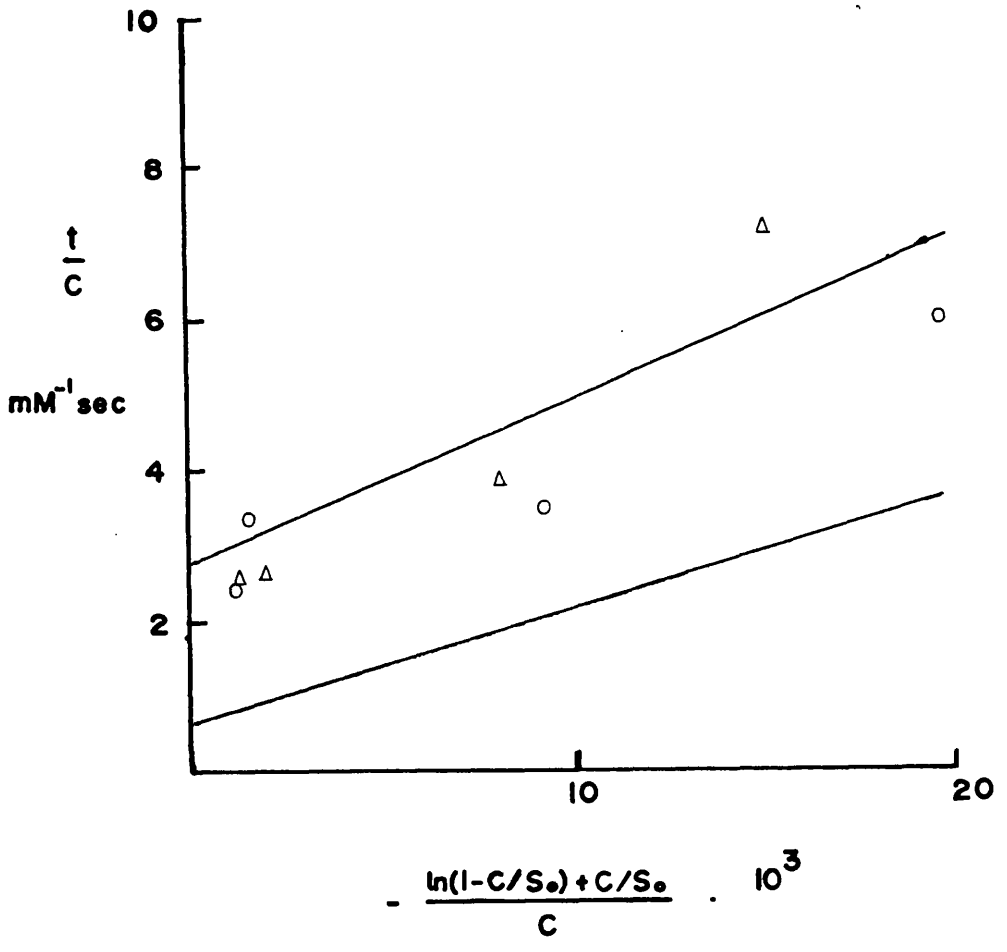


FIGURE 44

An Integrated Rate Analysis of Uptake in Ghosts.

Fig. 44: The points show the values obtained from transport in ghosts resealed with ATP (see Fig. 43) for basal (o) and insulin-treated (Δ) ghosts. The lines represent the infinite cis kinetics of ghosts resealed without ATP, as shown previously in Fig. 42.

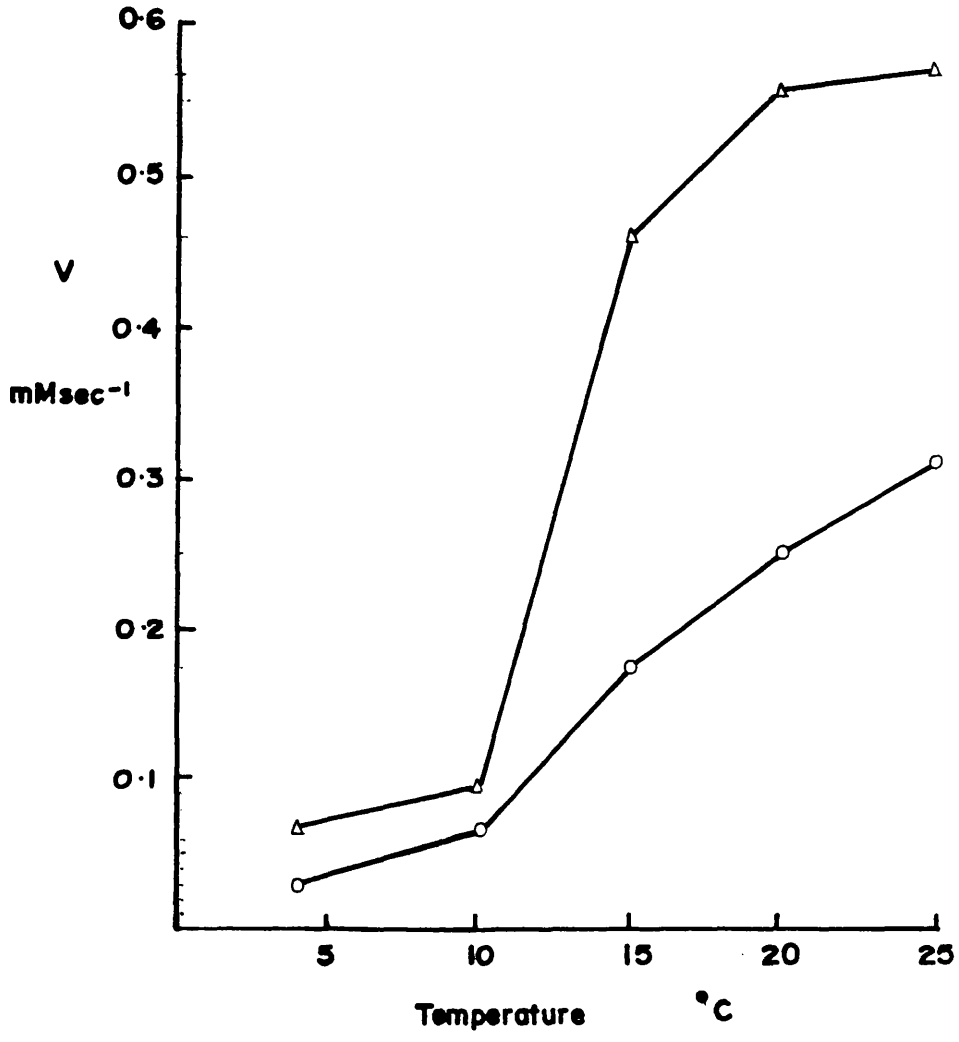


FIGURE 45

The Effect of Temperature on Basal and Insulin-stimulated Transport in Fat Cell Ghosts.

Fig. 45: The effect of temperature on the initial rate of transport of 3mM 3-O-methyl-D-glucose into adipocyte ghosts, where initial rates were measured over two seconds. The total number of experiments was six and points are the mean values of duplicates. (O, basal transport; Δ insulin-stimulated transport).

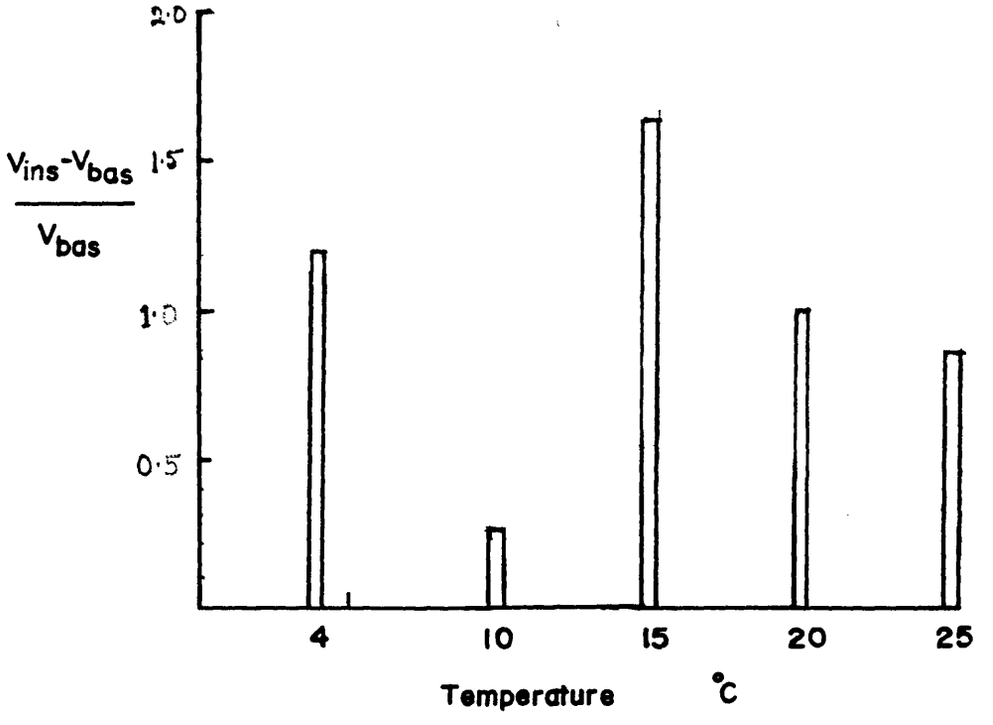


FIGURE 46

The Effect of Temperature on the Insulin Response of Transport in Adipocyte Ghosts.

Fig. 46: An analysis of the extent of stimulation by insulin of the 3mM 3-O-methyl-D-glucose zero *trans* entry in ghosts at varying temperatures. The number of experiments contributing to each bar was ≥ 3, where initial rate values (v) where measured in duplicate. The subscripts ins and bas refer to insulin-treated and basal ghosts.

that at 4°C.

The mean data in Figure 45 shows that insulin always stimulated the ghosts to take up 3-O-methyl-D-glucose more rapidly than basal ghosts. It would appear that there is a quite dramatic increase in stimulation between 10°C and 15°C, but that the increase of transport with temperature thereafter becomes less marked. However, a more detailed analysis of the actual data which comprises the means in Figure 45 is shown in Figure 46. In this figure, the extent of stimulation in these experiments has been calculated, and mean values for the size of the stimulation calculated. Figure 46 illustrates the scatter of the data contributing to the previous graph. It is nonetheless noteworthy that in only one cases out of eighteen comparisons was there no stimulation of transport by insulin (at 10°C). Thus, at the very least, the conclusion may be drawn that insulin stimulates 3-O-methyl-D-glucose transport in ghosts at all these temperatures.

3.7. Transport Kinetics of Erythrocyte Ghosts.

This series of transport experiments also conform to the model experiments described by Eilam & Stein (1974). Zero *trans* entry and exit, and infinite *cis* entry experiments were followed.

In the red blood cell ghosts, the transport of D-glucose was studied. Red blood cell ghosts do not metabolise this sugar, so there was no need to use a D-glucose analogue to study the sugar transporter. In all the experiments transport was measured at 20°C.

3.7.1. The Validity of Initial Rate Measurements.

The validity of using a particular time interval over which to assess the initial velocity of radioactive D-glucose transport was tested by

measuring a short time course at a low D-glucose concentration. Figure 47 shows the time course for uptake of 0.5mM D-glucose. The graph shows that at two seconds, the rate is initial. Therefore, at this, and higher concentrations of D-glucose, a two second time interval would be appropriate for the direct measurement of an initial rate of zero *trans* entry.

3.7.2. Zero trans Entry in Erythrocyte Ghosts.

In order to calculate values for the zero *trans* entry K_m and V_{max} (K_{zt}^{oi} and V_{zt}^{oi}), the initial rates of entry of a range of D-glucose concentrations, above and below the affinity constant, were measured. Data from nine such experiments is shown in Figure 48. Values of 1.56 ± 0.23mM, and 25.5 ± 3.2mMmin^{-1} were obtained for the parameters K_{zt}^{oi} and V_{zt}^{oi}, respectively.

3.7.3. Zero trans Exit in Erythrocyte Ghosts.

To measure the zero *trans* exit parameters, a time course for exit at 40mM D-glucose was measured from five seconds to forty seconds (Figure 49). The zero *trans* exit K_m (K_{zt}^{io}) was obtained using Equation 4. The integrated rate replot following from this analysis is shown in Figure 50; from this figure, $K_{zt}^{io} = 28.4 \pm 10.4$mM, and $V_{zt}^{io} = 262 \pm 59$mMmin^{-1}.

3.7.4. Infinite cis Entry in Erythrocyte Ghosts.

The infinite *cis* parameters, K_{ic}^{oi} and V_{ic}^{oi} were evaluated by following a time course at 40mM D-glucose (a concentration which was high relative to the affinity constant). The time course shown in Figure 51 was analysed with the integrated rate equation (Section 3.2.3). The data from the time course in Figure 51 was replotted in the form t/c vs. $-[\ln(1-C/S_o) + C/S_o]/C$ in Figure 52. This analysis gave K_{ic}^{oi} as 14.5 ± 2.2mM.

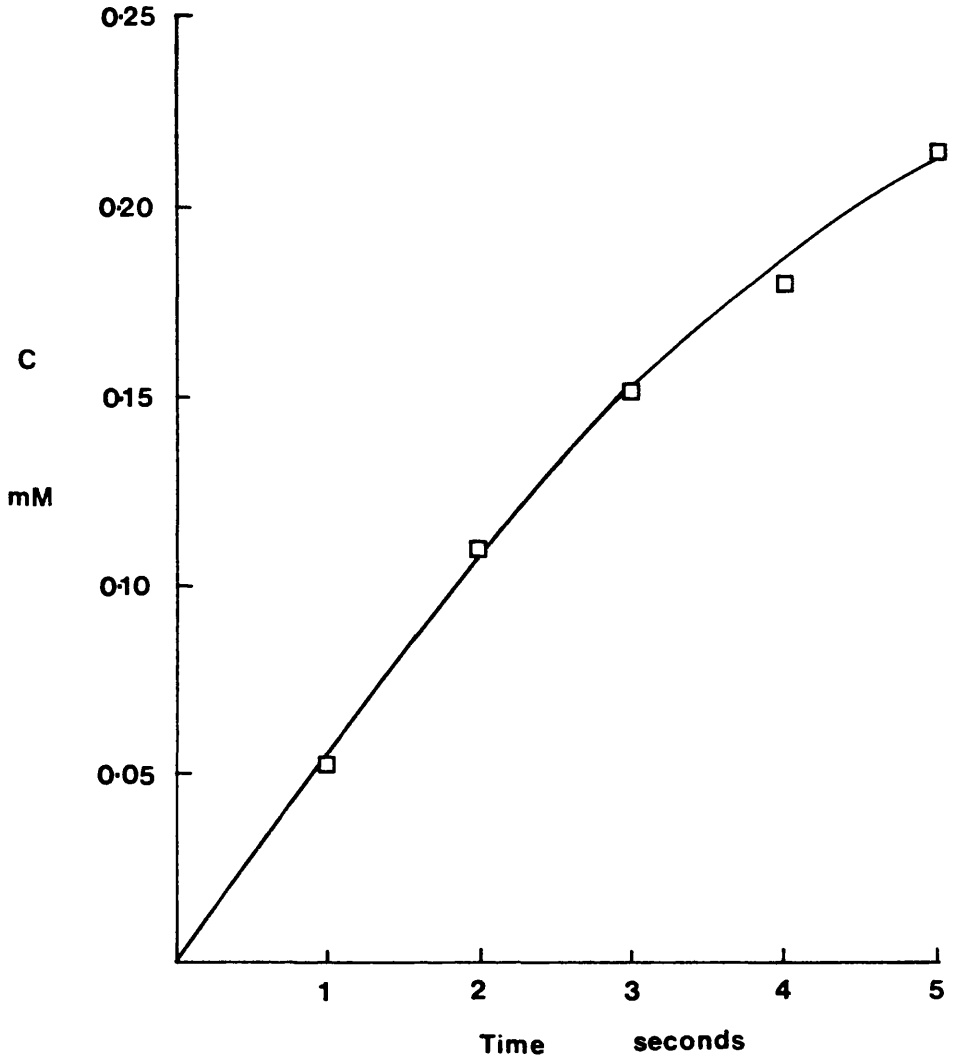


FIGURE 47

Entry of D-Glucose in Erythrocyte Ghosts.

Fig. 47: A time course for zero trans entry of 0.5mM D-glucose into erythrocyte ghosts at 20°C. Values are from a single experiment, where the number of observations was 19.

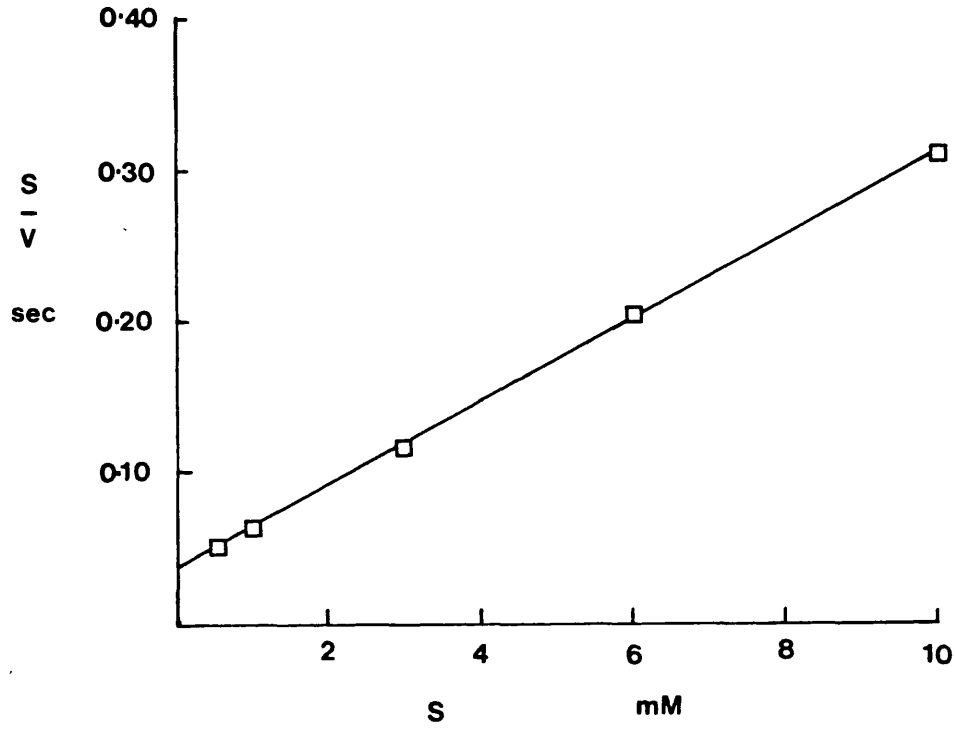


FIGURE 48

Zero trans Entry Kinetics in Erythrocyte Ghosts.

Fig. 48: The zero trans entry of D-glucose into erythrocyte ghosts at 20°C. Data is from nine experiments in which values were determined in duplicate. K_{zt}^{oi} = 1.56 ± 0.23mM, V_{zt}^{oi} = 25.5 ± 3.2mMmin^{-1}.

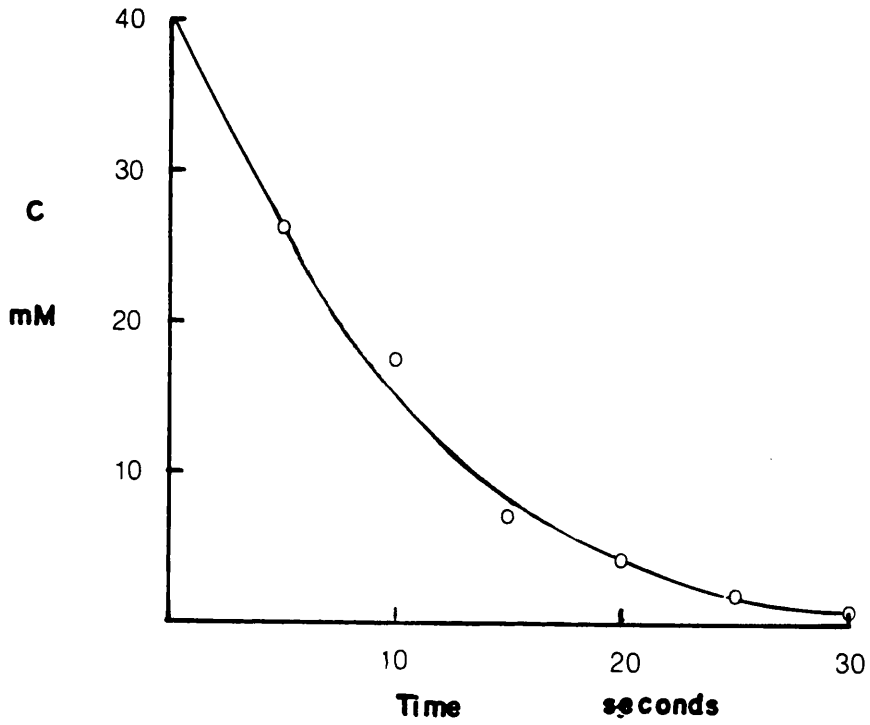


FIGURE 49

A Time Course for D-Glucose Exit from Red Blood Cell Ghosts.

Fig. 49: A time course for the efflux of 40mM D-glucose from erythrocyte ghosts at 20°C. Data was from 3 experiments where efflux was measured in duplicate at each time point.

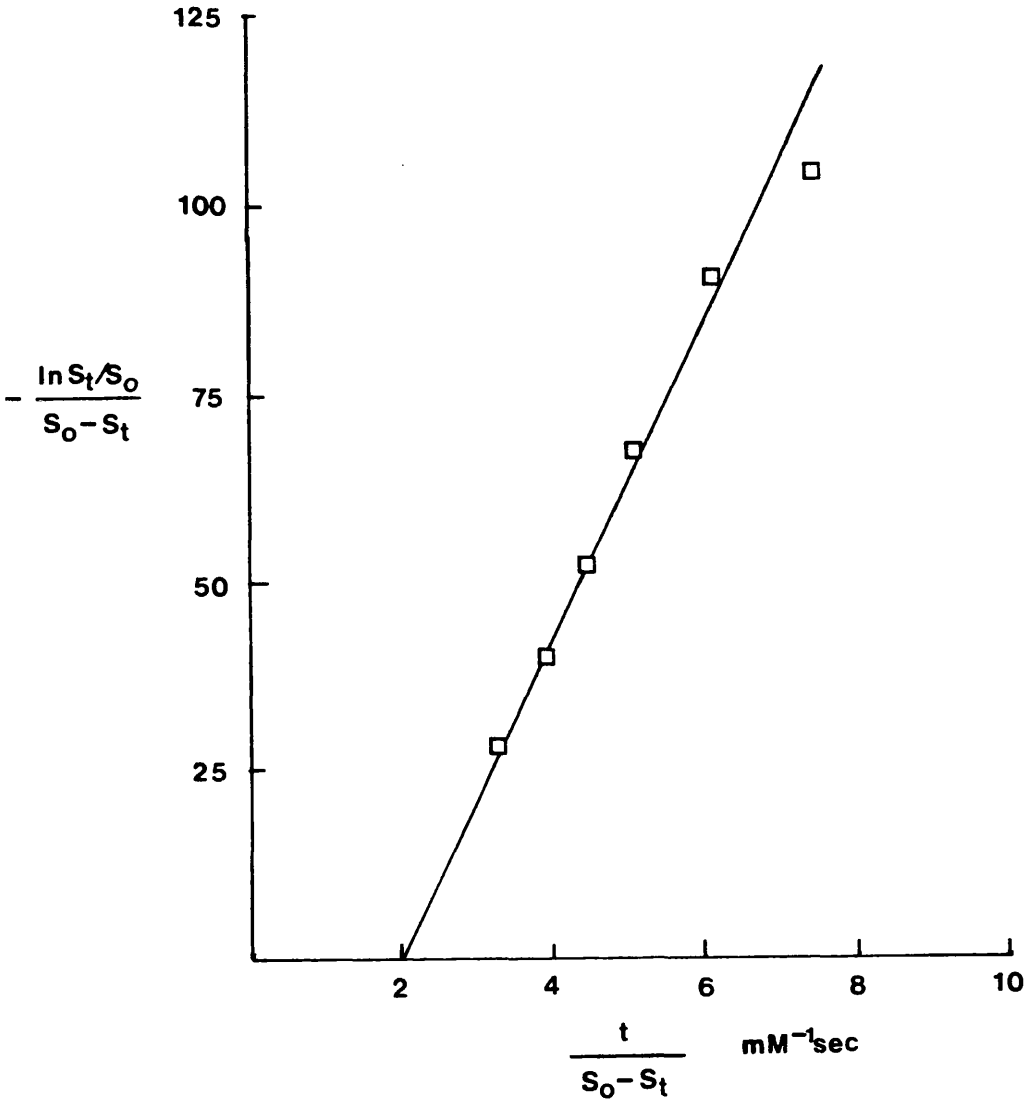


FIGURE 50

Zero trans Exit from Erythrocyte Ghosts: An Integrated Rate Analysis.

Fig. 50: An integrated rate equation replot of the zero trans exit from erythrocyte ghosts shown in the previous figuure (49). K^{io}_{zt} = 28.4 ± 10.4mM, V^{io}_{zt} = 262 ± 59mMmin^{-1}.

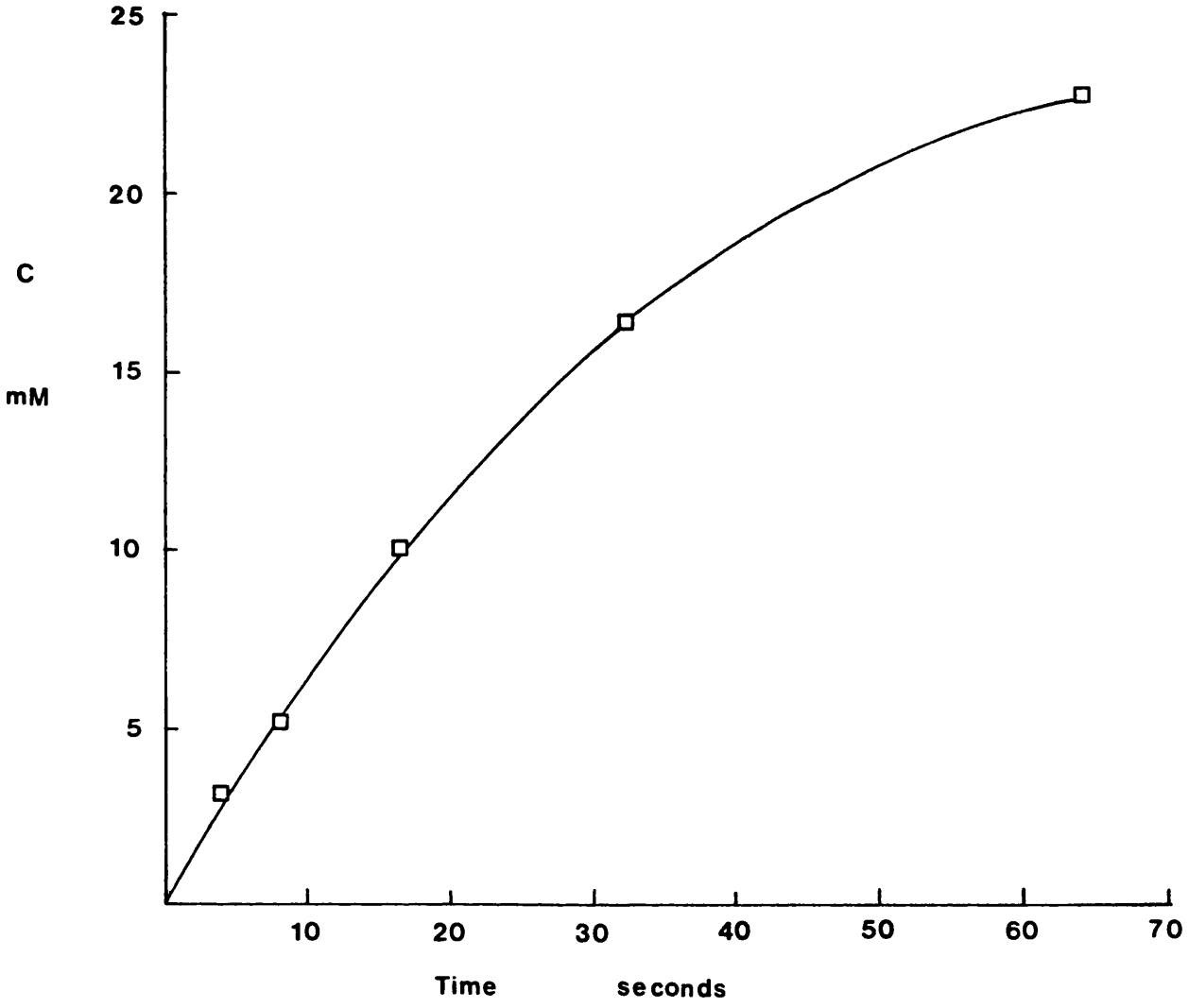


FIGURE 51

Infinite cis Entry into Red Blood Cell Ghosts.

Fig. 51: A time course for net entry of 40mM D-glucose by erythrocyte ghosts at 20°C. Data points are means from five experiments, where transport was measured in duplicate.

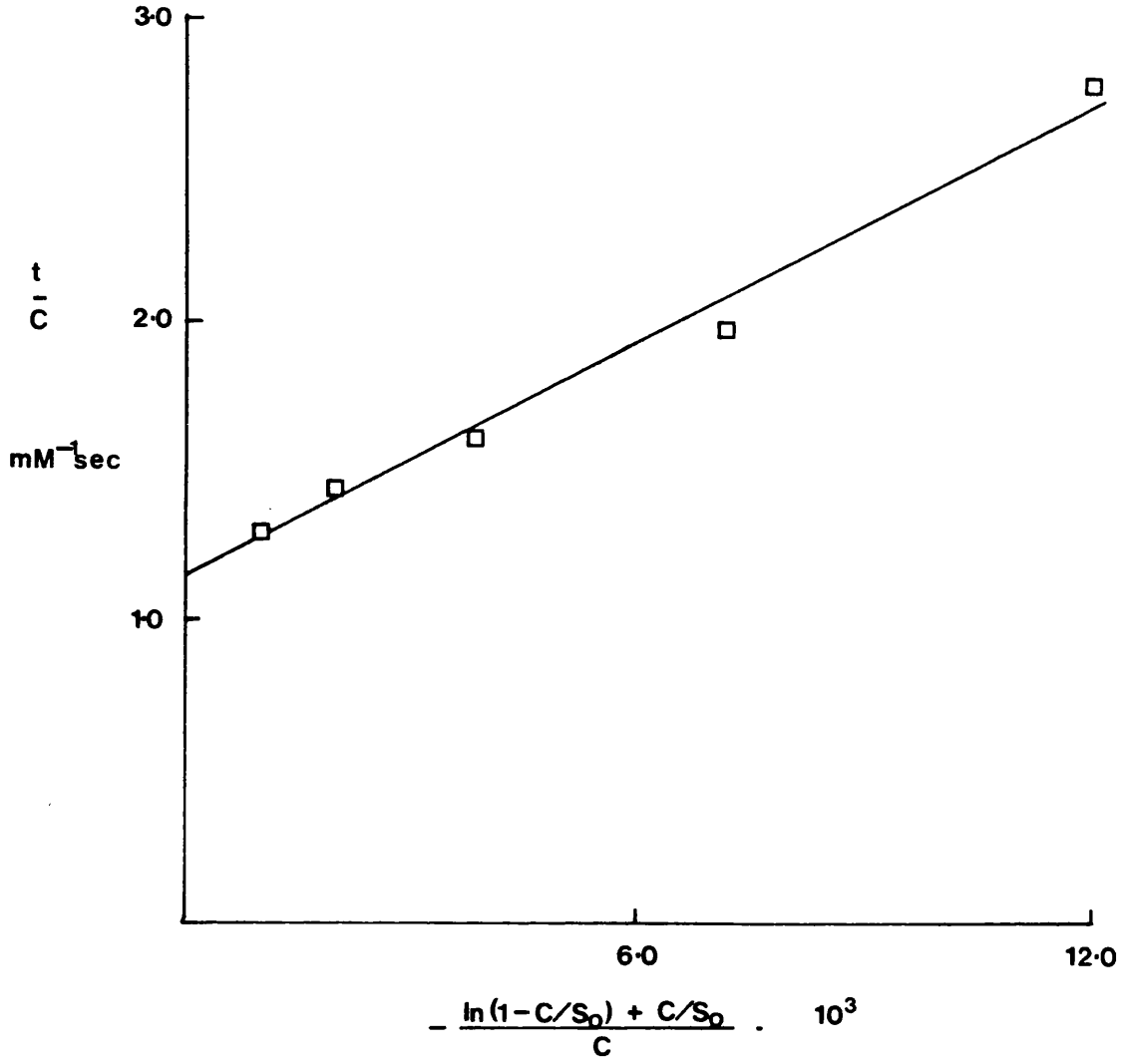


FIGURE 52

Infinite cis Analysis of D-Glucose Transport in Erythrocyte Ghosts.

Fig. 52: An integrated rate equation replot of the infinite cis data shown in Figure 51. K_{ic}^{oi} = 14.5 ± 2.2mM.

3.7.5. The Integrity of the Pink Ghost.

The integrity of the resealed ghosts relative to that of whole cells was assessed by analysis of the integrated rate replot of the infinite *cis* data. The "permeability" (K/V, on the ordinate) was approximately the same in pink ghosts as in whole cells (Challis *et al.*, 1980), showing that the leakage of D-glucose in both systems was similar; increased leakage in ghosts would be apparent in a lower K/V value.

In order to confirm the absence of any leakage significant enough to affect the K_m, inhibition of the uptake of 40mM D-glucose by cytochalasin B was measured. Cytochalasin B was shown to inhibit the uptake by 99%, thus indicating that an insignificant amount of sugar taken up could be accounted for by leakage.

3.8. Selective Effectors of Sugar Transport.

The asymmetric nature of the D-glucose transport kinetics illustrated in previous sections was studied further by analysis of the effect of 3-isobutyl-1-methylxanthine (IBMX) on the transport. A study of potential inhibitors of transport can provide useful information about the symmetry of the transport system. Ghost cells are ideally suited to this type of study since it is possible to selectively treat one side of the membrane only with a particular compound, because a compound to which the cell is usually impermeable may be placed within the membrane sac during the resealing process. The effect on the transport kinetics may be studied both before and after subsequent washing. Washing frees the outside surface of the compound. The effect on both internal and external kinetic parameters may then be determined. In whole cells, similar data can be obtained by using compounds to which the cell has a low permeability: short incubations can

ascertain effects on the outside of the membrane, and longer incubations, the effect from the inside.

3.8.1. The Effect of 3-isobutyl-1-methylxanthine on Zero trans Entry in Erythrocyte Ghosts.

The effect of 0.5mM IBMX on both sides of the ghost membrane on the zero *trans* entry of a range of D-glucose concentrations was studied in two experiments, at 20°C. Figure 53 shows that IBMX did not alter the basal level of transport.

3.8.2. The Effect of 3-isobutyl-1-methylxanthine on Zero trans Exit in Erythrocyte Ghosts.

Possible changes caused by 0.5mM IBMX on the zero *trans* exit of D-glucose from pink ghosts were initially studied by measuring a time course for the exit of D-glucose from ghosts preloaded with 40mM D-glucose (Figure 54). These experiments showed that 0.5mM IBMX significantly inhibited D-glucose exit when compared to basal values over the same time course (4 to 40 seconds). However, in order to allow the time course to fall below the inhibited K_{zt}^{io}, ghosts were preloaded with 10mM D-glucose. The basal time course at this concentration was consistent with the data obtained with a 40mM D-glucose loading. The time course in the presence of 0.5mM IBMX enabled analysis using the integrated rate replot (Figure 55) to be made. This indicated that the inhibition was uncompetitive with K_{zt}^{io} approximately 5mM and V_{zt}^{io} approximately 60mMmin^{-1}. K/V was similar to the basal value, indicating that the ghosts remained intact when treated with IBMX.

The conclusion may be drawn that IBMX substantially decreases the kinetic asymmetry of pink ghosts by reducing both of these zero *trans* exit parameters.

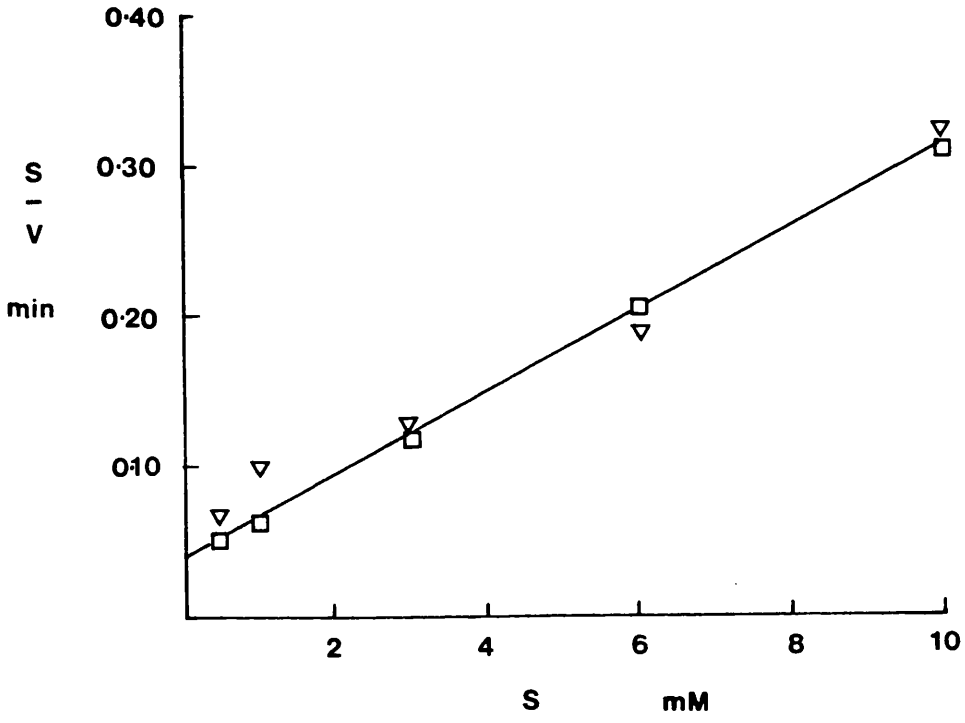


FIGURE 53

Zero trans Entry Kinetics for Isobutylmethylxanthine-Treated Erythrocyte Ghosts.

Fig. 53: Zero trans entry of a range of D-glucose concentrations into (▽) IBMX-treated and basal (□) red blood cell ghosts, at 20°C. Points represent data from nine experiments for basal ghosts where transport was measured in duplicate. The line represents basal transport (see Fig. 48). For IBMX-treated ghosts, data was from 2 experiments, where transport was measured in duplicate.

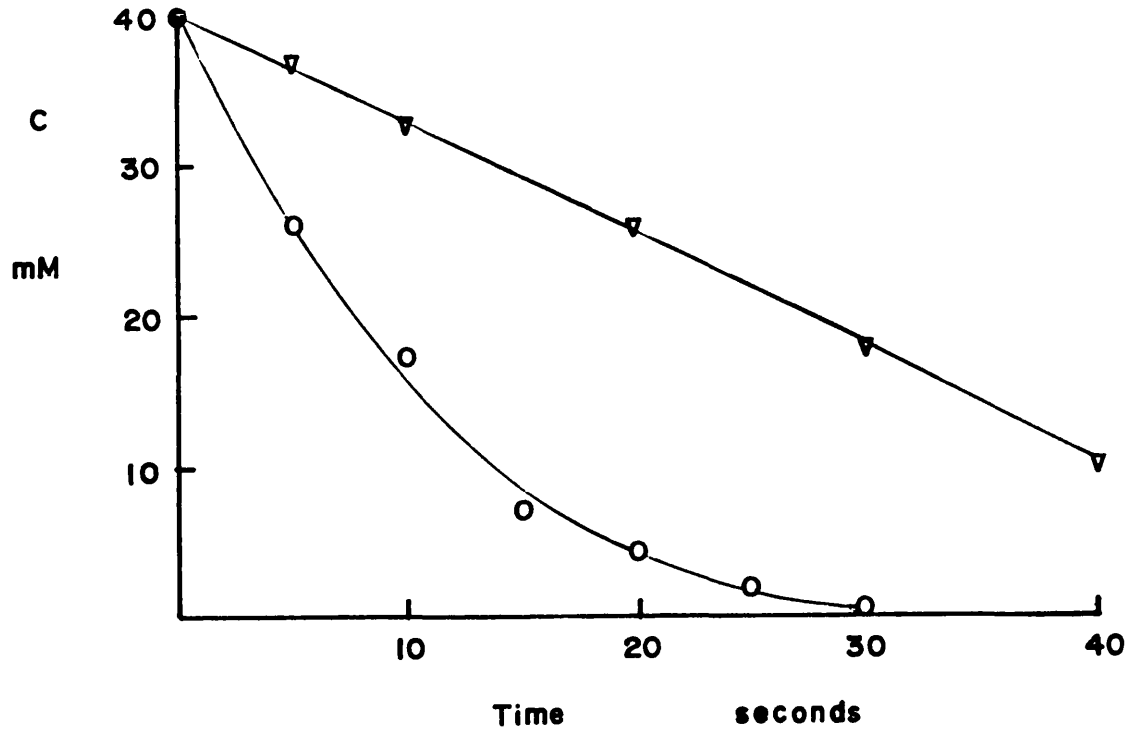


FIGURE 54

Zero trans Exit Kinetics for IBMX-treated Red Blood Cell Ghosts.

Fig. 54: Two time courses for zero trans exit of D-glucose at 20°C are shown. The first (O) represents basal efflux (see Fig. 49), from 3 experiments where values measured in duplicate. The second curve (▽) shows the effect of 0.5mM IBMX on the efflux, when IBMX was present both inside and outside the ghost. Data is from 2 experiments where values were measured in duplicate.

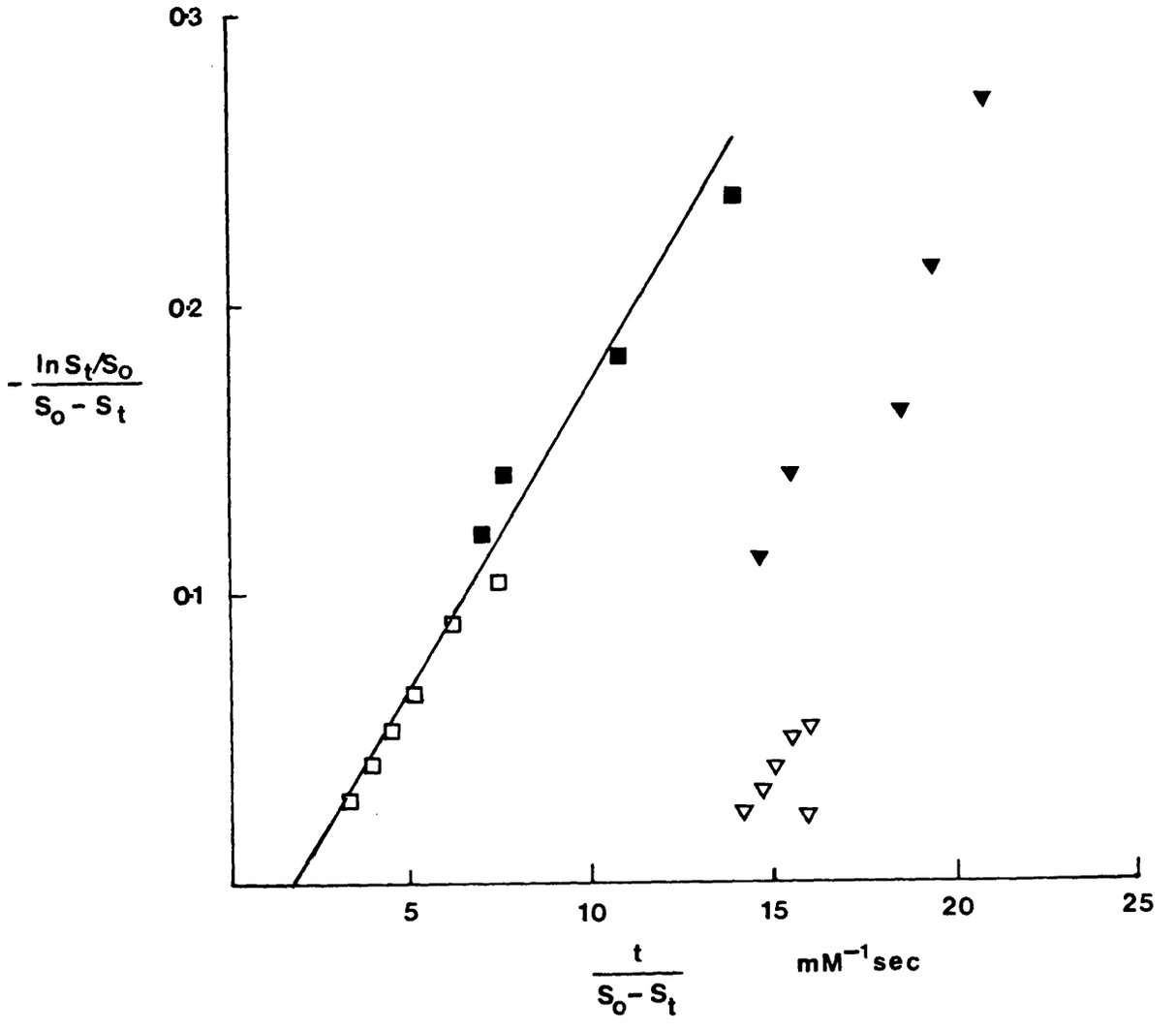


FIGURE 55

An Analysis of the Effect of IBMX on Zero trans Exit.

Fig. 55: An integrated rate equation replot of the data in Fig. 54. (▽) and (□), combined with values for exit at 10mM D-glucose, (▼) and (■), for +IBMX and -IBMX respectively.

3.8.3. The Effect of Cyclic AMP on Zero trans Exit in Erythocyte Ghosts.

To investigate the route by which IBMX can inhibit D-glucose exit but not entry in red blood cell ghosts, the effect of 10mM cyclic AMP on the exit of 40mM D-glucose was assessed over a time course of five to twenty-five seconds. It is generally assumed that there is neither adenyl cyclase (Rodan et al., 1976) nor phosphodiesterase activity within erythrocytes (Smoake et al., 1979). Thus, although IBMX has been shown to inhibit cyclic AMP breakdown, its effect on the D-glucose transport system could be unspecific and unrelated to phsophodiesterase inhibition. However, as shown in Figure 56, 10mM cyclic AMP appears to have no effect on the D-glucose exit, within the limits of experimental error (where the bar represents the single value nearest to the mean basal exit).

3.8.4. The Effect of Xanthines on Sugar Transport in Adipocytes.

Some of the class of compounds known as "Xanthines" have been shown to effect changes in the metabolic state of adipocytes (Taylor & Halperrin, 1979). By incubation with these compounds for at least thirty minutes, it was possible to show which of them had a significant effect on the adipocyte preparation.

Three questions were addressed in these studies: firstly, whether the xanthine inhibited basal transport; secondly whether the insulin-stimulated transport diminished; and thirdly whether the addition of the compound before the insulin treatment prevented the elevation of transport rates usually mediated by insulin.

The uptake of 1mM 3-O-methyl-D-glucose at 37°C at one second was used to assay effects on the insulin stimulated uptake and at fifteen seconds to measure effects on the basal level of transport. The adipocytes were incubated with the xanthine both before and after insulin treatment. Table

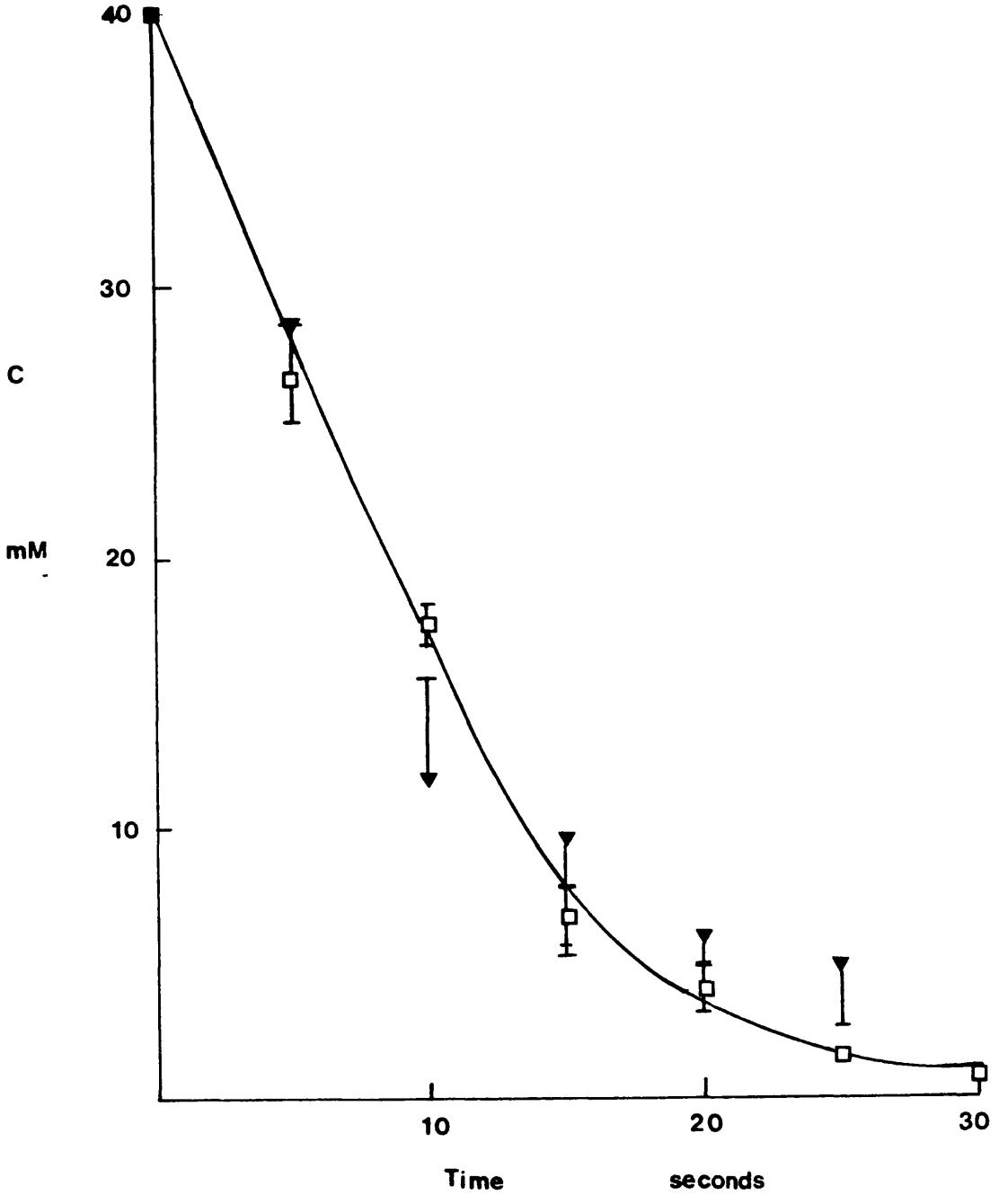


FIGURE 56

Zero trans Exit Kinetics for cAMP-treated Erythrocyte Ghosts.

Fig. 56: The effect of 10mM cAMP on the zero trans exit of 40mM D-glucose from red blood cell ghosts at 20°C. The basal data (□) represents three experiments (Fig. 49), the cAMP-treated ghost data is from a single experiment where values were measured in duplicate(▼).

18 shows that additon to insulin-stimulated cells of theophylline and possibly caffeine prevented the expected increase in the uptake on addition of insulin, but that xanthine had no significant effect. If the compounds were added before insulin stimulation, theophylline and caffeine significantly decreased the uptake to a small extent and again, xanthine had no effect. A further experiment (Table 19) showed that isobutylmethylxanthine behaved in a similar manner to theophylline, but that caffeine apparently had no significant effect when added to insulin-stimulated cells, although there was a slight (but statistically insignificant) decrease when it was added prior to insulin treatment. This second experiment also showed that hypoxanthine had no effect on the insulin-stimulated uptake. Only IBMX or caffeine appeared to significantly decrease the basal uptake, IBMX by 85%, caffeine by 78%.

The qualitative experiments described above show that IBMX causes the largest decrease in the uptake shown by both insulin-stimulated and basal adipocytes. It has been shown (Section 3.7.1 amd 3.7.2) that IBMX inhibits the D-glucose transport in human erythrocyte ghosts, and whole cells (Challis et al., 1980). Although phosphodiesterase inhibition may complicate the measurement of possibly similar effects in adipocytes, the direct effect on transport was studied by a very rapid treatment with IBMX, when contributions from secondary changes mediated via the intracellular phosphodiesterase may perhaps be minimized; IBMX was added only in the sugar uptake solutions.

Figure 57 (a) shows the effect of 0.75mM IBMX on basal adipocytes. There is a very significant decrease in the uptake in the presence of IBMX. Figure 57 (b) shows that a decrease in transport is also observed when IBMX is added to the insulin-stimulated cell suspensions.

TABLE 18

The Effect of Xanthine, Theophylline and Caffeine on Adipocytes.

Condition	*Incubation Time	**Addition	#Uptake in $mMsec^{-1}$
A	0-15	10nM insulin	0.134
B	0-60	None	0.0046
C	0-15 15-45	10nM insulin 0.25mM xanthine	0.112
D	0-15 15-30	0.25mM xanthine 10nM insulin	0.111
E	0-15 15-45	10nM insulin 0.25mM theophylline	0.0893
F	0-15 15-30	0.25mM theophylline 10nM insulin	0.0696
G	0-15 15-30	10nM insulin 0.25mM caffeine	0.0904
H	0-15 15-45	0.25mM caffeine 10nM insulin	0.0734

* Measured in minutes.

** see Table 14.

see Table 14.

TABLE 19

The Effect of IBMX, Hypoxanthine, and Caffeine on Adipocytes.

Condition	*Incubation time	**Addition	#Uptake in $mMsec^{-1}$
A	0-180	None	0.00935
B	0-30	10nM insulin	0.07006
C	0-15 15-75	10nM insulin 0.25mM IBMX	0.0521
D	0-15 15-30	0.25mM IBMX 10nM insulin	0.0254
E	0-30	0.25mM IBMX	0.00143
F	0-15 15-75	10nM insulin 0.25mM caffeine	0.0885
G	0-15 15-30	0.25mM caffeine 10nM insulin	0.0569
H	0-30	0.25mM caffeine	0.00193
J	0-30	10mM insulin 25mM NaOH	0.107
K	0-15 15-75	10nM insulin 0.25mM hypoxanthine in 25mM NaOH	0.103
L	0-15 15-30	0.25mM hypoxanthine in 25mM NaOH 10nM insulin	0.0887
M	0-30	0.25mM hypoxanthine in 25mM NaOH	0.00454

*, **, #, see Table 14.

FIGURE 57

The Effect of Insulin on the IBMX Inhibition in Adipocytes.

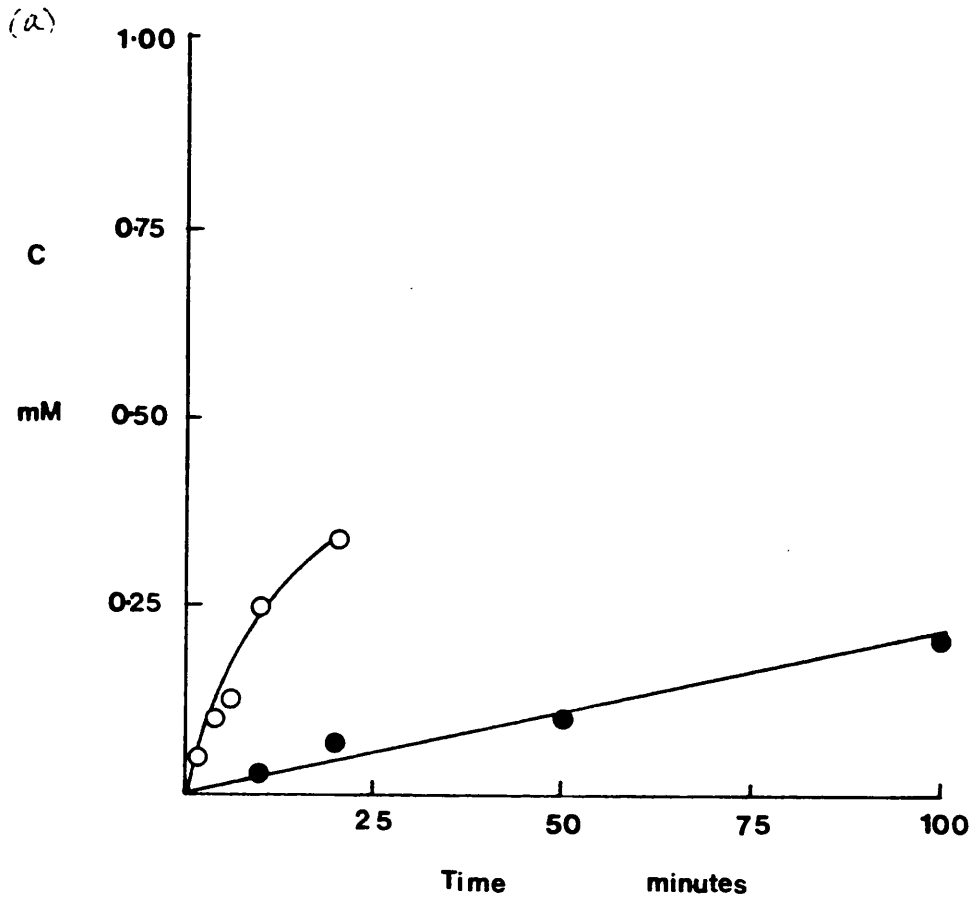
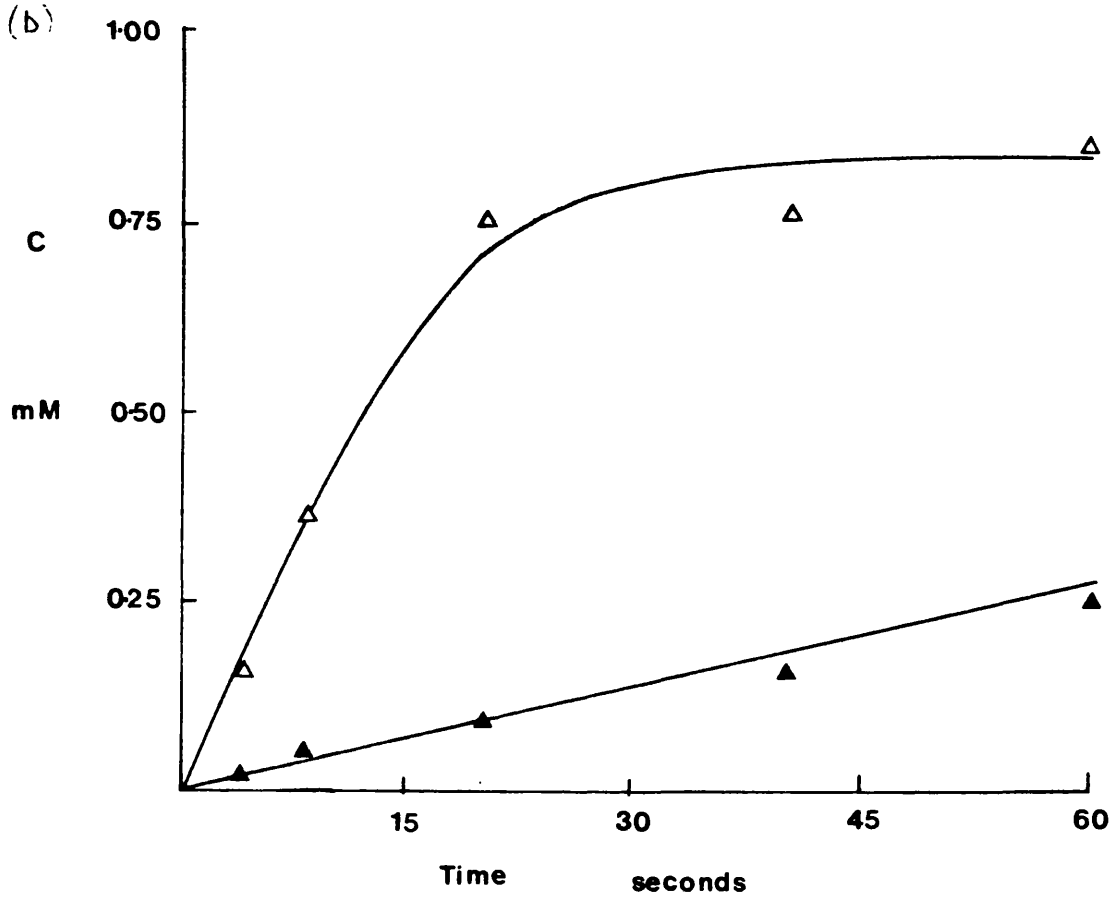


Fig. 57 (a): Basal cells. A time course for the zero trans uptake of approximately 0.9mM 3-O-methyl-D-glucose into adipocytes in the presence (●) and absence (o) of 0.75mM IBMX, at 37°C. Each point was the mean of two observations from one experiment.



(b): Insulin-stimulated cells. A time course for the uptake of approximately 0.9mM 3-O-methyl-D-glucose in the presence (▲) and absence (Δ) of 0.75mM IBMX, at 37°C. Each point represents means of two measured values.

3.8.5. The Kinetics of 3-isobutyl-1-methylxanthine Inhibition in Basal and Insulin-Treated Adipocytes.

More detailed kinetics of the IBMX inhibition were then assessed. The zero *trans* entry of a range of 3-O-methyl-D-glucose concentrations was measured at one second (Figure 58). For insulin-stimulated adipocytes in the presence of IBMX, $K_{zt}^{oi} = 10.82 \pm 1.62$ mM, $V_{zt}^{oi} = 0.866 \pm 0.089 mMsec^{-1}$. For the control (ie. insulin-stimulated) state, as measured on the same three cell preparations as the IBMX inhibited preparations, the $K_{zt}^{oi} = 3.95 \pm 0.72$ mM, $V_{zt}^{oi} = 1.002 \pm 0.191$ $mMsec^{-1}$. A comparison of these values shows that the IBMX inhibition is competitive.

The equilibrium exchange parameters were also measured in the presence and absence of IBMX for insulin-stimulated adipocytes (Figure 59). The IBMX-inhibited parameters are $K_{ee}^{oi} = 12.13 \pm 0.66$ mM, $V_{ee}^{oi} = 0.543 \pm 0.021 mMsec^{-1}$, those for insulin alone are $K_{ee}^{oi} = 3.47 \pm 0.50$ mM, $V_{ee}^{oi} = 0.61 \pm 0.042 mMsec^{-1}$. The type of inhibition is again seen to be competitive. In these experiments, rather low values for the insulin-stimulated (control) transport reflects prolonged insulin storage (see above).

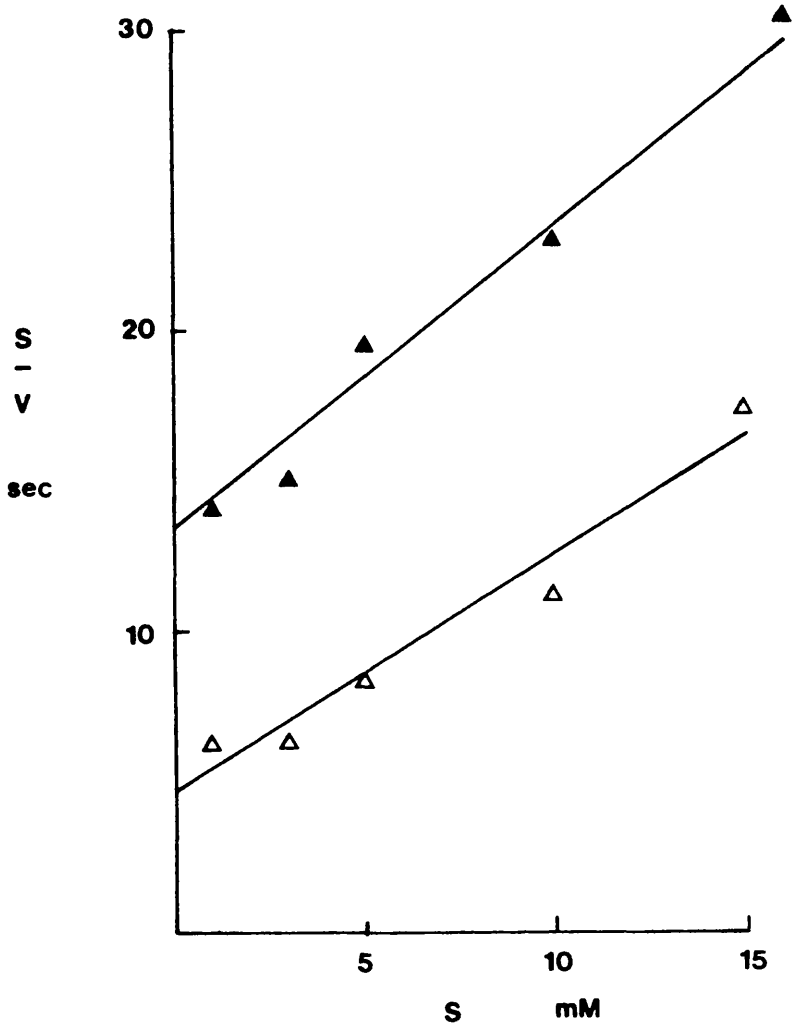


FIGURE 58

The Effect of IBMX on Zero trans Uptake in Insulin-treated Adipocytes.

Fig. 58: The effect of 0.25mM IBMX on the zero trans entry of 3-O-methyl-D-glucose in insulin-stimulated adipocytes, at 37°C. (Δ) represents uptake without IBMX, thirty observations from three experiments, K_{zt}^{oi} = 3.95 ± 0.72 mM, V_{zt}^{oi} = 1.002 ± 0.191 $mMsec^{-1}$. (▲) represents uptake with IBMX, sixty-three observations, three experiments, K_{zt}^{oi} = 10.82 ± 1.62 mM, V_{zt}^{oi} = 0.866 ± 0.089$mMsec^{-1}$.

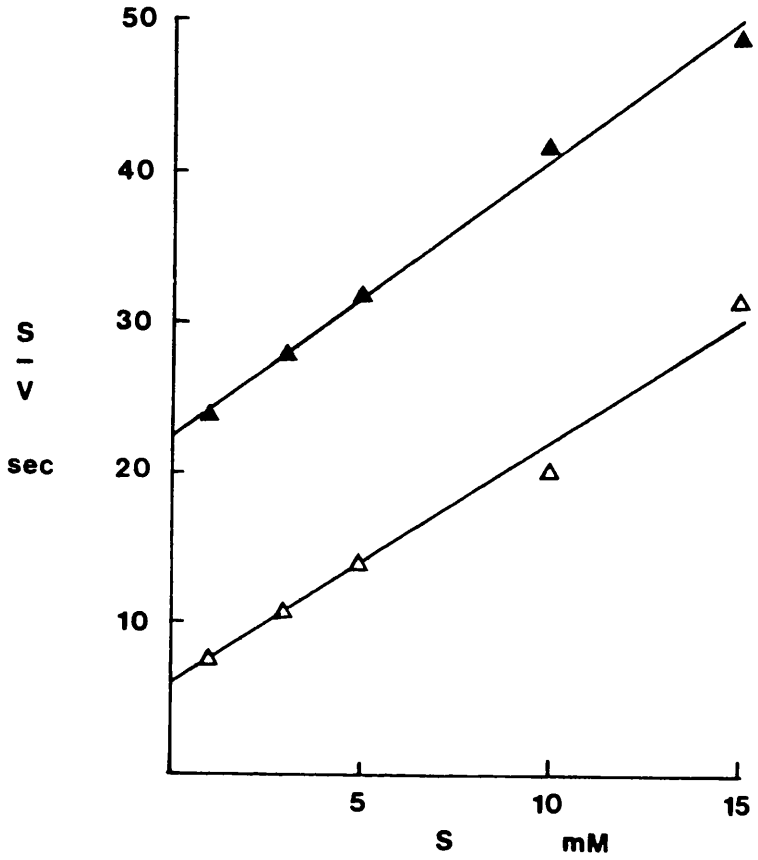


FIGURE 59

The Effect of IBMX on Exchange in Insulin-treated Adipocytes.

Fig. 59: The effect of 0.25M IBMX on the entry, under equilibrium exchange conditions, of 3-O-methyl-D-glucose into insulin-stimulated adipocytes at 37°C. (△) represents uptake in the absence of IBMX. The number of observations (n) per point was 4, except at 15mM, where n = 3 (one value gave <2% filling and was therefore rejected), K_{ee}^{oi} = 3.47 ± 0.50 mM, V_{ee}^{oi} = 0.61 ± 0.042 $mMsec^{-1}$. (▲) represents uptake with IBMX present, n = 38 from two experiments, where K_{ee}^{oi} = 12.13 ± 0.66 mM, V_{ee}^{oi} = 0.543 ± 0.021 $mMsec^{-1}$.

Chapter 4: Discussion.

4.1. The Transport Kinetics of Erythrocyte Ghosts.

When studying transport systems, one way of usefully perturbing and probing the system is to remove intracellular components, and to observe whether any kinetic changes have occurred. For red blood cells, removal of 95% of the haemoglobin by a single lysis step (producing pink ghosts) was undertaken. Residual haemoglobin was not removed by extensive washing because ghosts prepared in this manner appeared fragile and, as Jung et al. (1971) have pointed out, extensively purified membrane ghosts (with removal of > 99% of the haemoglobin) are much more leaky than intact erythrocytes, although they retain the glucose transporter with a comparable (high) K_m for exchange to that of whole cells. It was demonstrated that pink ghosts had no significant leakage pathway, by effective cytochalasin B inhibition of glucose entry.

Glucose transport has been studied in extensively purified human erythrocyte ghosts (white ghosts) in spite of their fragility. Whereas in whole cells the transport system appears to be asymmetric, the early reports suggest that white ghost transport might be symmetric. Taverna & Langdon (1973c), comparing uptake in ghosts and inverted membrane vesicles by estimating the activity of internally placed glucose oxidase, concluded that transport was symmetric. Benes et al. (1972) studied a sugar with a low affinity for the glucose transporter; by inhibiting its transport with phloretin from either side of the membrane, they reached the same conclusion. The asymmetry of whole cell transport has apparently been abolished by an almost complete removal of the haemoglobin from the erythrocytes to form white ghosts. By studying transport in pink ghosts, an intermediate stage in the purification, the role of tightly bound and total

haemoglobin in the kinetic asymmetry of whole cells has been investigated.

Removal of the bulk of the haemoglobin is a way of testing one of the recently proposed models for human red blood cell sugar transport. This is the gating pore model with internal sugar-haemoglobin complexes contributing to the transport (Section 1.7.9; Holman & Naftalin, 1975; Baker & Naftalin, 1979). In this model, the gating pore itself is symmetrical but the operational asymmetry observed in whole cells is due to an overall rate limitation imposed by the association of the entering sugar with the haemoglobin. If the haemoglobin is removed, this limitation would be lifted, and symmetric sugar transport kinetics or kinetics showing less asymmetry should be observed, to be consistent with this model.

Zero *trans* kinetics in human erythrocytes were therefore examined. Preliminary studies showed that an appropriate time interval was used to determine initial rates of entry; integrated rate equations were used to analyse exit curves.

TABLE 20

Experimental Design	Parameter	Units	Ghosts	[a]Whole Cells
Zero *trans* entry	K_{zt}^{oi}	mM	1.56±0.23	1.6±0.24
	V_{zt}^{oi}	$mMmin^{-1}$	25.5±3.2	30.8±2.2
Zero *trans* exit	K_{zt}^{io}	mM	28.4±10.4	22.9±6.3
	V_{zt}^{io}	$mMmin^{-1}$	262±59	218±33.5

Table 20: The zero *trans* parameters for D-glucose transport in erythrocyte ghosts and intact cells. [a]Taken from Challis *et al*. (1980) and Chapter 3 of this work.

Table 20 shows the zero *trans* entry and exit parameters obtained for pink ghosts at 20°C, compared to values obtained for whole cells in the same laboratory (Chapter 3 and Challis *et al.*, 1980). There is no significant change between these parameters in ghosts and in whole cells. Comparisons with previous work on intact erythrocytes (Karlish *et al.* 1972) show that the values for K_{zt}^{oi} are similar in cells and the pink ghosts, although their value for V_{zt}^{io} for the whole cells (129 $mMmin^{-1}$) was lower than values obtained for either the ghosts or the whole cells in the present investigation. Baker & Naftalin (1979) have not obtained this lower value either.

The infinite *cis* entry experiment was assessed. The infinite *cis* entry K_m was determined as 14.5 ± 2.2mM in the pink ghosts. This estimate of the internal transport site affinity is different from the internal K_m determined in the zero *trans* exit experiment (K_{zt}^{io}); the pink ghost transport system is therefore not symmetric.

Thus these kinetic measurements lead to the conclusion that the transport in whole cells is not adequately described by the gating pore model, where the operational asymmetry arises from association of the bulk of the haemoglobin with the transported sugar. Removal of the residual haemoglobin or the loss or structural alteration of other possibly regulatory components of the system could account for the loss of asymmetry in white ghosts. It is also possible that the fact that the white ghosts were so leaky would give rise to apparently symmetric kinetics. Further experiments would be necessary to distinguish between these possiblities.

In the Introduction (Section 1.7) a number of models were outlined, with modifications to explain observations made on intact erythrocytes. It is immediately obvious that the symmetric carrier model cannot account for the pink

ghost data since the zero *trans* affinity constants and the maximum rate constant for the internal and external sites differ by ten-fold: the transport is asymmetric. In fact, the zero *trans* parameters would strongly suggest that thes ghosts are kinetically identical to whole cells, and that the discussion of models in Section 1.7 would apply equally to pink ghosts as to the intact erythrocyte. However, the infinite *cis* K_m is apparently higher in ghosts than in whole cells: a value of 2mM was determined in a similar manner to the ghost value, for whole cells (Holman, G.D., unpublished) from data reported by Hankin *et al*. (1972). The K_{ic}^{oi} value for the ghosts was still significantly lower than that expected in several asymmetric models.

One such model, the "easy-off" mobile carrier hypothesis, may be rejected because it does not explain differences between infinite *cis* and zero *trans* affinity constants (Section 1.7.2). The asymmetric carrier model proposed by Geck (Section 1.7.3) and the unstirred layer modifications (Section 1.7.4) also would not explain why the infinite *cis* K_m is lower than the zero *trans* exit K_m, since the single internal site was predicted to be of low affinity in both cases.

Other models are based not on a rotating transport carrier, but on movement through a protein-bounded, hydrophilic pore. In the tetramer model (Section 1.7.5), where four protein subunits are postulated, the low infinite *cis* entry K_m fits the model, but although the high zero *trans* exit K_m and low infinite *cis* entry K_m adequately describe the two sites of low and high affinity on the inside, the zero *trans* entry K_m describes only high affinity external sites, and the proposed low affinity sites are not apparent. On the basis of the low infinite *cis* K_m obtained for erythrocyte ghosts, the modified tetramer model, having two asymmetric antiparallel subunits, can also be rejected. None of the tetramer, lattice membrane pore or introverting hemiport models can explain the observed

differnces in V_{max} values for zero *trans* entry and exit, because either only one class of binding site is postulated or the low and high affinity sites are arranged symmetrically. The allosteric pore model can account for the asymmetric net flux and symmetric infinite *cis* transport in ghosts, as well as in the intact cells.

In conclusion, the removal of the cellular contents to form pink ghosts does not lead to substantial changes in the zero *trans* entry or exit parameters; the infinite *cis* K_m may be higher than it is in intact erythrocytes, but not as high as the zero *trans* exit K_m. Several models for ghost glucose transport may be rejected, in particular the gated pore model with sugar-haemoglobin complexes causing asymmetry; other models which are inappropriate for whole cells do not accord with the pink ghost data.

4.2. 3-O-Methyl-D-glucose Transport in Basal Adipocytes.

4.2.1. The Kinetic Constants.

In this research, the use of direct initial rate measurements, and integrated rate analyses enabled the kinetic constants for 3-O-methyl-D-glucose transport in unstimulated adipocytes, at a physiological temperature, to be evaluated. The constants for all the experiments described as critical for testing models for sugar transport were determined (see Chapter 1). The kinetic parameters for adipocytes were of about the same order of magnitude as those for human erythrocytes, although the actual values were different.

For basal adipocytes, the affinity constants for entry were 5.41 $\pm$ 0.98mM and 4.54 $\pm$ 1.32mM for the zero *trans* entry and infinite *cis* exit experiments, respectively. Those for exit were 4.09 $\pm$ 1.05mM and 9.03 $\pm$ 3.28mM for zero *trans* exit and infinite *cis* entry respectively. The affinity constant under equilibrium exchange conditions was 4.22 $\pm$ 1.24mM. The maximum rates for entry were 0.034 $\pm$ 0.014mmoles/l cell water/s and

0.066 ± 0.013mmoles/l cell water/s for the zero *trans* entry and infinite *cis* entry experiments respectively, whereas for exit, values of 0.0153 ± 0.023mmoles/l cell water/s and 0.106 ± 0.026mmoles/l cell water/s were obtained from the zero *trans* exit and infinite *cis* exit experiments respectively. The maximum rate under equilibrium exchange conditions was 0.058 ± .0001mmoles/cell water/s.

For the affinity constants, values for the entry measured directly (zero *trans* entry) and confirmed by indirect methods (infinite *cis* exit) were identical within the limits of the errors. The zero *trans* exit affinity constant would appear to be similar to these, as was the infinite *cis* entry value. The exchange experiment shows a slightly higher affinity constant, but problems were encountered in this measurement. In the exchange experiments, the scatter of the data points was poor for basal cells. The variations probably arose from reduced cell viability with incubation times several fold longer than were used in experiments with insulin-treated cells. Thus, for basal cells, the least reliable estimates of the parameters were the exchange measurements, and to a lesser extent, the zero *trans* exit design, where long incubations were also necessary prior to transport sampling.

The same problem of viability with different experimental designs might also account for the higher values of the maximum rate parameters for exit relative to the entry determinations. If the exit V_{max} (V^{io}_{zt} ; V^{io}_{ic}) was significantly greater than that for entry (V^{oi}_{zt}; V^{oi}_{ic}) it is surprising that the exchange rate parameter does not reflect the slowest "maximum rate", ie. that measured for the external site, but approximates more closely to the values for the internal site. This suggests that these small variations are not significant.

A common variable found with transport measurements in intact fat cells is

the variation found between rats of a different size (Czech, 1980) and different cell pools prepared on separate occasions. Gliemann & Vinten (1974) found a coefficient of variation of 0.6 between the lipogenesis of single fat cells from one cell pool in identical incubations. To minimize this variation, rats of a constant size were used, and more replicates than those required to provide accurate data in human red blood cell ghost studies (only duplicates) were used. Since the number of replicates used varied from day to day (up to five for some experiments) each value was assessed individually when calculating the mean values for transport rates from several experiments.

For whole cells, in order to provide enough cells for many replicates to be used, kinetics on basal cells were measured on separate cell pools to those used to measure the insulin-stimulated transport: the number of samples measured was limited by the lifetime of the isolated cells. When the insulin-responsiveness of carbon dioxide evolution was used to assess cell viability, cells survived for at least 2.5 hours after isolation. On some occasions, one cell pool was used for experiments of two different designs. The results of these experiments did not differ noticably from mean values.

In no other study have kinetic parameters for both the internal and external sites of transport in fat cells been measured and confirmed by different experimental designs, as in the present investigation. Nonetheless several groups have measured the kinetic constants for zero *trans* uptake, and equilibrium exchange experiments. It is interesting to compare the values obtained here with these others, in the context of the different transport techniques (see Chapter 1), before considering models for the mechanisms firstly of transport, and secondly of activation by insulin.

Table 21: 3-O-methyl-D-glucose Transport in Basal Adipocytes.

Experimental Design	Temperature °C	K_m mM	V_{max} mmoles/l cell water/s	Reference
Exchange	37	5	0.07 to 0.2	Vinten et al. (1976)
Exchange	22	3.5±0.4	≅0.13	Whitesell & Gliemann (1979)
Zero trans entry***	22	2.5 to 5	--	Whitesell & Gliemann (1979)
Zero trans entry	24	6.6	≅*1.3x10^{-3}	Siegel & Olefsky (1980)
Zero trans entry	37	≅1.5	**--	Czech (1976c)

*Calculated from the value of 0.4 ± 0.1nmol/min/10^5cells given in the reference. It was assumed that 1ml of a packed cell suspension contained 10^6 cells and that the intracellular 3-O-methyl-D-glucose space was 2µl per 100µl packed cells (Whitesell & Gliemann, 1979).
**The value was not calculated, but it varied by about five-times in two representative experiments.
***The affinity constant was calculated from the inhibition of 3-O-methyl-D-glucose on [^{14}C]-3-O-methyl-D-glucose uptake, over 1.5 seconds.

Table 21 shows these other reports of kinetic constants determined at a variety of temperatures, in experiments where both oil-centrifugation and filtration methods were used. Although several of the values of K_m are not statistically rigorous estimates, the affinities are all obviously of the same order as the values for both zero trans entry and exchange transport measured in the present investigation. Although estimates of the maximum rate parameter are variable, values obtained by Vinten et al. (1976) and Whitesell & Gliemann (1979) are of the same order as those obtained for exchange experiments in the present research. Only Siegel & Olefsky (1980) give a value for V_{zt}^{oi}. When this value is converted into the same units as those used by Vinten et al. (1976), Whitesell & Gliemann (1979), and the present author, it is two orders of magnitude lower than that found here.

Czech (1976c) did not give enough information for a V_{zt}^{oi} value to be estimated from Fig. 4 of this reference, but two representative experiments show a five-fold difference in apparent V_{zt}^{oi} values. In conclusion, the general observation that the affinity constant for zero <u>trans</u> entry and exchange experiments remains fairly constant with different cell pools, but that the maximum rate constant may vary, fits well with the present research (see Results).

These other results will be considered in more detail, in order to see whether they were obtained under conditions suitable for the determination of accurate kinetic constants. The first requirement for such studies is that the initial rates of uptake or efflux were measured. That initial rates were measured in the present study was confirmed by measuring time courses at low 3-O-methyl-D-glucose concentrations (relative to the K_m) and applying the integrated rate equation analysis to that data, to calculate the initial rate, before deciding upon a suitable time interval over which to measure initial rates. For basal cells, a fifteen-second interval was appropriate.

Although neither Czech (1976c) nor Olefsky (1978) (the latter using oil-centrifugation methods, without stopping solutions) demonstrated that rates were linear over the ten seconds they used, our research suggests that such a time interval would be appropriate for measuring initial rates in basal cells. Czech's technique may be criticised in that the filter separation of cells from their medium took ten to fifteen seconds and therefore the time for the end of the transport was imprecisely defined. Dilution of cells into an ice-cold buffer solution may have retarded influx, but the dilution may also have increased efflux, and therefore errors, during the filtration.

Vinten et al. (1976)'s exchange experiments differed from all the others mentioned so far in that efflux and not influx was measured and initial rates were maintained beyond the very short times noted for influx. For the transport estimates, separation of cells and medium was complete in five seconds with an oil-centrifugation method. Since a diluting buffer was not used, further efflux during separation was unlikely for this reason, but the lack of addition of an inhibitory solution (eg. phloridzin, which was shown to significantly inhibit 3-O-methyl-D-glucose equilibrium exchange by Vinten et al.) could again lead to errors in accurately determining the point at which transport ceased. To determine the exchange parameters time courses were measured and used to calculate several efflux rates from which the concentration dependence was ascertained.

Siegel & Olefsky (1980) measured initial rates of entry over four seconds with an oil-centrifugation technique in which phloretin was employed to stop transport after a defined time interval. A similar method was used by Whitesell & Gliemann (1979) who fitted exponential curves to influx data obtained at one to forty seconds, and determined initial rates from these curves. These were used to calculate several initial rates, from which the concentration dependence of methylglucose entry under equilibrium exchange conditions was determined.

The second requirement for the accurate estimate of kinetic constants is that the initial rate measurement does not include a large component of non-mediated diffusion. Whitesell & Gliemann's study fulfills this requirement. They showed that, in basal cells, L-[^{14}C]-glucose, or non-mediated permeability was maximally not more than 2 to 3% of the total permeability of basal cells. The rate of uptake of L-[^{14}C]-glucose was <1% of that for [^{14}C]-3-O-methyl-D-glucose. They also estimated that the non-mediated diffusion component was maximally 7%. This was apparent

because the half-time for exchange efflux of the 20mM 3-O-methyl-D-glucose in the presence of 0.3mM phloretin was much greater (at least 60 to 120 minutes) than the corresponding half-time (4 minutes) for 20mM 3-O-methyl-D-glucose exchange efflux recorded by Vinten et al. (1976).

Using Whitesell & Gliemann's method for the experiments studied in the present research, contributions from a "leakage" or simple diffusion pathway possibly available to 3-O-methyl-D-glucose would have been detected in deviations from the exponential curves describing the relationship between V and S (or as deviations from the linear S/V vs. S plots). Siegel & Olefsky (1980) relied on similar assumptions for their measurements of 3-O-methyl-D-glucose zero trans entry, but subtracted the values for [^{14}C]-L-glucose uptake to correct for "extracellular trapping" of sugar; the extracellular water space was always <5% of the total intracellular water content. Vinten et al. showed that L-glucose was transported fifty-five-fold more slowly than 3-O-methyl-D-glucose. The contribution of simple diffusion of 3-O-methyl-D-glucose to the efflux would not have been detected as a separate component in their efflux measurements. On the other hand, Czech (1976c) reported the presence of a large non-mediated component. He subtracted the contribution of this cytochalasin B-insensitive component from the transport in the absence of cytochalasin B (a competitive inhibitor of 2-deoxy-D-glucose transport; Czech et al., 1973) in order to calculate the constants pertaining only to mediated transport. Olefsky (1978) used a similar method to correct the uptake values for contributions from simple diffusion. Like Czech (1976c), Olefsky used an oil-separation without a stopping solution and ten second incubations to measure entry under zero trans conditions. Both these workers found that in addition to the previously described (Table 21) transport, a non-saturable (high K_m) process which was inhibited by cytochalasin B accounted for a large fraction of the methylglucose uptake.

The apparent heterogeneity of the cytochalasin B-sensitive uptake obviously complicates the calculation of the contribution of non-saturable processes to the transport of 3-O-methyl-D-glucose in the preparations of Olefsky (1978) and Czech (1976c).

4.2.2. Kinetic Models for Basal Hexose Transport.

The most striking feature of the basal 3-O-methyl-D-glucose transport kinetics (Table 21) is that the transporter has symmetric affinity and rate constants. These were apparent in the zero *trans* parameters and confirmed with the infinite *cis* constants. This immediately shows that the transporter is kinetically dissimilar to the intact human erythrocyte and erythrocyte ghost systems. Interestingly, isolated rat hepatocytes also exhibit symmetric 3-O-methyl-D-glucose transport (Section 1.9). However, this transport differs from that of the adipocyte in that the affinity constants are high (eg. K_{ee} = 18.1 ± 5.9mM). In other tissues, both symmetric and asymmetric systems have been found. The rabbit erythrocyte transport is symmetric (Regen & Morgan, 1964), and in rat thymocytes, two types of asymmetric transport are found. In "active" thymocytes the K_m and maximum rate parameters for zero *trans* entry were lower than those for exchange (Whitesell *et al.*, 1977). In "quiescent" cells, internal sugar increased the entry K_m (Whitesell & Regen, 1978). On stimulation of quiescent cells, the zero *trans* influx parameters changed, to resemble those of active cells.

Since there are no differences between net uptake (or efflux) and equilibrium exchange measurements in adipocytes it is not possible to reject the simple, symmetrical, mobile carrier model (Section 1.7.1) for the transport. Neither can the basic lattice pore model without unstirred layer assumptions be rejected (1.7.7). The asymmetry and unstirred layer

modifications to the mobile carrier model were both designed to explain the asymmetry of D-glucose transport in human erythrocytes and are therefore not applicable to the symmetric adipocyte 3-O-methyl-D-glucose transport. The asymmetry inherent in the modified tetramer model (1.7.6) eliminates this hypothesis. The introverting hemiport model (1.7.8) which predicts accelerated exhcange is also an inadequate description of 3-O-methyl-D-glucose transport. Although the gating pore with sugar-haemoglobin complexes is obviously not applicable to adipocytes, other models devised for human erythrocytes could have a general relevance to other transport systems. However, the complexity of the tetramer model (1.7.5) is unnecessary: four symmetrically arranged subunits of high and low affinity do not have to be invoked.

Recently, Foley *et al.* (1980a) have suggested that the simple carrier model does not account for adipocyte hexose transport because differences observed between the zero *trans* entry rates (V_{zt}^{oi}) for 2-deoxy-D-glucose and 3-O-methyl-D-glucose are not explained. When fitting models to their transport observations, these authors considered that neither the particular extensions and modifications of the simple carrier model in human erythrocytes, nor the other hypotheses substituted for it, were relevant to adipocytes. However, they had examined the relevance of the carrier model (formulated to explain erythrocyte D-glucose transport; Section 1.5). They considered it unlikely that a carrier loaded with one of these glucose analogues would move through the membrane at a rate different to that of the carrier loaded with the other hexose because there was no evidence to suggest that empty carriers moved at a different rate to 3-O-methyl-D-glucose-loaded carriers. They therefore rejected the simple carrier model, and suggested a model similar to the introverting hemiport model (1.7.8), differing solely in that both the affinity and rate constants at either

barrier may differ.

In this model, the "two microcarriers in series" model, an external "microcarrier" protein bound to a hexose molecule rotates and deposits the sugar in an aqueous pore. The sugar diffuses through the pore and binds to another microcarrier at the inner membrane interface which moves the sugar into the cell. The latter microcarrier was considered to have a lower affinity or "capacity" for 2-deoxy-D-glucose than for 3-O-methyl-D-glucose and to be a "partially rate limiting step" in the transport: in the case of 3-O-methyl-D-glucose, the two microcarriers showed apparently equal affinities and rates of transport.

A simpler asymmetric carrrier model, where the movement of the transporter-sugar complex (ES; Section 1.7) was rate limiting, would explain their observations. In 2-deoxy-D-glucose transport the characteristics of the rate determining step could change. This hypothesis could be tested by inhibition experiments because in such a case, 2-deoxy-D-glucose would act not only as a competitive inhibitor of transport, but would be an uncompetitive inhibitor, since the V_{max} for transport of the two sugars would differ. Foley *et al*. need not have rejected the single carrier type of model.

Although Foley *et al*. were reluctant to compare fat cells to red cells, kinetic studies of either system must take into account other similarities of the transporters, for example in specificity and perhaps size, between the two cell types. Previous reports (Section 1.4.2) have suggested that the adipocyte transporter had a different molecular weight to that of the erythrocyte wherein it has been shown to be a glycoprotein (probably Band 4.5; with a monomer weight of about 55000 (Section 1.4.1). However, recent reports of purifications using similar techniques for both cell types (Shanahan *et al*., 1982), where [^{3}H]-cytochalasin B was used to photolabel

the transporter, have shown that glucose-sensitive bands on SDS gels of similar molecular weight were photolabeled, in both cases (bands at 55000 and 46000 for human erythrocytes, and at 50000 and 46000 for rat adipocytes). An antibody to the isolated human erythrocyte transporter cross-reacts to some extent with a polypeptide in rat adipocytes that binds cytochalasin B. Insulin-sensitive bands of 45000 daltons on SDS gels were identified from plasma and microsomal membrane preparations (Wheeler et al., 1982; Lienhard et al., 1982).

Holman & Rees (1981) have examined the specificity of the adipocyte transport system. They determined which sugar hydroxyl groups were most important in hydrogen bonding to the transporter by testing sugar analogues as inhibitors of the transport of D-allose (a low affinity sugar). They measured the relative inhibition of several D-glucose epimers, of deoxy-D-glucose and of fluoro-substituted D-glucoses. The important binding features were the ring oxygen, a β position at C-1, a hydroxyl in the equatorial position at C-3 and to a lesser extent a hydroxyl at C-6. Hydroxyls at C-2 and C-4 were relatively unimportant.

When compared to the human red blood cell transport specificity (Section 1.5.3) it is obvious that there is a lack of specificity in the adipocyte at C-4 and C-6; loss of the C-6 hydrogen bond was of less importance than in red blood cells. This flexibility with regard to the C-4 and C-6 positions was accompanied by more imprecise spatial requirements in adipocytes than in the erythrocytes; this was demonstrated with inhibition by bulky substituents (Rees & Holman, 1981).

In view of these similarities it seems reasonable to suggest (Holman et al., 1981) that the allosteric pore model (an adequate description of human erythrocyte transport; Section 1.7.10) can as well be applied to adipocytes as to human red blood cells and that differences between the two systems are

due to more flexible subunit interactions at the inner surface of the erythrocyte transporter.

4.3. 3-O-Methyl-D-glucose Transport in Insulin-Stimulated Adipocytes.

4.3.1. The Kinetic Constants.

The complete kinetic characterisation described and discussed above for basal adipocytes was entirely repeated for insulin-stimulated adipocytes also at 37°C. Again both zero *trans* determinations and infinite *cis* confirmations were used to define the parameters for 3-O-methyl-D-glucose transport. In addition, for the insulin-treated cells, where transport was very rapid, initial rates were determined by integrated rate analyses of time courses at several hexose concentrations. These were then used to show that initial rates measured over one second would provide acceptable estimates of the inital rates for kinetic determinations, but that longer time intervals would result in a significant underestimation of initial rates at the lower sugar concentrations.

The affinity constants for the external site were determined as 6.10 ± 1.65mM and 3.60 ± 1.33mM for the zero *trans* entry and infinite *cis* exit experiments respectively. For the internal site, values of 2.66 ± 0.26mM and 6.51 ± 0.83mM for the zero *trans* exit and infinte *cis* entry parameters were obtained. The affinity constant under equilibrium exchange conditions was 4.45 ± 0.26mM. The maximum rate constants for entry were 1.20 ± 0.19mmoles/l cell water/s and 0.98 ± 0.09mmoles/l cell water/s for zero *trans* and infinite *cis* entry respectively. The internal site exhibited maximum rate constants of 1.19 ± 0.07mmoles/l cell water/s for the zero *trans* and 1.76 ± 0.63 mmoles/l cell water/s for the infinite *cis* exit experiments. The equilibrium exchange maximum rate constant was 0.84 ± 0.002mmoles/l cell water/s.

From studies of the literature, the most difficult measurements to make

in a satisfactory manner for adipocytes were thought to be those of the zero *trans* entry design (Olefsky, 1978; Ludvigsen & Jarett, 1979). This was most applicable to cells exhibiting rapid transport, in particular, cells stimulated dramatically with insulin. In the earlier work this lack of success was probably due to inaccurately timed termination of entry of the labeled sugar when inhibitor solutions were not used to stop transport, or separation techniques were insufficently rapid (Czech, 1980). These two problems were overcome in the present work by the use of specific transport inhibitors, rapid separation of the cells from the external medium, and very short transport incubation times (Whitesell & Gliemann, 1979; Ludvigsen & Jarett, 1979, 1980). Thus zero *trans* entry measurements in insulin-stimulated cells were not noticably prone to high errors, once the intricacies of the timing procedure had been mastered.

Unlike basal cells, insulin-stimulated cells did not need long equilibrations before equilibrium exchange or zero *trans* and infinite *cis* exit measurements were made: relatively brief incubations sufficed because of the rapidity of the transport. Thus errors specifically related to prolonged cell incubations (such as cell deterioration) were not significant. However, in the procedure for measuring zero *trans* exit, difficulties arose. In view of these methodological difficulties the observation that the affinity constant for the internal site measured by the zero *trans* exit techniques is slightly lower than the corresponding infinite *cis* entry value is not considered to be very significant.

For the insulin-treated cells, the affinity constant for the external site measured by the zero *trans* entry procedure, and that measured by the infinite *cis* experiment fall well within the standard error of each other. The infinite *cis* exit K_m has the larger error. This is attributed to the problems of measuring exit (see earlier).

The internal and external affinity constants are identical (except perhaps for the zero trans exit value). The exchange K_m is consistent with these values.

For the maximum rate parameters, the two values for the external site agree well, and those for the internal site are statistically not significantly different from one another. The maximum rate constant for exchange is similar to these values.

Table 22: 3-O-Methyl-D-glucose Transport in Insulin-Stimulated Adipocytes.

Experimental Design	Temperature	K_m	V_{max}	$V_{max}^{insulin}/V_{max}^{basal}$	Reference
	°C	mM	mmol/l cell water/s		
Equilbrium Exchange	37	5	1.7	8.5 to 24	Vinten et al. (1976)
Equilibrium Exchange	22	3.1±0.3 (n=5)	0.8±0.3	6	Whitesell & Gliemann (1979)
Zero trans entry	24	6.6±1.2 (n=5)	*7.2×10^{-3}	5.5	Siegel & Olefsky (1980)
Zero trans entry	37	≅1.5	**--	--	Czech (1976c)

*Calculated from the value of 2.2 ± 0.3nmol/min/2 x 10^5cells given in the reference. The assumptions in this calculation are described in a footnote to the previous Table.
**this value was not calculated but was described as variable, although increased relative to the basal value.

Table 22 shows the kinetic constants for 3-O-methyl-D-glucose transport in insulin-stimulated adipocytes, as measured in other laboratories. As with basal cells, although also not statistically rigorous, the values for the affinity constants are of the same order as both the zero trans and exchange parameters measured in the present study. For the equilibrium exchange experiment, the maximum rate parameters (although variable) were

similar to those obtained in the present investigation. Vinten et al. (1976) attribute their differences to the variation in conditions in successive collagenase isolations and washes, which were also reflected in the basal glucose metabolism. Again, as with their value for basal cell transport, Siegel & Olefsky's value appeared to be about 100-fold lower than the value obtained in the present research. A general conclusion may be drawn from these results, that although not precisely defined in some cases, the general trend fits that shown in the current work.

As with basal cells, these values are dependent on the conditions of their measurement. With the rapid transport observed when cells are stimulated with insulin, the determination of the initial velocities of transport with accuracy requries more emphasis and verification.

Evaluating their oil-centrifugation method, Whitesell & Gliemann (1979) measured the limits of efficiency of phloretin as an inhibitory agent in the stopping solution, and showed that ice-cold buffer was an ineffective agent for this purpose. They assessed the period of stability of the preparation and the thoroughness of the mixing procedure, showing that retardation by hypothetical unstirred layers would be negligible. The maximum permeabilities, determined for the exchange experiment were calculated at one second from several uptake curves. They verified that the timing of intervals of one second was as accurate as that when measuring transport at two seconds, by comparing coefficients of variation at both times: the values were both around 10%.

As noted earlier, Vinten et al. (1976) measured their earliest time point at five seconds, a rather nominal time, since this was also the time required to visibly separate the cells from the medium by oil. However, they did not rely solely on this measurement to determine initial rates of transport, but on a progression of longer time intervals of efflux.

Like Whitesell & Gliemann (1979), Siegel & Olefsky (1980) also found that half of the maximal amount of uptake had occurred in insulin-stimulated cells within a few seconds. This confirmed Olefsky (1978)'s earlier conclusion that the apparent rate of entry of a low concentration of 3-O-methyl-D-glucose entering sugar-free cells had rapidly decreased by ten seconds. At 24°C, the time taken was five seconds and at 37°C, three seconds. In the latter cases, trans-membrane equilibration had occurred in one minute. Although the insulin-stimulated 3-O-methyl-D-glucose uptake was so rapid, Siegel & Olefsky only noted that it was necessary to use "very early time points" to measure initial rates, and do not show data supporting their apparent contention that, from uptake measured over four seconds, K_m and V_{max} parameters could be determined. The present research, using integrated rates, would suggest that the underestimation of the initial rates would lead to a significant underestimation of V_{max}, but a minimal error in the estimate of the affinity constant.

In the cells isolated by Czech (1976c), transport (measured by filtration assay) at 37°C and in the presence of insulin appeared to be much slower than in cells prepared by other groups. Czech found that "tracer" [^{3}H]-3-O-methyl-D-glucose uptake was linear for at least thirty seconds. This is in direct contrast to the studies of Whitesell & Gliemann (1979) who found that, under similar conditions, the "half-time" for equilibration was only two seconds; the maximal permeability was at least twenty-times higher in the latter study. Comparisons of initial rates of uptake of 100μM 3-O-methyl-D-gluocose showed that Whitesell & Gliemann were measuring rates that were one hundred-times greater that Czech (1976c). Czech (1980) attributes these differences to collagenase preparations, although Whitesell & Gliemann find that such differences usually only account for about three-fold variations; they suggest that Czech

underestimates transport in insulin-stimulated cells. Several factors may contribute to this underestimation of transport with the filtration method, according to Whitesell & Gliemann: mixing of cells and labeled sugar was unlikely to be instantaneous; uptake over fifteen to thirty seconds would not be at the initial velocity; ice-cold buffer was an inadequate inhibitor of efflux. These problems would have involved a much larger percentage error in insulin-stimulated compared to basal cells, due to the rapid intake of sugar.

It may be concluded that in several of the earlier studies of 3-O-methyl-D-glucose transport, initial rates were underestimated. The second requisite factor in the determination of accurate initial rates, namely, elimination of contributions from "leakage" or simple diffusion pathways has been discussed above for basal cells, and applies also to insulin-stimulated data.

From previous studies (Table 22) the general conclusion had been drawn that insulin affected D-glucose transport by raising the maximum rate constant, but not changing the affinity constant. These studies were kinetically incomplete descriptions of the transport system. However, the present research confirms this general statement in the detailed analysis of the kinetics.

4.3.2. Kinetic Models for Insulin-Stimulated Hexose Transport.

Insulin stimulates 3-O-methyl-D-glucose transport completely symmetrically and therefore kinetic models which are relevant to the basal transport apply equally to the insulin-elevated rates.

The symmetry of the stimulated transport is particularly interesting, and was not obvious from previous studies of insulin-treated cells (Section 1.10). It had been thought that activation might have been due to an

asymmetric increase in inward, unidirectional transport, or a decrease in transporter activity in the outward direction, or a combination of both. However, symmetrical increases in both directions were observed in the present work. This has to be taken into account when considering the manner in which insulin activates transport (see Section 4.5).

4.4. The Adipocyte Ghosts.

4.4.1. The Composition and Morphology of Adipocyte Ghosts.

The usefulness of comparing the transport kinetics of ghost cells to their intact counterparts has been amply demonstrated with human erythrocytes where kinetic measurements have led to the rejection of particular transport models; specifically, rejection of the contribution of bulk haemoglobin-sugar complexes to transport asymmetry (Section 4.1). It was therefore of interest to compare the kinetics from intact fat cells with those found in adipocyte ghosts.

As regards the intact fat cell, isolated by a collagenase digestion, results of some kinetic studies were available; notably the zero *trans* exit and exchange parameters determined by Vinten *et al*. (1976) and Czech (1976c). However, major problems were associated with kinetic determinations in intact fat cells. In particular, monitoring initial rates of hexose transport had proved very difficult due to the small aqueous cytoplasmic volume, compared to the surface area, most of the internal volume being filled by the fat globule (Czech, 1980). This led to rapid equilibration and therefore backflux quickly became significant. Until techniques were developed which would accurately measure initial rates over only seconds (ie., rapid filtration; see Chapter 2), accurate kinetic determinations were impossible. One might imagine that transport in the ghost fat cells would be of a similar rapidity but Illiano & Cuatrecasas (1971) in a brief study of glucose transport found that uptake rates were

apparently linear for five minutes in ghosts derived from insulin pre-stimulated cells. Filtration techniques were available in our laboratory and had been tested for measuring rates over two seconds in human red blood cell ghosts: it was felt that whole fat cells would be too fragile (as initially prepared in this laboratory) to be subjected to the rigours of filtration for a sufficiently long time after preparation for enough transport samples to be processed, but that the method would be suitable for the more resilient ghosts (Illiano & Cuatrecasas (1971) report transport activity in ghosts after considerable periods of storage).

The aim of the first studies of the glucose transporter in fat cell ghosts was to reproduce the kinetic results obtained by Illiano & Cuatrecasas, and then to extend the studies into a more complete kinetic description of the transport system, in the basal and insulin-stimulated states, with an analysis of the dependence of the transport activity on temperature.

To this end, the ghosts were initially prepared for this research in the same way as those used by Illiano & Cuatrecasas, namely according to the details described by Rodbell (1967a) (see Chapter 2). Rodbell and Illiano & Cuatrecasas relied on marker enzyme analyses to establish the distribution of the plasma membrane within their ghost preparations. The enzyme studies showed that the ghosts were fat-free and contained a large fraction (25%) of the mitochondria. Since D-glucose was used for Illiano & Cuatrecasas' transport study, it was interesting to note that, although only 2% of the soluble glycolytic enzymes were retained, 15% of the hexokinase activity was found in the ghost fraction (Rodbell, 1967a). The hexokinase does not appear to be active to any significant extent during the first four minutes of incubation with [^{14}C]-D-glucose (<1% was modified) but Illiano & Cuatrecasas found that "significant amounts" of the radiolabel was insoluble

in water after five minutes. This finding must throw doubt on the reliability of their efflux measurements. It also suggested that a non-metabolisable glucose analogue would be a more suitable tool for the kinetic studies attempted here, where it was essential to obtain equilibrium measurements of the sugar (see Chapter 2).

Rodbell also used phase contrast light microscopy to watch the expulsion of fat droplets with the formation of apparently spherical ghosts. He observed that although only some ghosts retained the cell nucleus, all contained particles and interestingly, vesicles. Other workers, using different methods for preparing fat cell plasma membrane vesicles (eg. Brenner & Kahlenberg, 1977) have visualised their preparations with electron microscopy to determine the physical structure of the membrane pieces - ie. sheet or vesicle formation, and the presence of vesicles within ghosts. This procedure also allows a qualitative estimation of the contamination of the plasma membrane fraction by other membranes (eg. nuclear, mitochondrial and endoplasmic reticular). In the present study, the preparative method having been established by the enzymatic characterizations of Rodbell (1964), and similar phase contrast micrographs having demonstrated that superficially similar ghosts were formed (data not shown), the morphology of the ghosts was analysed by transmission electron microscopy.

In most respects, these results were as one would expect from Rodbell's studies, although slight modifications to the preparative procedure had been made (Chapter 2). Ghosts were not sheet-like but were sac-like, although the sacs were not obviously spherical, probably due to distortion during the brief centrifugation at 10000xg prior to fixation for sectioning; similar size sacs ($\cong$10μm and larger) were observed, containing cell "debris" (Section 3.5). The electron micrographs showed the varied nature of the

ghost contents, in particular in the formation of vesicles of mitrochondria, rough endoplasmic reticulum and plasma membrane. One major difference was the additional appearance of apparently "empty" plasma membrane sacs. These would be ideal vehicles for sugar transport studies, but there was no apparent reason for their production, and they also appeared as part of a conglomeration of the more conventional "Rodbell ghosts". The preferential selection of these structures over the conventional ghost would obviously be an interesting future line of research, since most other preparations of pure plasma membrane are long procedures involving high speed and differential centrifugation (McKeel & Jarett, 1970).

The use of disrupted adipocytes for transport studies has one major drawback, lack of control of the sidedness of the resultant vesicle. We attempted to obviate the necessity for fractionating the sacs into "right"-side-out and "wrong"-side-out vesicles by using the gentle disruption procedures advocated by Rodbell. If the detailed kinetic studies had showed more promise, it would have been interesting to attempt a rigorous characterisation of this feature: methods for separating red blood cell ghosts according to their sidedness have been developed (Steck, 1974).

4.4.2. Transport Kinetics in Adipocyte Ghosts.

In this work, the early experiments on 3-O-methyl-D-glucose entry in ghosts, under zero *trans* conditions showed that (A) the sugar was transported and (B) that this transport was saturable. It was interesting to find that under basal conditions, the internal concentration of labeled 3mM 3-O-methyl-D-glucose appeared to have reached a maximum at two minutes, and was constant at least until thirty minutes (Fig. 39). This contrasts markedly with Illiano & Cuatrecasas' finding that zero *trans* uptake remained linear for up to five minutes, even at a lower glucose

concentration (1mM D-glucose) and in the presence of insulin. However, the transport methodology used in the present study differed in several respects from that used by Illiano & Cuatrecasas. Most importantly, sugar transport inhibitors (sodium iodide, mercuric chloride and phoretin) were present in the transport "stopping solutions". If efflux was rapid, a significant fraction of the influxed sugar may have flowed from the ghost during the washing procedures: although Illiano & Cuatrecasas used an ice-cold washing buffer to reduce transport, they could disregard any leakage during the separation because of the rapidity of the filtration process relative to their apparent transport rates.

Although Illiano & Cuatrecasas observed slower transport than that reported here, in the highly purified preparation of McKeel & Jarett (1970) (modified by an additional sonication step after membrane isolation, to improve the integrity of the vesicle) the transport was also rapid (Ludvigsen & Jarett, 1979). That this preparation exhibited transport of D-glucose and not sugar binding, was shown by a study by Carter et al. (1972): the transporter showed a stereospecific preference for the D-sugar; countertransport of D-glucose occurred; D- and L-glucose eventually reached identical equilibrium levels; the analogues 3-O-methyl-D-glucose and 2-deoxy-D-glucose inhibited D-glucose transport, as did a low concentration of phlorizin.

It appears, therefore, that the preparation of ghosts described here, although similar to that of Illiano & Cuatrecasas, shows certain of the characteristics of a purified plasma membrane preparation, whose general transport characteristics have been documented. The details of the kinetics observed in the different preparations will be compared and discussed.

In the present study, values for the kinetics parameters for influx were determined by the zero trans and exchange experiments. Under zero

trans conditions, at 25°C, the affinity constant is of the order of 5 to 6mM for both insulin-elevated and basal transport. The zero *trans* entry parameters accord well with the comparable parameters measured in intact cells in that (1) K_{zt}^{oi} is of the same order of magnitude, (2) it is unchanged by insulin treatment and (3) the V_{max} increases on insulin stimulation of the cells, although the increase is only about four-fold in ghosts (basal value is about $0.3mMs^{-1}$, insulin prestimulated level is 1.3mMs, c.f., whole cell results in Table 12). For zero *trans* entry parameters, Illiano & Cuatrecasas, with initial rates measured at four minutes, and at the same temperature, values were observed for K_T of 3.3mM and V_{max} of 12.3mmoles/mg protein/min. The affinity constant is similar to that obtained in the present research; the insulin effect on the kinetic parameters was not determined.

The equilibrium exchange K_m (K_{ee}) determined here was at least 6mM, if not greater (Figs. 39 and 40) under basal conditions. This affinity constant is therefore of the same order as that observed by Ludvigsen & Jarett (1980), with initial rates measured at 1/2 and one second (26mM): for this experiment, Ludvigsen & Jarett used a fifteen minute pre-incubation of the cells. This was not used prior to ghost preparation in the present study and when Ludvigsen & Jarrett ommit this step, a lower K_m (9mM) was observed.

Although efflux measurements were not successfully made for ghosts, infinite *cis* entry experiments were used, giving estimates of the internal affinity constant (K_{ic}^{oi}) of about 5.20mM, and of the external V_{max} (V_{ic}^{oi}) of about 0.37mmoles/l ghost water/sec for 3-O-methyl-D-glucose under basal conditions. The latter value confirms the zero *trans* entry V_{max} (V_{zt}^{oi}). The internal affinity constant is very similar to the external constant, suggesting that the transporter is kinetically symmetrical under basal conditions, as observed in intact cells. Since the relative populations of

right- and wrong-wide out ghosts was not known, no conclusions can be drawn from this symmetry. However, the internal affinity constant differs markedly from the value obtained by direct measurement of D-glucose efflux by Illiano & Cuatrecasas (K_T = 0.2mM, with a much slower apparent initial rate). Illiano & Cuatrecasas also showed about a three-fold apparent asymmetry in the maximum rates for transport influx and efflux (V_{zt}^{oi} = 12.3mmoles/mg protein/min; V_{zt}^{io} = 3.8mmoles/mg protein/min), which is not apparent in whole cells.

The infinite <u>cis</u> entry parameters show an unusual feature in ghosts fractionated from insulin-stimulated cells. Namely, the affinity constant apparently falls to ≅1.52mM with an increase in V_{max} to about 1.89mmoles/ l ghost water/sec. Thus, insulin appears to cause a six-fold increase in the external maximum rate constant, confirming the value for zero <u>trans</u> entry, but with a concomitant three-fold decrease in the internal affinity constant of the transporter for 3-O-methyl-D-glucose.

If this value is not merely an experimental artefact, produced by the variablity and lumpiness of the ghost preparations, it is interesting to consider the reproducible variation observed by Ludvigsen & Jarett in their pure membrane preparation: the equilibrium exchange K_m increased from 9 to 26mM in basal cells merely on the introduction of a fifteen-minute cell incubation prior to fractionation. This suggests that some factor, capable of modifying the transporters' affinity for hexose is active during this time; such a factor may also affect the transporter in the crude ghost preparation.

Insulin therefore appears to act on the entry parameters of ghosts in a similar manner to that seen in whole cells, ie. by an increase in V_{max}, with an unchanged K_m, although the size of the effect is smaller in ghosts. However, it appears to show a different effect on the internal

K_m, when compared to intact cells; the K_m is decreased. Unfortunately, the full range of kinetic experiments was not successful, due to the problems of the preparation of ghosts, in particular the difficulty of dispersing the ghosts. Thus the single estimate of the internal affinity was not confirmed. It is interesting to speculate that a residual portion of the insulin response is all that is observed in ghosts, and that this exhibits an asymmetry in response to insulin which is normally masked by the larger response in intact cells. However, the result should be treated with caution in view of the possible occurence of "inside-out" ghosts, although the formation of these sacs would seem less likely in the gently disrupted, crude ghost preparation, than in the other vesicle preparations.

In conclusion, the kinetic parameters in fat cell ghosts appear to be symmetrical under basal conditions for the experiments used, with similar affinity constants to those measured in whole cells. However, insulin may induce an asymmetry in ghosts which is not observed in whole cells, although the effect on the external parameters appears to be of a similar type to that in whole cells (constant affinity constant, with an increase in the maximum rate).

4.4.3. The Insulin Response of Adipocyte Ghosts.

The insulin-induced stimulation of sugar transport was elusive in ghosts. In about one half of the ghost preparations, insulin had no effect on the transport. When stimulation occurred, it was at most a four-fold stimulation of the initial rate of transport. Before discussing the significance of this response it is important to point out that the basal rates of transport may, in some cases be overestimated: equilibration of the sugar may not have taken place in the thirty minutes used for the estimation (see Fig. 37). This time interval was chosen to minimize ghost

deterioration: because of the crude nature of the preparation, degradative enzymes may have been released into the ghost "cytoplasm." Other studies (Illiano & Cuatrecasas, 1971) also indicated that equilibrium should have been reached by thirty minutes even in basal ghosts.

This low hormonal sensitivity of the ghosts was also observed by Illiano & Cuatrecasas (1971) and Rodbell (1967b) in their crude ghost preparations. In these cases insulin was not added until after preparation of the ghosts, and since highly purified plasma membranes from untreated adipocytes are unresponsive to insulin (Czech, 1980) some losses of hormonal sensitivity might have occurred during the partial purification to produce ghosts. However, the crude ghost preparation was chosen for the kinetic studies because it was thought that the gentle disruption of the fat cells, allowing expulsion of the fat globule was most likely to produce ghosts reflecting the high hormonal sensitivity of the intact cells (up to 15-fold, Whitesell & Gliemann, 1979).

In other more highly purified membrane preparations the stimulation of transport was a mere doubling. If this was due to the loss of some water-soluble factor, of importance in the mediation of the insulin response, then a less extensive purification might lead to a larger insulin effect. An example of a highly purified preparation of plasma membrane in which the glucose transport derived from insulin-stimulated cells was elevated only two-fold over the basal level was recently published by Ludvigsen & Jarett (1980). These membranes were prepared by a method described in detail in earlier papers (Ludvigsen & Jarett, 1979; McDonald et al., 1976a). Briefly, it involved isolation of fat cells with only one half of the collagenase concentration, and with a shorter incubation in a larger volume, than Rodbell used. The isolation of the plasma membranes was by a completely different method, not involving hypotonic lysis, but instead, cell

disruption was by homogenisation, with high speed centrifugations and differential centrifugation to collect the plasma membrane fragments.

In conclusion, it appears that both highly purified plasma membranes and crudely prepared ghosts show only a two- to four-fold insulin response. If one assumes that the extents of stimulation are genuine measurements and not artefacts of the transport procedure (see below), there are several possible explanations for the observations. Firstly, since some stimulation remains, it may be either a residual remnant of the larger response seen in the intact cells, the major part of which has disappeared with the loss of some mediator, or a minor component of the insulin effect with a different mechanism, normally hidden by the larger response, may have been unmasked.

If a major cytoplasmic mediator were missing, this would accord with one hypothesis where an undefined second messenger mediates the insulin response of sugar transport (Section 1.3) and most, but not all, of this messenger has been lost in the hypotonic lysis. An alternative plausible hypothesis is that the translocation of a cytoplasmic pool of completely or partially preformed transporter molecules to the cell surface to increase the rate of transport in response to insulin has been disturbed. Such a mechanism for the insulin-induced stimulation of D-glucose transport has recently been proposed (Suzuki & Kono, 1980; Cushman & Wardzala, 1980; Section 4.5.2).

In an earlier paper, Vega & Kono (1979) showed that the transport activity of basal whole cells could be increased by mechanical agitation (and that the reversal of this effect was glucose- or pyruvate-dependent). Since disruption of the cells to form ghosts inevitably involves "mechanical agitation", even in the gentler methods of forming a crude ghost preparation (Rodbell, 1964;1967a) and certainly in the highly purified preparations (McKeel & Jarett, 1970) it is not surprising that the stimulation by insulin

in ghosts is small: the transport in the ghosts is already partially stimulated. A direct comparison of the rates of transport in whole cells as compared to ghosts would be a useful way of showing whether or not the basal rate in ghosts is greater than that in whole cells. In ghosts, the basal initial rate for zero trans entry of 3mM 3-O-methyl-D-glucose at 20°C was about 0.19mmoles/l "ghost" water/second (Fig. 36); for whole cells, the insulin stimulated rate was about 0.4mmoles/l cell water/second at 37°C (Fig. 7). However, a direct comparison between these units is not valid, since a larger aqueous volume is contained by a ghost membrane than the intact cell, the fat globule having been lost. Comparisons of estimates of initial rates related to the weight of cell protein would not account for losses of protein during ghost preparation, or provide a specific marker for plasma membranes versus internal membranes. An enzyme marker for each structure would perhaps provide better "universal units" for cell and ghost transport. At least, the weight of integral membrane proteins could be assessed.

It is therefore proposed that the insulin stimulation in ghosts is low because "mechanical agitation" has triggered the translocation response, so that a substantial proportion of the internally stored transporters have moved to the plasma membrane. The variability of the D-glucose transport and hormone response observed in ghosts could then be due to variation in the extent of stimulation of the translocation.

4.5. Mechanisms for the Kinetic Changes Induced by Insulin in Whole Adipocytes.

That an effector of transport stimulation such as insulin does not act on the affinity of the transport molecules has implications for the mechanism of transport stimulation. Both Ludvigsen & Jarett (1980) and Czech (1980) have summarized three hypothetical ways in which transport may

be activated by insulin. Firstly, that the number of transport sites of a similar nature to molecules in the membrane already effecting D-glucose transport is increased. Secondly, that the overall number of active transporters in the membrane increases, but that the nature of the extra sites is different from the original sites. Thirdly, that a change in existing sites occurs, making them more efficient at transporting hexoses through the membrane, without altering the total number of sites.

In classical terms, V_{max} is described as equal to k_2E_0, where k_2 is the rate constant describing intermediate dissociation to products, and E_0 is the total enzyme concentration in the system. K_m is defined as $(k_{+2} + k_{-1})/k_{+1}$, where k_{-1} and k_{+1} represent the backward and forward rate constants for the formation of intermediates from enzyme and substrate. If the existing enzyme (the transporter) does not undergo changes in any rate constant described by K_m, then k_2 must remain constant (Olefsky, 1978). Thus, from simple enzyme kinetics, if V_{max} increases the total enzyme (transporter) concentration (E_0) may increase, either by the insertion of more transporters into the cell membrane, or by the release of a non-competitive inhibitor of the transporter, but not by a change in the sugar affinity of existing membrane transporters. Thus the precise determination of the kinetic parameters in whole fat cells could lead to the conclusion that insulin increases hexose transport by increasing the number of effective transport molecules in the cell membrane, of the same type as those active under basal conditions.

However, if k_{+2} was small compared to k_{-1}, a V_{max} change could also be due to a k_{+2} change with an undetectable change in K_m. In addition, as with some other kinetic systems, the K_m determined may include more rate constants than in this simple case with just k_{-1}, k_{+2} and k_{+1}, particularly if the reactions are readily reversible. Therefore the applicability of a simple Michaelis-Menton analysis may not be strictly relevant.

4.5.1. Models for Transport Stimulation: Aggregation, Peptide and Calcium Ions as Mediators, the Thiol Redox Model, and Receptor Phosphorylation.

Several models for the activation of hexose transport have been described (Section 1.3). It is interesting to consider the application of some of these hypotheses to the activation of hexose transport in the light of the kinetic conclusions.

The kinetic results rule out the possibility (described in Section 1.2.2) that previously scattered, functioning glucose transporters may aggregate in the plasma membrane in respone to insulin to form a multimeric complex, further facilitating transport: the additon of a different route for glucose transposition would lead to a change in the affinity constant, as well as an increase in E_0 in response to insulin. For an interpretation consistent with the kinetics, it must be assumed firstly that scattered transporters are non-functional and secondly that there are a small number of active, aggregated complexes of the transporter under basal conditions. Insulin would then cause scattered transporters to aggregate and join the small number of previously active complexes, being indistinguishable from them. In this way, the number of identical, effective transporters (E_0) would be increased in response to insulin.

The release of a non-competitive inhibitor, or binding of a non-competitive enhancer of the transport activity is consistent with the kinetic conclusions: an intracellular polypeptide or calcium ions (Sections 1.3.3 & 1.3.4) could perform this function; the thiol redox model of transport activation would also be tenable. If a non-competitive regulatory protein component of the transporter in a reduced, inactive form blocking the transport channel, could be effectively moved from the transport site by the formation of disulphide bonds, then the number of transporters would increase. Again, basal transport would be mediated by the same type of

transporter, already in the oxidised form.

The observation has been made that insulin promotes the phosphorylation of membrane proteins (Chang et al., 1974; (Belsham et al., 1980; Benjamin & Clayton, 1978) and that this may be a stage in the activation of other insulin-sensitive cell components. There is some controversy in the identification of these membrane proteins: Belsham et al. have demonstrated in rat adipocytes that four phosphoproteins are found of between 67000 and 20000 daltons. These were not glycoproteins. Benjamin & Clayton found only two phosphoproteins (Chapter 1). An intriguing observation has recently been made that insulin causes the phosphorylation of its own receptor (a glycoprotein). In intact hepatoma cells the serine and tyrosine residues of the β(95000 dalton) subunit are phosphorylated (Kasuga et al., 1982); in a partially purified plasma membrane fraction of rat liver cells, both the β and α(135000 dalton) subunits where phosphorylated, but only phosphotyrosine is found. Insulin modulated insulin receptor phosphorylation has also been observed in intact rat adipocytes and in a cell-free system (Haring et al., 1982). The role of protein phosphorylation in the hormonal control of cellular activity has recently been reviewed (Cohen, 1982). Further studies on the insulin receptor might lead to the elucidation of the intermediate steps in insulins' stimulation of heoxse transport in fat and muscle cells.

4.5.2. The Translocation Model for Transporter Stimulation.

In the current understanding of the biogenesis and homeostatic maintenance of the plasma membrane, intrinsic membrane proteins are initially synthesised within the rough endoplasmic reticulum, and then transferred to the smooth endoplasmic reticulum where sugars found proximal to the protein are added. The nascent glycoproteins are then transferred to

the Golgi apparatus where the addition of terminal sugars occurs, followed by translocation in intracellular vesicles to the plasma membrane, where they are inserted, or release some of their membrane into the plasma membrane. Redundant glycoproteins may be returned to the cell interior by invagination and "pinching off" of vesicles containing the glycoprotein (Palade, 1975; reviewed by Morre et al., 1979).

This brief general description is of particular relevance to a new model of hexose transport activation, the "translocation hypothesis", recently proposed by Cushman & Wardzala (1980) and Suzuki & Kono (1980).

There had been some indications by Carter & Martin (1969), using a less highly purified preparation than that of McKeel & Jarett (1970), that D-glucose transport activity was occurring in the endoplasmic reticulum vesicles, although the latter and the plasma membrane had not been entirely separated by the purification: they recorded an insulin-induced acceleration of the transport (Martin & Carter, 1970).

Suzuki & Kono (1980) also detected two locations of glucose transporters with the rat fat cell: one was the plasma membrane, the other was found at an "intracellular storage site" co-sedimenting with the Golgi apparatus. These findings were based on the fractionation of fat cells on sucrose gradients: measurement of D-glucose transport activity in the resultant fractions by insertion into phospholipid vesicles was correlated with the distribution of marker enzyme activities in these fractions. Suzuki & Kono showed that in cells which had been pre-treated with insulin (1nM) the glucose transport activity in the plasma membrane fraction increased about two-fold for non-initial rates of uptake, with a reproducible, concommitant drop in the activity observed in the Golgi-associated fraction.

On the basis of these observations, Suzuki & Kono hypothesised that

insulin stimulates D-glucose transport by facilitating the translocation of the "activity" from the internal storage site to the plasma membrane. This hypothesis is supported by the studies of Cushman & Wardzala (1980) who independently found that D-glucose-inhibitable cytochalasin B binding in a similar plasma membrane fraction was increased by three-fold with a corresponding halving of that found in a microsomal fraction, in response to 7nM insulin: cytochalasin B had previously been shown to bind competitively to the D-glucose transporter (Wardzala et al., 1978). Suzuki & Kono noted that their plasma membrane stimulation was only two-fold compared to the five- to ten-fold they had found in whole cells (Kono, 1969) and, indeed, the fifteen-fold effect found during the present research (Chapter 3). In a further study, Kono et al. (1982) attempted to separate the plasma membrane and Golgi-associated fractions more completely and were able to demonstrate larger insulin effects. With this separation, involving differential centrifugation and resolution of the fractions on a linear sucrose density gradient, 1nM insulin increased the D-glucose transport activity of the plasma membrane fraction by 6.3- to 8.6-fold, concommitant with a halving of the Golgi-associated transport. Several insulin-mimetic agents also increased the plasma membrane glucose transport rates with a corresponding decrease in the intracellular activity.

However, Kono et al. (1982) once again used non-initial rates to estimate transport under zero trans entry conditions, for example measuring insulin-stimulated 1mM 3-O-[^{14}C]-methyl-D-glucose uptake at 37^{o}C over ten seconds. It is apparent from the research described in this thesis that both the basal transport rate (measured over forty seconds) and the insulin-stimulated transport rate (measured over ten seconds) would be considerable underestimates of the initial rates: a comparison of the two rates is invalid since there is no reason to believe that they are taken from

equivalent points on the kinetic curve.

In an important experiment, Kono et al. (1982) demonstrated that translocation between these two sites was reversible: if insulin was proteolytically removed from whole fat cells, the transport activity in the Golgi fraction increased, as that in the plasma membrane fraction decreased. However it is difficult to rule out proteolytic damage to the external transporters. Cushman and coworkers (Karnieli et al., 1981) recently examined the reversibility of the D-glucose-inhibitable [^{3}H]-cytochalasin B binding of their intracellular and plasma membrane fractions. If maximally stimulated cells were exposed to a large excess of anti-insulin antibody, the previously observed 5.5-fold increase in the number of cytochalasin B binding sites was decreased almost completely whilst the three-fold decrease in the binding sites of the microsomal membrane fraction was also reversed. The overall movement between the "membrane compartments" was on a time scale suitable for transport activation, and effected by an appropriate insulin concentration.

Although Karnieli et al. (1981) and Kono et al. (1982) have shown that insulin causes increases in plasma membrane glucose transport activity with concomitant decreases in the activity of an intracellular "storage" pool, Carter-Su & Czech (1980) find no such relationship. Using only differential centrifugation separation of cholate-solubilized membranes these authors measured the cytochalasin B-sensitive glucose transport activity in both plasma membrane and microsomal fractions. They found intracellular transport activity which showed the same sugar and inhibitor specificity as the plasma membrane transporter when reconstituted, although the apparent transport activity was three- to ten-fold higher than in basal plasma membranes. Under the influence of insulin, their reconstituted plasma membrane fraction increased its transport activity to between five- to eight-

fold, but the microsomal fraction showed small and inconsistent changes. The overall basal transport activity of the reconstituted, combined two fractions was only one half of that found in the combined insulin-treated system. So, in contrast to Kono et al. (1982), Carter-Su & Czech concluded that translocation of intracellular glucose transporters alone is not the sole way in which hexose transport is augmented.

In Cushman and coworkers' model, the activation of latent transporters in the plasma membrane is not ruled out. They postulated two mechanisms for the translocation process (Karnieli et al., loc. cit.). In the first, they suggested that the transporters move between their intracellular pool and the plasma membrane via vesicles which continuously shuttle back and forth to the plasma membrane, regardless of whether insulin is bound to the cell surface or not. Insulin acts by altering the rate of backward or forward shuttling; if this occurred at the plasma membrane, no "second messenger" would be required. In the second model, consistent with the membrane flow hypothesis outlined at the beginning of this section, the movement of vesicles bearing the transporter occurs only in response to insulin, possibly via a second messenger. Deactivation of transport occurs in response to insulin dissociation from its receptor, by invagination of areas containing the transporter and translocation to the intracellular storage pool from whence they can be recycled. The kinetics of insulin binding and dissociation (see Introduction) are consistent with this latter hypothesis.

One way to test the translocation hypothesis is to try to selectively inhibit the one of the many steps involved in the flow of transporters between the cellular compartments (Karnieli et al., 1981). Since the storage site has been found to be "Golgi-associated" (Kono et al., 1981), it seemed logical to try to inhibit the glycosylation, one of the post-translational steps which is a possible control point for membrane component

movement. To this end, the inhibitor of glycosylation, tunicamycin, was tested. Unfortunately inconclusive results were obtained (Section 3.4.1), but further experiments along these lines should prove an interesting way to test the hypothesis. De novo translation was ruled out by the lack of effect of the protein synthesis inhibitor, puromycin or RNA synthesis inhibitor cordecypin, confirming earlier (Fain, 1964) and more recent (Kono et al., 1981) studies. In any event, the time scale for the production of a new protein would be too long for this to be the major way glucose transport is activated in response to insulin.

4.6. The General Characteristics of Adipocyte Transport.

Apart from a consideration of kinetic parameters, other features define the characteristics of transport. Thus, although the previous kinetic measurements support the conclusion that the number of similar transporters is increased in response to insulin, other evidence for models of transport activation does not. The responses of insulin-stimulated and basal hexose transport to changes in other parameters such as pH sensitivity, temperature dependence,inhibition by cytochalasin B, the effects of sulphydryl reagents and divalent cations (discussed by Ludvigsen & Jarett (1980) and Czech (1980)) have been compared. Some of these studies point to functional differences in the hormone-stimulated and basal transport. On the basis of these studies, support has been given to the other hypotheses for mechanisms of transport stimulation, and it has also been suggested by Czech (1980) that the D-glucose transport system may show heterogeneity. In these studies, it must be noted that a change in, for example, pH may cause gross morphological changes to occur in the cell membrane, which might vary in the presence or absence of insulin. The precise kinetic measurements are more accurate reflections of changes in the glucose transporter. The effects of

secondary changes in the cells on the transport is especially relevant to studies on temperature changes in intact cells, where, as mentioned by Ludvigsen & Jarett (1980), changes in metabolic processes not specifically related to the cell membrane (Lawrence et al., 1977) could alter the transport data artifactually. This would be particularly apparent in whole cells because long incubations would be required to obtain insulin effects at low temperatures. Although by using pure plasma membranes, Ludvigsen & Jarett avoided the necessity for these long incubations (by using uniform incubations at low and high temperatures) general effects of different temperatures on the membrane rather than the transport molecules per se are not ruled out.

A full discussion of the effects of temperature changes on transport is found in the next section.

4.6.1. The Effect of Temperature on Fat Cell Ghosts.

In the ghost preparation, the temperature dependence of both basal and insulin-stimulated transport was assessed. The results, although exhibiting some variability showed that both the basal and insulin-elevated initial transport rates increased with increasing temperature, and that the degree of stimulation by insulin was always about one-fold at the higher temperatures, but was apparently smaller at 10°C. The basal transport does not show any sharp change in rate with increasing temperature, although the insulin-stimulated transport does show a disjunction at 10°C. Above this temperature, the rate increases quite suddenly. The apparent size of the stimulation at 10°C was only about 0·3-fold as compared to about one-fold at other temperatures. This may reflect inaccuracies in measuring rates, or perhaps increased inhibition of the insulin response at this

temperatures.

It is consistent with these temperature effects on the initial rate of transport, that the transporter is not a fixed channel, but is sensitive to the mobility of the membrane. Other workers have shown that the glucose transport in fat cell membranes, assayed in vesicles of reconstituted membrane protein with a variety of lipids, is sensitive to changes in phospholipid fluidity. Activation of transport was associated with melting of the bilayer, in experiments where the transition temperature was modulated by the addition of cholesterol, or the formation of vesicles of different lipids around the membrane proteins (Melchior & Czech, 1979).

If insulin activated transport by increasing the efficiency of the existing transport molecules, the profile of rate dependence on temperature would differ in the stimulated and non-stimulated ghosts or cells, and the energy of activation would be greater in basal cells or ghosts. On the other hand, if activation of transport was mediated by the "unmasking" of previously inactive transporters at the membrane, or insertion of additional identical transporters at the cell surface, then the activation energy should not change. More sensitive measurements than those used in the present research gave conflicting results in whole cells and in purified membrane preparations.

For pure membranes (Ludvigsen & Jarett, 1980) an Arrhenius analysis of the effect of different temperatures of the energy of activation of transport was undertaken. Ludvigsen & Jarett showed that the temperature dependence of both basal and insulin-stimulated vesicles was the same, with a phase transition for both between 33°C and 37°C. This implies that the rate determining step is the same in both cases.

Several workers have considered the effect of temperature changes in intact cells. Olefsky (1978) submitted both basal and insulin-stimulated

intact cells to an Arrhenius analysis. The parallel slopes of the Arrhenius graph indicated that insulin had not altered the activation energy, so E_0 must increase.

This conclusion may not be applicable to all D-glucose analogues. For his study, Olefsky used the analogue 2-deoxy-D-glucose. Before rapid transport techniques were developed, 2-deoxy-D-glucose had a major advantage over 3-O-methyl-D-glucose as a D-glucose analogue. The determination of initial rates of 2-deoxy-D-glucose entry does not require measurements to be made over seconds; longer time intervals suffice because efflux is minimal over a period of several minutes even at the higher substrate concentrations (five minutes at 20mM; Olefsky, 1978). This occurs because, unlike 3-O-methyl-D-glucose, intracellular 2-deoxy-D-glucose is a high affinity substrate for hexokinase and is phosphorylated as fast as it enters the cell, under conditions where hexokinase is not rate limiting. The main product, 2-deoxyglucose-6-phosphate does not compete for the transporter, nor is it metabolized further.

There is some evidence to suggest that 2-deoxy-D-glucose and 3-O-methyl-D-glucose exhibit different transport characteristics. Foley et al. (1980) showed that initial rates of "tracer" 2-deoxyglucose were less than half as fast as those for 3-O-methyl-D-glucose, although the inhibition constants (K_i) for [^{14}C]-3-O-methyl-D-glucose uptake were similar; Olefsky (1978) found different inhibition constants for D-glucose and 3-O-methyl-D-glucose on [^{14}C]-2-deoxy-D-glucose entry, but these kinetics may be complicated by several points raised by Foley et al. (1980a). The latter employed similar transport techniques, but used phloretin as a transport stopping agent. Although they reproduced Olefsky's experiment for measuring the inhibition constant for D-glucose on 2-deoxy-D-glucose uptake, they noticed a time-dependence of K_i for D-glucose or 2-deoxy-D-glucose on [^{14}C]-2-deoxy-D-

glucose transport, and found that hexokinase become rate limiting for the analogue entry after a few minutes in the presence of D-glucose or unlabeled 2-deoxy-D-glucose. In addition, they found that [^{14}C]-2-deoxy-D-glucose was cleared from the cell water so rapidly after removal of the extacellular sugar, that they concluded that Olefsky would not have detected intracellular levels (and by implication, efflux), so his conclusion that these levels had a negligible effect on the entry (ie., initial) rate was invalid. Thus, although this study was consistent with the kinetic changes induced by insulin, it was invalid. However, from other experiments, similar conclusions have been drawn (Vinten, 1978). Arrhenius analyses between 18°C and 37°C were derived from efflux experiments measuring methylglucose exchange: the Arrhenius graphs were again linear, and parallel for stimulated and basal transport. Ciaraldi & Olefsky (1979) quantitated the temperature dependence of the time of coupling of the insulin receptor to glucose transport by an Arrhenius analysis and concluded that the activation of transport was not affected by membrane fluidity between 15°C and 37°C.

In contrast, several other studies show conflicting data. In one such study, Amatruda & Finch (1979) used electron spin resonance spectroscopy to evaluate temperature-induced alterations in membrane fluidity in pure adipocyte plasma membranes, containing the spin probe 2-doxylstearate. These were compared to the effect of temperature changes on both insulin binding and hexose uptake, measured with an oil-centrifugation assay and long (45 to 60 seconds) uptake times, in intact cells. Phase transitions in the temperature dependence of D-glucose, 2-deoxy-D-glucose and 3-O-methyl-D-glucose uptake occurred at 30°C to 32°C in both stimulated and basal cells. Transitions in membrane fluidity occurred at 25°C to 26°C, 30°C to 31°C and 33°C to 34°C. Thus one of the phase transitions

observed in the fluidity of pure membranes corresponded to a phase transition of the temperature dependence of hexose uptake. However, at 36.5°C to 37°C, another transition in hexose uptake occurred, but only in insulin-treated cells; this was not reflected in fluidity changes of the membrane, but it was concomitant with a transition in insulin-receptor binding. In agreement with this finding, Whitesell & Gliemann (1979) found that tracer 3-O-methyl-D-glucose uptake showed a different temperature dependence at 37°C as compared to 22°C. Czech (1976c) supported this conclusion.

In addition to the difference in temperature dependence observed between basal and insulin-stimulated uptake, Amatruda & Finch found that although basal uptake increased little between 15°C and 37°C, insulin-stimulated transport rates increased more than three-fold. Kono et al. (1977) recorded the same effect with 3-O-methyl-D-glucose transport. By showing that incubating the cells at 37°C, then rapidly cooling them to 15°C did not prevent stimulation, these authors showed that incubation at the higher temperature was necessary to elevate transport. However, incubations with insulin were for only five minutes, a time which was probably insufficient to fully activate the cells at the lower temperature: Siegel & Olefsky (1980) reported that maximum activation with high insulin concentrations does not occur for twenty minutes at 24°C. Kono et al.'s study cannot therefore be said to support Amatruda & Finch's conclusion as to possible models for transport stimulation. These models suggest that insulin may enhance a process of transport different from basal transport, or have an allosteric effect on the system, or increase the binding affinity of glucose for the transporter. All these possibilities are, of course, inconsistent with the kinetic data.

Thus the studies of temperature dependence apparently point to

conclusions which conflict with each other and the kinetic conclusion. In an attempt to reconcile the conflicting results, Czech (1980) has suggested that insulin action may have a more complex role than simply that of increasing the number of effective transport molecules in the cell membrane: certain properties of the transporters induced by insulin may differ from the basal properties, or the new or unmasked transporters may be located in different regions of the plasma membrane, so that the temperature dependence, but not the sugar affinity may appear to be altered by insulin.

4.6.2. The Effect on the Infinite cis Kinetics of Resealing Adipoycte Ghosts in the Presence of ATP.

The energy requirement of the insulin-activation of transport was investigated in fat cell ghosts (Chapter 3). Preliminary investigations suggested that 1mM ATP, when added to the ghosts during the resealing incubation, apparently inhibited the insulin response of the sugar transport in ghosts. Illiano & Cuatrecasas (1971) have reported that ATP additions did not affect basal tranport in prepared ghosts.

Previous studies had indicated that the insulin effect was dependent on "energy"; agents which depleted intracellular ATP in intact cells were associated with inhibition of the insulin stimulation of sugar transport (Kono et al., 1977; Chandramouli et al., 1977; Siegel & Olefsky, 1980). Also, the high activation energy (E_{act} = 24 kcal/mol) of the coupling step between the insulin receptor and the transport system suggests an energy-requiring mechanism. Siegel & Olefsky, using 2,4-dinitrophenol to deplete fat cells of ATP, showed that endogenous ATP is only required for the maximum insulin effect to be achieved and subsequent ATP depletion did not inhibit the effect. Thus the activated transporter is not dependent on ATP to maintain activation, and it also appears that deactivation is an energy requiring process (Ciaraldi & Olefsky, 1980). One interpretation of the

present results is that ATP which has entered the ghosts during the resealing process has stimulated the deactivation of the insulin response, although no obvious trigger has been applied to encourage deactivation.

However, there are also reports that even small amounts of exogenous ATP may block the insulin response of intact cells (Chang & Cuatrecasas, 1974). It seems that although endogenous ATP is required for insulin activation of transport, exogenous ATP opposes this effect, perhaps acting solely at the plasma membrane. However, this report was of a prevention of stimulation by insulin, whereas our results show suppression of stimulated transport. The transporters would have been fully activated before disruption of the fat cells. Crude ghosts or pure plasma membranes do not show insulin-elevation of transport rates unless the intact cells are treated with insulin prior to disruption (Martin & Carter, 1970). It is possible that external ATP triggers the deactivation, which may occur because extra ATP has entered the ghosts during resealing. Although it would be surprising if the remnants of the cell cytoplasm within the ghosts were able to function efficiently to "deactivate" the transporters, in view of the disruption of the internal cell structures during lysis: Cushman (1970) shows, by transmission electron microscopy, the predominance of sheets of internal membranes in the intact cell, which vesicularize after hypotonic lysis and resealing (see Figs. 29 to 33), potentially disturbing the compartmentalization of metabolites.

It must of course be noted that the insulin response shown in ghosts was variable, and the lack of a response in three experiments is perhaps not conclusive evidence for an effect. It is interesting to find that this would be the only successful attempt to deactivate the "fossilized" insulin response in plasma membranes. Treatment with anti-insulin serum (Avruch *et al.*, 1972) or by removal of extrinsic membrane proteins (Pillion *et*

al., 1978) have been unsuccessful.

4.7. The Effect of Xanthines on Hexose Transport Kinetics.

4.7.1. Inhibition in Erythrocytes.

Studies on the effect of isobutylmethylxanthine (IBMX) in human red blood cell ghosts were carried out in conjunction with similar experiments on intact erythrocytes (reported by Challis et al., 1980). Whole cells were used to show that IBMX exerted its inhibitory effects on transport from the external face of the membrane. Influx was inhibited even when 0.75mM IBMX was added with the transport substrate. Measurements of the IBMX in whole cell lysates by monitoring the absorbance at 273nm, the λ_{max} of IBMX, showed that 0.75mM IBMX was fully equilibrated within thirty minutes, and that its effect was not dependent on internal IBMX since the inhibition (in this case, of influx in whole cells) did not increase with incubations of up to an hour: when IBMX was only inside in whole cells, influx was not inhibited, thus showing that the inhibitor acted only from the outside.

Table 23

Experimental Design	Parameter	Units	Ghosts	[a]Whole Cells
Zero *trans* entry	K_{zt}^{oi}	mM	[b]--	2.3 ± 0.15
	V_{zt}^{oi}	$mMmin^{-1}$	--	18.8 ± 0.7
Zero *trans* exit	K_{zt}^{io}	mM	≅5	32.9 ± 7.3
	V_{zt}^{io}	$mMmin^{-1}$	≅60	83 ± 11

Table 23: The zero *trans* parameters for D-glucose transport in IBMX-treated intact human erythrocytes and ghost at 20°C.
[a]Taken from Challis et al. (1980).
[b]No effect on basal rate.

Table 23 shows the effect of IBMX on the zero *trans* entry and exit parameters in whole cells. Resealing the IBMX into ghosts does not give different kinetics to whole cells, so the general remarks about IBMX access in whole cells probably apply equally to ghosts. However, from the Table it is seen that IBMX does not have similar inhibitory effects in red cells and ghosts: IBMX inhibits net efflux in both intact cells and in ghosts but net influx only in whole cells and not in ghosts. The effect on the whole cell influx parameters is small as is the increase caused in the efflux (internal) K_m (K_{zt}^{io}). In ghosts, there is a larger decrease in K_{zt}^{io}. In both instances, the largest effect of IBMX was on the depression of the efflux V_{max} (V_{zt}^{io}). In whole cells, the inhibition was noncompetitive, but uncompetitive in ghosts. For whole cells, internal IBMX had almost no effect on exit, but when outside of the cell (in the efflux buffer) or on both sides of the membrane, the inhibition of exit was marked.

Inhibitor kinetics provide additional evidence for particular transport models: the kinetics can be considered in terms of particular models. In terms of the asymmetric carrier model, one could consider that methylxanthines combine with the free carrier outside and free carrier inside and therefore retard net sugar flux by a reduction of empty carriers returning through the membrane. The effect of IBMX on the asymmetric carrier equation was described by Holman (in Challis *et al.*, 1980). V_{max} and K_m would both be affected (V_{max} more than K_m) by an inhibitor combining with unoccupied carrier. Exit would be more inhibited than entry if the inhibitor had a greater affinity for the carrier when it was outside than when it was available inside ($K_i > K_0$). However, the limitations of the asymmetric carrier model (namely the infinite *cis* parameters) apply equally to IBMX inhibited as to basal transport, although

infinite *cis* parameters were not measured in this present study.

The allosteric pore model (Section 1.7.10) can also describe the asymmetric inhibited transport. If the inhibitor combines preferentially with an external site, but only when the pore is doubly occupied from inside, with a vacant site outside, then uncompetitive inhibition of exit may be expected. Both V_{max} or K_m for exit will be decreased equally with no effect on entry. This was observed in the pink ghosts with IBMX (Results).

In intact red cells, a less specific effect appears to occur although it is not clear why there is this difference. Methylxanthines may combine with external sites in the pore both when the internal sites are unoccupied and also when the internal sites are occupied by substrate. It may also combine with or inactivate vacant internal sites in the presence and in the absence of substrate bound outside. If the inhibitor affinity for external sites is greater than that for internal sites, then greater inhibition of exit than of entry will result. For this less specific type of inhibition the inhibitor may combine with or inactivate internal and external sites simultaneously providing neither site is occupied by substrate. This will result in the mixed inhibition kinetics observed, since bound substrate will reduce the number of inhibitor molecules bound to each pore and some protection from inhibition will occur at high substrate concentrations.

4.7.2. Xanthine Inhibition in Adipocytes.

The type of inhibition by a particular compound observed in adipocytes, if similar to that in erythrocytes may lend more weight to suggestions that the transporter in both systems is the same entity (Holman *et al.*, 1981). IBMX inhibits zero *trans* entry and exchange in adipocytes (Chapter 3). This immediately suggests that the erythrocyte and adipocyte systems vary, since

zero *trans* exit and entry inhibition is not the same in either red blood cells or pink ghosts. The type of inhibition of zero *trans* entry observed in adipocytes is competitive, again different to that observed in human erythrocytes. This is the same as that for exchange of 3-O-methyl-D-glucose. Thus even the inhibition in adipocytes appears symmetrical.

Insulin-stimulated and basal transport is inhibited, and in both states the inhibition is competitive, once again highlighting the similarity in the type of transport in the basal and insulin-treated cells.

Since not all of the xanthines have the same effect on 3-O-methyl-D-glucose entry into adipocytes (Chapter 3), it would be interesting to compare their structure and to consider whether this correlates with their inhibitory nature, for example indicating where the important sugar transporter inhibition binding sites are located.

Firstly, IBMX, which has the largest inhibitory effect on basal and insulin-stimulated transport, will be examined. The most noticable structural features of this compound are the bulky isobutyl group at the N-3 position and a methyl group at N-1. The possibility of hydrogen bonding exists through the hydrogen accepting groups at C-2 and C-6. The major difference between the xanthines with inhibitory effects on the transport and those without is in the alkyl substituents. Theophylline (1,3-dimethyl xanthine), caffeine (1,3,7-trimethyl xanthine) and IBMX (3-isobutyl-1-methylxanthine) differ from xanthine and hypoxanthine in that methyl groups occur at N-1 in the former cases suggesting that this region may be important in transport inhibition. All three compounds also have alkyl substituents at the N-3 position and the size of the inhibition correlates with the size of this group: in theophylline and caffeine methyl groups occur at N-3, whereas in IBMX, the larger isobutyl group is present. Since theophylline and caffeine exhibit similar effects, the N-7 position methyl

group in caffeine (not found in IBMX) is probably unimportant.

With regard to the potential hydrogen bonding, only hypoxanthine lacks the C-2 position oxygen. Although hypoxanthine has small or negligible effects on the transport, it cannot be concluded that this oxygen is important for the transport inhibition because xanthine, whose sole difference is the presence of this C-2 position oxygen, also has no inhibitory effect on the transport. Neither does the C-6 position oxygen alone play a significant role in the transport inhibition because it is present in all of the compounds studied, both those with and those without inhibitory effects; it may be important in conjunction with other features.

The conclusion to be drawn from these studies is that the isobutyl group at N-3 is the most important feature of IBMX inhibition of hexose transport inhibition in adipocytes, although the N-1 methyl group may contribute to the inhibition. The importance of the overall spatial configuration of the alkylxanthine molecule to the inhibition has not been established; further studies with a wider range of nucleotide anologues are needed.

4.8. A Comparison of the Erythrocyte and Adipocyte Hexose Transporters.

The human red blood cell and rat adipocyte glucose transporters appear similar but not identical in several respects, for example in substrate specificity and antibody cross-reactivity (see Section 4.2.2). The adipocyte transporter has not been purified and unambiguous determinations of molecular weight have not yet been made. Thus its size and oligomericity cannot be compared to the human erythrocyte transporter, and it is not yet possible to definitively conclude whether or not the two transport systems are the same gene products.

These two transporters differ primarily in that only the rat adipocyte

transporter is regulated by insulin. The major kinetic difference lies in the symmetry of the adipocyte transport system for both the basal and insulin-stimulated 3-O-methyl-D-glucose transport (documented in this thesis) and the asymmetry observed in the human erythrocyte transporter. However, it is not the case that only regulated transporters are symmetrical because the facilitated diffusion of 3-O-methyl-D-glucose in the liver is not regulated by insulin but is also kinetically symmetric (Section 1.9).

It is unlikely that the kinetic differences in symmetry are brought about by modulation of the transport by extra subunits since IBMX inhibition also shows different effects in the two systems. It would therefore seem that the control of sugar transport by insulin is related to the production of different transporters in different classes of cells.

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