THE RELATIONSHIP BETWEEN INSULIN CONCENTRATION AND ITS EFFECT ON GLUCOSE UTILISATION AND METABOLISM IN THE ISOLATED RAT ADIPOCYTE

by

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Chapter 1

INTRODUCTION

It has been known for some considerable time that diabetes is associated with some loss of pancreatic function. In 1890 von Meering and Minkowsky showed that pancreatectomy caused diabetes in the dog and Schaffer (1895) suggested that the pancreatic control of carbohydrate metabolism might be mediated through an internal secretion of the islets of Langerhans. Studies involving pancreatic duct ligation and blockage subsequently confirmed the importance of the islet tissue in diabetes and Opie (1900-01) concluded from a study of pancreatic lesions that diabetes was always associated with a degeneration of the islet tissue.

Numerous workers attempted to demonstrate the involvement of a pancreatic hormone in the control of carbohydrate metabolism by isolating and characterising the active material. Scott (1912) tried to prevent inactivation of the material by heat or pancreatic enzymes and produced an extract which apparently reduced the sugar to nitrogen ratio in the urine of depancreatised dogs. However, Murlin and Kramer (1913) concluded that a pancreatic extract did not correct the deficiency in carbohydrate metabolism which followed pancreatectomy, though these workers used a boiled extract. The work of Knowlton and Starling (1912) provided clear evidence that a pancreatic hormone was indeed involved in the control of glucose uptake. They showed that the heart-lung preparation from a diabetic dog exhibited an impaired glucose utilisation and that perfusion of a preparation from a normal dog with blood from a diabetic animal was accompanied by a fall

in glucose utilisation. Conversely, perfusion of the diabetic preparation with blood from a normal dog restored the glucose utilisation towards the normal range. Finally, Knowlton and Starling (1912) prepared an acid extract of pancreas which increased the glucose utilisation of a diabetic dog heart.

This work culminated in the discovery by Banting and Best (1922) that doses of an acid-ethanol extract of a pancreas in which exocrine tissue degeneration was brought about by ligation of the duct of Wirsung could sustain depancreatised dogs. In the same year this group reported the successful control of a case of human diabetes (Banting et.al., 1922).

The activity of the pancreatic extract was attributed to insulin, a name first used by de Meyer (1909). Insulin was found to be a large molecular weight compound which could withstand dialysis through a vegetable membrane (Piper et.al., 1923). It was stable at low pH but was rapidly destroyed at high pH or by treatment with pepsin or trypsin (Dudley, 1923).

The first crystalline preparation of insulin was obtained by Abel (1926) and the first x-ray diffraction studies (Crowfoot, 1935) closely followed the demonstration by Scott and Fisher (1934) of a requirement for zinc or a similar divalent metal ion in the crystallisation process.

From a study of the physical properties of the insulin crystal, Crowfoot (1935) reported a molecular weight of 37,600, which was similar to the value of 35,100 obtained by Sjogren and Svedberg (1931) from ultracentrifugation studies. Furthermore, Crowfoot observed that the unit rhombohedron in the insulin crystal contained three equivalent subunits of approximately 12,000 molecular weight. The osmotic pressure studies of Gutfreund (1948a,b and 1952) and ultracentrifugation studies (Fredericq and Neurath, 1950; Oncley et.al., 1952

and Jeffrey and Coates, 1965, 1966) revealed the ability of insulin to undergo reversible association in solution. The state of association was found to depend upon such factors as the insulin concentration and the pH and ionic strength of the buffer. At concentrations below 0.167 mM in the pH range 2.0 to 2.7 insulin was found to dissociate completely to give subunits of approximately 6,000 molecular weight (Fredericq, 1956 and Jeffrey and Coates, 1966a,b). This figure corresponds closely with the formula weight of 5,733 established by the structure determination of beef insulin by Sanger and his colleagues (Ryle et.al., 1955).

Intensive efforts have since been made to determine the underlying mechanisms by which insulin regulates cellular metabolism.

Studies with purified preparations of insulin have shown that the hormone exerts diverse effects on many different cell types, including adipose tissue, bone and cartilage, lens, liver, mammary gland, muscle, skin and uterus (for review, see Krahl, 1961).

Much of the early work was concerned with the effect of insulin on glucose utilisation by muscle tissue. For example, Soskin and Levine (1940) showed that the glucose utilisation of the eviscerated dog was increased by the infusion of insulin at low glucose concentrations. Wick and Drury (1953) subsequently showed that the glucose utilisation of the eviscerated rabbit was enhanced by insulin even at high glucose concentrations. In quantitative terms the most important tissue in this preparation is muscle and it may be argued that this work provides evidence of an increased glucose utilisation by muscle in response to insulin.

Several workers have shown that the glucose utilisation of diaphragm muscle is increased in the presence of insulin (Gemmill, 1940; Chain, 1959 and Norman et.al., 1959) and an effect of insulin on the glucose utilisation of some other tissues has also been demonstrated (see Krahl, 1961). However, it became apparent that insulin did not merely increase the availability of glucose to tissues. For example, it has been claimed that insulin specifically increases glycogen deposition in diaphragm muscle (Gemmill and Hamman, 1941), CO_2 production by heart muscle (Cruickshank and Startup, 1934) and amino acid incorporation into diaphragm muscle protein (Krahl, 1952, 1953 and Sinex et.al., 1952).

Levine et.al. (1949) and Levine and Goldstein (1955) argued that all the effects of insulin which had been observed could be ascribed to a single, primary effect of the hormone on glucose transport. They suggested that glucose entry into muscle was normally rate-limiting for glucose utilisation (Levine et.al., 1949) and this was subsequently confirmed (Park et.al., 1956, 1957, 1959). This unitary hypothesis attributed the increased synthesis of proteins, polysaccharides and lipids to the increased supply of glucose carbon and energy available under the influence of insulin.

However, this attractive hypothesis was soon shown to be inadequate. Chain et.al. (1956) presented evidence of a directive effect of insulin on glycogen synthesis which was not apparently due to the elevated glucose utilisation. Norman et.al. (1959) subsequently showed that the increase in glucose utilisation observed with insulin was associated with a proportionately greater glycogen synthesis than a similar glucose utilisation obtained by manipulation of the glucose

concentration in the buffer. These reports are consistent with the demonstration by Villar-Palasi and Larner (1960) that treatment of diaphragms with insulin leads to an increase in the activity of glycogen synthetase in the tissue.

Furthermore, Manchester and Young (1958) observed a stimulation by insulin of amino acid incorporation into protein of diaphragm incubated without glucose, confirming earlier reports by Krahl (1952, 1953).

Wool (1964) has also shown that the effect of insulin on protein synthesis is independent of the hormone's actions on glucose and amino acid transport.

Furthermore, experimental data obtained with adipose tissue cannot be readily accommodated by the unitary hypothesis of Levine and his colleagues. A change in the proportions of pyruvate flowing into fatty acids and lactate was observed when insulin was added to adipose tissue incubated in glucose free buffer (Fain, 1964; Denton and Halperin, 1968; Halperin, 1970; Halperin and Robinson, 1970 and Saggerson and Greenbaum, 1970). In adipose tissue from fasted-refed rats insulin increased fatty acid synthesis and decreased the output of pyruvate into the medium when no glucose was present (Halperin, 1970). This enhancement of fatty acid synthesis can be correlated with the increased activity of pyruvate dehydrogenase (PDH) which has been observed in adipose tissue treated with insulin (Jungas, 1970; Denton et.al., 1971 and Weiss et.al., 1971). This activation of PDH does not appear to require the presence of substrate (Coore et.al., 1971) though Jungas and Taylor (1972) only observed full activation when glucose or fructose was present.

The epinephrine-stimulated release of free fatty acids from adipose tissue was shown to be inhibited by insulin in

the absence of glucose (Perry and Bowen, 1962 and Jungas and Ball, 1962).

An effect of insulin on electrolyte transport which was independent of the stimulation of glucose transport has also been observed (Gourley and Bethea, 1964). In the face of this body of evidence the hypothesis that all the effects of insulin arise from a stimulation of glucose transport is clearly no longer tenable.

The absence of a tenable unitary hypothesis makes a detailed study of the primary site of action of insulin difficult in view of the diversity of effects which have been observed. Nevertheless, insulin must first associate with some cellular receptor in order to produce these effects, though it is recognised that there may be more than one type of receptor (Rudman et.al., 1968).

Although all the various effects of insulin are no longer believed to be due to a stimulation of glucose transport there is much evidence of changes in the plasma membrane in response to insulin. Consideration of this evidence has led a number of authors to postulate unitary hypotheses of insulin action in which the diverse effects of the hormone are attributed to a primary change in membrane structure or function (Hechter, 1961; Hechter et.al., 1964; Hechter and Lester, 1964; Krahl, 1957; Levine, 1966; Rodbell et.al., 1968 and Ungar and Kadis, 1959). This evidence may be summarised as follows.

Many effects of insulin are only observed when the hormone is added to the intact cell and not to broken cell preparations. Examples of this are the stimulation of pyruvate dehydrogenase (Coore et.al., 1971) and glycogen

synthetase (Blatt and Kim, 1971) and changes in the concentration of 3',5'-AMP (cAMP) (Jungas, 1966).

Krahl (1957) envisaged an induced fit of a receptor located in the plasma membrane to insulin and proposed that this induced fit caused a propagated disturbance throughout the cell which preferentially directed glucose and other precursors to anabolic systems. Barrnett and Ball (1959, 1960) observed pronounced changes in the fine structure of adipocytes exposed to insulin in the absence of glucose. These changes included the formation of cytoplasmic vacuoles, an attenuation of the cytoplasmic matrix and changes in the structure of mitochondria suggesting an increased activity or growth of these organelles. However, Swift (1956) was unable to observe any changes in the structure of rat diaphragm following incubation with insulin. Beigelman and Hollander (1962) reported a change in the membrane potential of muscle following the addition of insulin to the incubation medium. This change did not require the presence of glucose or other exogenous substrate and it may indicate a fundamental change in the membrane structure in response to insulin.

Rodbell et.al. (1968) proposed that a primary effect of insulin on the adipocyte was to produce a fundamental change in the conformation of the plasma membrane lipoproteins from a laminar to a micellar form. Such a change has been observed following the addition of insulin to model membrane systems (Bangham and Horne, 1964).

In this context it is interesting to note that perturbations of the plasma membrane of the adipocyte have been shown to mimic some aspects of the action of insulin on these

cells. Kuo et.al. (1966a, b) showed that incubation of adipocytes with low concentrations of proteases led to an increase in glucose uptake which was not accompanied by marked cell damage. Indeed the activity of pronase in this system was probably not due to its hydrolytic activity, since an acid-denatured preparation of the enzyme, though no longer able to lyse adipocytes, retained its ability to enhance glucose uptake. Knno and Barham (1971b) subsequently showed that low concentrations of trypsin not only increased glucose uptake into adipocytes but also reduced epinephrine-stimulated lipolysis.

Furthermore it has been shown that phospho-lipases A and C (PHL-A and -C) at low concentrations increased glucose uptake and metabolism and reduced lipolysis in adipocytes (Blecher, 1965, 1966; Rodbell, 1966a and Rodbell and Jones, 1966). The activity of these enzymes in promoting glucose utilisation was found to be proportional to the extent of hydrolysis of phospholipids in the plasma membrane and incubation of adipocytes with high concentrations of these enzymes led ultimately to cell lysis. However, the carrier-mediated glucose transport system remained intact after mild digestion with PHL-C and the observed stimulation of glucose uptake was shown not to be due to the introduction of holes into the membrane (Rodbell, 1966a).

PHL-A hydrolyses the ester group on the -β-position of glycerophosphatides yielding lysophosphatides and fatty acids. It is interesting to note, therefore, that Blecher (1967) observed an increase in glucose uptake into adipocytes treated with low concentrations of lysophosphatidylcholine and ethanolamime. Rodbell et.al. (1968) have suggested that the insulin-like actions of PHL-A may result from a combination of

the hydrolysis of membrane lipids and a detergent action of the lysophosphatides produced.

Blecher (1966) concluded that insulin and the phospholipases affected a common receptor material in the plasma membrane and that this was probably a phospholipoprotein. He proposed that a change in the conformation of the phospholipoprotein in response to various treatments might modify the permeability of the plasma membrane to glucose and the metabolic distribution of the sugar within the cell. Electron microscopic evidence of a change in the conformation of the lipoproteins of a purified rat liver plasma membrane preparation from a laminar to a micellar form in response to insulin has been presented (Benedetti and Emmelot, 1966). Furthermore lysolecithin has been shown to induce micelle formation in a model membrane system (Bangham and Horne, 1964).

The nature of the signal which is propagated throughout the cell when insulin binds to its receptor remains to be discovered. Cuatrecasas (1971b) has shown that PHL-C digestion of the adipocyte under certain conditions destroys the ability of the cell to respond to insulin without reducing the capacity or affinity of the cell for ^{125}I-insulin. In addition Cuatrecasas (1972) has isolated a high molecular weight protein from liver and fat cell membranes of the rat which displays some of the properties expected of a receptor. This putative receptor displays a high affinity for insulin and has similar properties to the intact membrane with respect to the specificity and kinetic parameters of binding.

The search for a primary action of insulin on the cell received a strong stimulus with the discovery that some of the actions of insulin could be correlated with changes in the concentration of cAMP within the cell (Butcher et.al., 1966).

The mediation of the effects of insulin on cell metabolism through variations in the level of cAMP is consistent with a primary action of insulin on the plasma membrane since adenyl cyclase and phosphodiesterase, enzymes which determine the level of cAMP within the cell, are both located in the plasma membrane (Marinetti et.al., 1972 and Vaughan, 1972). These workers showed that insulin could inhibit adenyl cyclase (Marinetti et.al., 1972) and stimulate phosphodiesterase (Vaughan, 1972). A direct interaction of insulin with these enzymes may be excluded by the report of Jungas (1966) that insulin did not affect cAMP levels when it was added to cell homogenates.

An effect of insulin on cAMP levels has been observed in the presence of caffeine (Butcher and Baird, 1968), which inhibits phosphodiesterase and thus prolongs the lifetime of cAMP, and in the absence of methylxanthines (Sneyd et.al., 1968). Sutherland et.al. (1968) have shown that glycogenolysis is stimulated in conditions which elevate the intracellular level of cAMP and lipolysis rates have been correlated with cAMP levels within the cell (Butcher, 1970; Manganiello, Murad and Vaughan, 1971 and Rizack, 1964).

The original observation of Sneyd et.al. (1968) that insulin could depress the level of cAMP in the rat fat pad was confirmed by Knight and Cliffe (1973) and Soderling et.al. (1973). There appears to be no evidence to contradict these reports. Insulin has been shown to depress cAMP levels in free fat cells by several groups (Butcher et.al., 1966, 1968; Kuo and DeRenzo, 1969; Manganiello, 1971; Desai et.al., 1973 and Kono and Barham, 1973). However, several authors have failed to observe such an effect in free adipocytes (Fain,

1971; Fain and Rosenberg, 1972; Jarret et.al., 1972 and Khoo et.al., 1973).

Evidence against a central role for cAMP in the metabolic regulation of the adipocyte has been presented recently. Robison et.al. (1971) have shown that the effect of insulin on glucose transport into the adipocyte is independent of the cAMP level within the cell. Jungas (1971) has observed no change in the activity of PDH over a wide range of cAMP concentrations, an observation which was recently confirmed (Hucho, 1974). Thus two important effects of insulin on the adipocyte are not mediated through cAMP.

Curtis (1972) concluded from theoretical considerations that changes in the charge distribution across a membrane could lead to profound changes in the membrane structure. It has been shown that some of the effects of insulin on glucose metabolism can be reproduced by suitable manipulation of the ionic composition of the buffer (Hales and Perry, 1970). Furthermore, the incubation of adipocytes with ouabain has been shown to stimulate glucose uptake, pentose cycle activity and fatty acid synthesis and to suppress lipolysis and glycogenolysis (Ho and Jeanrenaud, 1967; Ho et.al., 1967). The effects of ouabain were mimicked by omitting K^+ from the medium and these results suggest that the distribution of certain ions across the plasma membrane might regulate membrane permeability to glucose and the pattern of glucose metabolism within the cell. These workers concluded that the actions of ouabain were due to its ability to reduce the activity of adenyl cyclase in the tissue (Ho et.al., 1967), though it is now clear that at least one of these actions (the stimulation of glucose uptake) cannot be so caused.

However, a primary action of insulin on electrolyte

transport appears unlikely, since an effect of insulin on glucose transport in adipocytes has been observed either with only Na^+ (letarte and Renold, 1967) or K^+ (Rodbell, 1965) in the buffer, whereas the stimulatory effect of ouabain requires the presence of both ions.

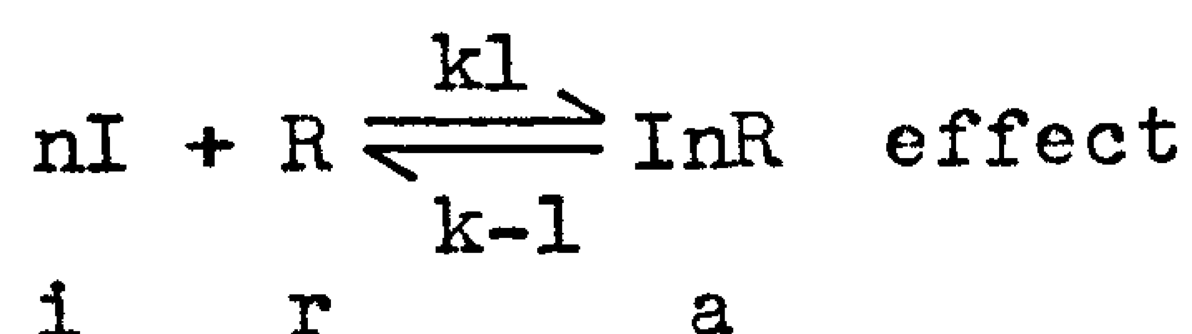
A unitary hypothesis has recently been proposed to account for results obtained with Sepharose-insulin derivatives. Oka and Topper (1971) have shown that mammary epithelial cells of mice responded to Sepharose-insulin but not to the native hormone. The response to Sepharose-insulin was blocked by preincubation with free insulin. These authors concluded that Sepharose-insulin stimulated the cell by binding to a specific receptor and simultaneously bringing into close contact the solid support and the cell surface close to the receptor. They suggested that the changed microenvironment might then produce charge effects (Nernst diffusion layers, Goldman et.al., 1971) or physical contacts which could elicit the biological response. This hypothesis was tested by attaching insulin to the Sepharose support with hydrocarbon chains of different lengths. Separation of the insulin molecule from the solid support by more than 10 to 12 A produced inactive derivatives, providing some support for this hypothesis.

Thus there is a widely held belief that the diverse effects of insulin follow some primary action at the membrane level (for a recent review see Fritz, 1972) though none of the hypotheses cited has suggested a molecular basis for the interaction between insulin and the cell. If this is so then it should be possible to demonstrate a relationship between insulin binding to the plasma membrane and its effect on

Stadie et.al. demonstrated an association between radioactively labelled insulin and diaphragm tissue (Stadie et.al. 1952a,b, 1953) and showed a quantitative relationship between the amount of insulin bound and its physiological activity (Stadie et.al., 1952b, 1953). However, Newerly and Berson (1957) disputed the physiological significance of the insulin binding. Garratt and his colleagues (1966a, b) demonstrated a linear relationship between ^{131}I-insulin binding to diaphragm and glucose utilisation until a limiting value of insulin association was attained. These workers concluded that a fraction of the observed binding was to an active receptor and the remainder to an inactive storage site.

The relationship between insulin concentration and its association with the cell might govern the form of the relationship between insulin concentration and the response of the cell to the hormone.

The relationship between insulin concentration and glucose utilisation in the adipocyte is apparently sigmoid and the observation has been made that such curves are frequently associated with co-operative binding systems (Garratt et.al., 1970). Garratt et.al. (1970) proposed that the sigmoid dose-response relationship might result from the co-operative binding of two or more insulin molecules to activate each receptor. The model proposed was



$$\underset{i}{nI} + \underset{r}{R} \underset{k-1}{\overset{k1}{\rightleftharpoons}} \underset{a}{InR} \quad \text{effect}$$

where I is insulin

R is receptor

i is the insulin concentration

a is the number of occupied receptors

$$\frac{i^n kl}{k-l} = \frac{a}{R-a}$$

The proportion of the total receptor binding $\left(\frac{a}{R-a}\right)$ cannot be readily determined. However, if it is assumed that the observed insulin effect is directly proportional to receptor binding (Garratt et.al., 1966a)

$$i^n K = C\left(\frac{e}{E-e}\right)$$

where C is a constant

e is the observed effect

E is the maximum effect

and a plot of log i versus $\log\left(\frac{e}{E-e}\right)$ will be a straight line with a slope of n, the Hill coefficient of co-operativity.

There is still uncertainty over the active species of insulin in solution (Blundell et.al., 1972). If the binding species was a dimer then this model allows the attractive hypothesis that the activated receptor contains an insulin hexamer (n = 3), which can exist in solution under appropriate conditions. If this were so and if the receptor-bound hexamer closely resembled that which exists in solution and in the crystal, then the groups involved in receptor binding and co-operative interactions between insulin molecules might be accurately defined and an understanding of the binding in molecular terms achieved.

There have been few attempts to describe in molecular terms how the initial interaction of insulin with the cell might produce the many effects which can be observed. However, the detailed knowledge of the conformation of insulin in the crystal (Blundell, 1972) and of its ability to aggregate in solution (Grant et.al., 1972) which is now available must stimulate thought in this direction. The major difficulty

encountered in the formulation of such a molecular hypothesis is the diversity of insulin effects which have been reported and hence the choice of a particular effect for study.

However, there is evidence that insulin can produce some of its characteristic effects on the cell without penetrating the plasma membrane. Insulin attached to bulky Sepharose beads has been shown to produce the characteristic effects of free insulin on glucose metabolism and lipolysis in the adipocyte (Cuatrecasas, 1969) and on RNA synthesis (Turkington, 1971) and α-aminoisobutyrate accumulation (Oka and Topper, 1971) in isolated mammary cells. In these studies no free insulin could be detected in the medium and no evidence of penetration of the beads into the cells was obtained.

Thus a study of the intracellular effects of insulin is unlikely to be rewarding, since any such effect must be several steps removed from the primary interaction with the cell if insulin does not penetrate the cell.

In contrast, a study of the effect of insulin on some aspect of membrane function might reasonably be expected to reveal a primary effect of insulin at the membrane level. A fundamental change in the plasma membrane in response to insulin could affect glucose utilisation if glucose transport was affected by the membrane change, providing glucose transport was rate-determining for utilisation. This condition excludes liver as a suitable tissue for study since the rate of glucose utilisation in liver is not determined by the rate of transport. Transport does appear to be the rate determining step for glucose utilisation in muscle under normal conditions (Park et.al., 1957). However, the rate of inactivation of insulin which is observed with muscle (Garratt et.al., 1966a) makes it an unsuitable tissue for the

study of the concentration dependence of insulin effects.

The isolated fat cell preparation first described by Rodbell (1964) has several important advantages for such a study. In contrast with muscle, a large number of replicate samples may be obtained from each isolated adipocyte preparation. This facilitates statistical treatment of the data. The connective tissue matrix and basement membrane are destroyed in the isolation of the cells. Thus uptake studies are not complicated by the need to estimate the rate of penetration of substances into the extracellular space, a difficulty which has been encountered with intact fat pads (Crofford and Renold, 1965). Although insulin is degraded at a finite rate by isolated adipocytes the loss of insulin over the concentration range within which glucose utilisation is sensitive is trivial with the density of cell suspension normally used (Gammeltoft and Gliemann, 1973). In addition, isolated adipocytes are extremely sensitive to small amounts of insulin (Gliemann, 1967).

Thus isolated adipocytes are suitable for the type of study envisaged providing conditions can be established under which glucose transport is rate determining for glucose utilisation.

The fat cell ghost preparation first described by Rodbell (1967) has been used to study glucose transport under initial velocity conditions (Illiano and Cuatrecasas, 1971). This study showed that transport of glucose was stereospecific, D-glucose being taken up much more rapidly than the L-form, and displayed saturation kinetics with respect to substrate concentration. Energy-producing metabolism was not linked to the transport process and no accumulation of glucose against a concentration gradient occurred. Furthermore, a unidirec-

tional efflux of glucose into medium with a negligible glucose concentration was accelerated by the addition of 3-O-methyl glucose (3-OMG) to the medium. These findings are consistent with the widely accepted hypothesis that glucose enters the adipocyte by a process of facilitated diffusion involving a mobile carrier mechanism.

In this context it is interesting to note that Walaas et. al. (1960a,b) succeeded in isolating a glucan-peptide complex from diaphragm muscle incubated with ^{14}C-glucose. Addition of insulin to the medium during incubation led to an increase in the specific activity of the complex, associated with an increase in the glucose utilisation of the tissue. The identity of this complex with a sugar-carrier complex has been suggested (Walaas et.al., 1960a).

A measurement of glucose utilisation by adipose tissue is a measurement of net glucose transport, since the carrier system can move glucose in both directions. Under conditions in which the concentration of free glucose within the cell is negligible the outward transport of glucose would be insignificant and glucose utilisation would be equivalent to the inward transport of glucose.

MacLeod et.al. (1960) claimed that the addition of insulin to intact rat fat pads led to a fall in the intracellular glucose concentration and an increase in the concentration of glucose-6-phosphate within the cells. They concluded, therefore, that insulin increased the rate of phosphorylation of glucose and that this increase in rate was probably due to an activation of hexokinase. However, Hernandez and Sols (1963) demonstrated the existence of a stereospecific hexose transport step before phosphorylation and showed that the addition of insulin to fat pads did not significantly increase hexokinase

activity, even when it was reduced after a period of fasting. Furthermore, these workers showed that the activity of the hexokinase isolated from adipose tissue was greater than that required to account for the maximum rates of glucose utilisation observed with insulin. They concluded that phosphorylation was not rate-limiting for utilisation even with the highest uptake rates observed in their studies, which were obtained by incubating adipose tissue in 30 mM (5.4 mg/ml) glucose buffer in the presence of insulin.

This conclusion is supported by the reports of several workers. Froesch and Ginsberg (1962) observed an inhibition of fructose metabolism by glucose in fat pads when both sugars were present at a concentration of 8.0 mg/ml in the presence of a maximally stimulating insulin concentration. At lower sugar concentrations there was no inhibition. The observed inhibition was attributed to a competition for hexokinase and Froesch and Ginsberg concluded that there was only substantial free glucose within the cell at the highest concentration used (that is to say at 8.0 mg/ml), since in cell-free systems the affinity of hexokinase for glucose is 5 to 10 times greater than its affinity for fructose. DiPietro (1963) drew essentially similar conclusions. Crofford and Renold (1965) were subsequently able to demonstrate directly that glucose uptake is rate-limiting for utilisation and is the principle site of insulin action in the fat pad. They were only able to observe an increase in free intracellular glucose in the fat pad with a medium glucose concentration of 80 mM (approximately 14.5 mg/ml) in the presence of insulin.

The studies cited above show that at low glucose concentrations (below 5.0 mg/ml) the concentration of free glucose within the adipocyte is negligible and therefore the outward

transport of glucose would be expected to be negligible. Under these conditions a measurement of glucose utilisation would therefore be equivalent to a measurement of the inward transport of glucose.

In spite of the detailed knowledge referred to above of the structure and conformation of insulin and of its ability to aggregate in solution, few of the current hypotheses of insulin action make use of this information. An interpretation of the sigmoid relationship between insulin concentration and glucose utilisation in the adipocyte suggests one way in which this knowledge might be used to describe the process by which insulin associates with the cell. This interpretation of the dose response curve permits the formulation of a hypothesis which describes the initial interaction of insulin with the cell in molecular terms.

This is likely to be an especially productive approach because evidence is presented to support the hypothesis that a primary effect of insulin is exerted at the membrane level. It therefore seemed that such an effect of insulin might be revealed by a study of the effect of insulin on some aspect of membrane function, such as glucose transport.

The work described in this thesis was designed to use the relationship between insulin concentration and glucose utilisation by isolated adipocytes to study a possible primary effect of insulin at the membrane level. It was thought that a careful study of the dose-response relationship might permit a description of the initial interaction between insulin and the cell in molecular terms and lead to an increased understanding of the way in which this interaction affects the membrane.

In order to carry out these studies it was necessary to develop a technique for accurately measuring glucose utilisation in the adipocyte.

Chapter 2

EXPERIMENTAL PROCEDURES

2.1 Introduction

In all the experiments described in this thesis the isolated rat epididymal adipocyte preparation first described by Rodbell (1964) was used. The uptake and metabolic fate of glucose under various experimental conditions was determined by means of radioisotopic tracer techniques.

This chapter contains details of the procedures used to extract and estimate ^{14}C-labelled metabolites of glucose. A simple, accurate technique is also described for the determination of total glucose utilisation using 5-^{3}H-glucose which overcomes many of the difficulties encountered with the use of conventional techniques.

Procedures used to estimate the loss of insulin from solutions exposed to adipocytes are described.

The isolation and purification of a sample of guinea pig insulin is also described.

All reagents used in this work were of recognised analytical grade unless otherwise stated, and all solutions were prepared using deionised distilled water. The animals used in these studies were male Wistar rats of 100-120g drawn from a breeding colony maintained at the University of York.

2.2 Buffer Preparation

Reagents used

Water: all water used was glass distilled and then deionised.

Bovine serum albumin: Fraction V (Sigma Chemical Company).

The necessary amount of BSA was dissolved in water to give an 8% solution and dialysed against water for 18 hours.

Buffer salts (All of analytical grade)	Final concentration in buffer
	mM
NaCl	240
KCl	9.7
$CaCl_2.2H_2O$	2.6
KH_2PO_4	2.4
$MgSO_4.7H_2O$	5.0
$NaHCO_3$	47.2

Glucose: analytical grade.

Insulin: 10 times recrystallised beef insulin, a gift from Novo Industries, AS. A stock solution was prepared by dissolving 60 mg in 10 ml of 0.01 N hydrochloric acid.

The exact concentration of the solution was determined by the spectrophotometric method of Harrison and Garratt (1969) and it was adjusted to be 1 mM if necessary. 200 µl aliquots of the solution were stored frozen (-15°) in plastic tubes.

U-^{14}C-glucose: specific activity 3 mCi/mmol (obtained from the Radiochemical Centre, Amersham). The lyophilised sugar was dissolved in water to give a stock solution containing 0.25 mCi/ml. This solution was stored frozen. The

U-^{14}C-glucose used for some experiments was first purified (see preparation of Buffer containing U-^{14}C-glucose).

5-^{3}H-glucose: specific activity 1 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, as a sterile, aqueous solution. This solution was diluted to 0.25 µCi/ml with water and this stock solution was stored frozen. 5-^{3}H-glucose was routinely purified for each experiment (see Buffer containing 5-^{3}H-glucose).

Collagenase: a crude bacterial enzyme, specific activity approximately 125-200 U/mg, was obtained from Worthington Chemical Company.

Basic Buffer

The Basic Buffer used in these experiments was a modified Krebs Ringer bicarbonate buffer (pH 7.4). It differed from the standard Krebs Ringer buffer (Krebs and Henseleit, 1932) in having half the specified concentration of calcium dichloride and BSA was added to give a final concentration of 2% (w/v). The salts were mixed in the proportions indicated and dissolved in water. The dialysed BSA was added and the buffer was diluted to volume with water. The buffer solution was then gassed carefully with 95% O_2/5% CO_2 to bring the pH to 7.4.

The various buffers needed for an experiment were then prepared from this Basic Buffer as described below.

Buffer containing glucose

If a single glucose concentration was required for an experiment it was prepared by adding the appropriate weight of the sugar directly to the Basic Buffer. In the dose response and transport studies (Chapters 3 and 5 respectively)

a single glucose concentration of 0.1 mg/ml (approximately 0.55 mM) was used (see 2.8.2).

In the metabolite studies (Chapter 4) a range of glucose concentrations was prepared as follows. A 5 mg/ml glucose solution was prepared by dissolving 125 mg of glucose in 25 ml of basic buffer. A ten-fold dilution was then made by transferring 1 ml to 10 ml volumetric flask and diluting with Basic Buffer. The required range of glucose concentrations was then prepared by adding appropriate volumes of these working solutions to the incubation vials.

Buffer containing insulin

Working insulin solutions were prepared by serial dilution of the stock insulin solution with Basic Buffer, giving a range from 5×10^{-12} to 5×10^{-9}M in factors of ten. Tubes of stock insulin solution, once thawed, were not returned to the freezer for further use.

Buffer containing U-^{14}C-glucose

A sample of the stock solution was diluted with Basic Buffer to give an appropriate working solution, the activity of this solution being varied according to the aims of each experiment. The activity was typically of the order of 2.5 µCi/ml. In most experiments described in this thesis the stock U-^{14}C-glucose was used without further purification. However, when glucose incorporation into total anionic metabolites was being studied the U-^{14}C-glucose was found to require further purification (see below). The glucose was purified by ascending paper chromatography the day before it was required and stored dried on paper in the cold.

The U-^{14}C-glucose was applied to a 1 x 20 cm strip of Whatman No. 1 paper under a stream of air and developed in

T-But-Form solvent (t-butanol: methyl ethyl ketone: water: formic acid; 44:44:11:0.26 by volume). The solvent front was allowed to run 15 cm and the glucose, which runs with an Rf of 0.1, was eluted with the appropriate volume of Basic Buffer. Recovery was typically of the order of 85%.

Purification of the U-^{14}C-glucose used for total anionic metabolite determination was necessary to bring the blank values within acceptable limits. Labelled anions were separated from unmetabolised U-^{14}C-glucose by anion exchange chromatography on Sephadex A-25-120 (see Section 2.5). Standards of U-^{14}C-glucose (prepared from the U-^{14}C-glucose stock solution) and U-^{14}C-lactate were therefore prepared in 500 µl of Basic Buffer to which 500 µl of 20% trichloroacetic acid was added (see Section 2.4, Adipocyte incubation) to estimate the separation and recovery of these compounds under appropriate conditions.

Samples were centrifuged at 2000 xg for 15 minutes to ensure complete precipitation of the albumin. 500 µl aliquots of the supernatant medium were diluted to 10 ml with degassed water and the whole was applied to the column. Unreacted glucose and uncharged metabolites were washed through the column with 40 ml of degassed water, the effluent being collected into weighed test tubes. Anions were eluted with 15 ml of 0.5 M hydrochloric acid, fractions again being collected into weighed test tubes. 1 ml aliquots were removed from the tubes for counting in 6 ml of Bray's. The acid effluent caused severe and variable quenching and so the quench was determined in each vial by internal standardisation.

Washing the columns with water did not lead to the

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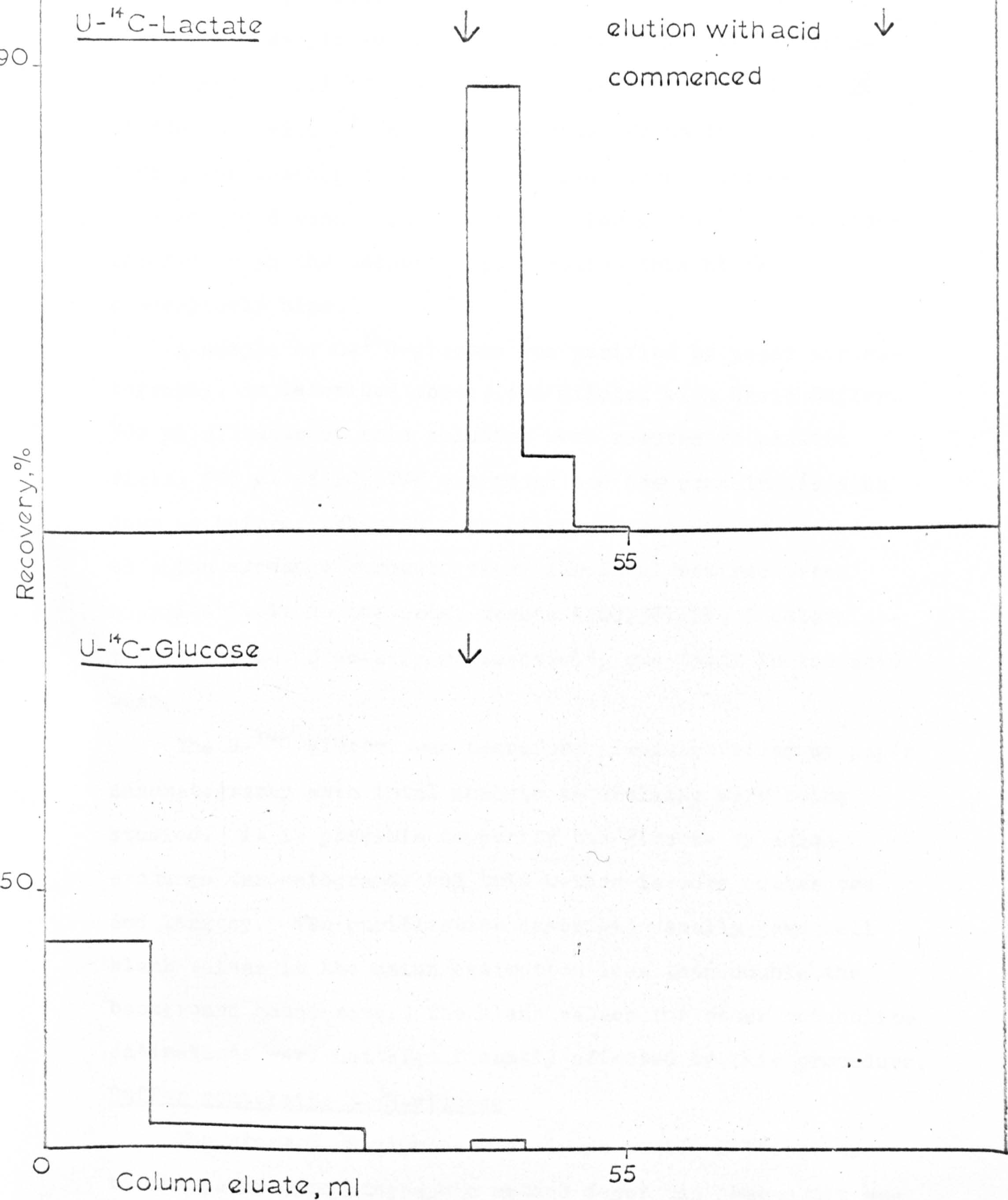
Figure 2.1 Anion exchange chromatography of U-^{14}C-glucose and U-^{14}C-Lactate



elution of significant amounts of lactate (Fig. 2.1). The lactate was quantitatively recovered in the acid wash ($106.2 \pm 2.2\%$, 4 determinations), the large correction factors for sample volume and quenching probably contributing substantially to the slight overestimation. About 2% of the applied U-^{14}C-glucose was retained on the column during the washing with water and was subsequently eluted with the acid wash. In view of the low glucose utilisations observed with the adipocyte preparation this blank value was unacceptably high.

A sample of U-^{14}C-glucose was purified by paper chromatography, as described above, and diluted with Basic Buffer. 500 µl aliquots of this solution were removed to plastic vials, 500 µl of 20% TCA was added and the protein was spun down as before. When this purified glucose was subjected to anion exchange chromatography the label was recovered quantitatively in the water washes ($100.5 \pm 1.2\%$, 5 determinations) and no detectable radioactivity was found in the acid wash.

The U-^{14}C-glucose was therefore always purified by paper chromatography when total anionic metabolites were being studied. It is possible to purify the glucose by anion exchange chromatography but this method is more cumbersome and lengthy. The purification described usually gave cell blank values in the anion estimation less than double the background count rate. The blank values for other metabolite estimations were not significantly affected by this procedure.

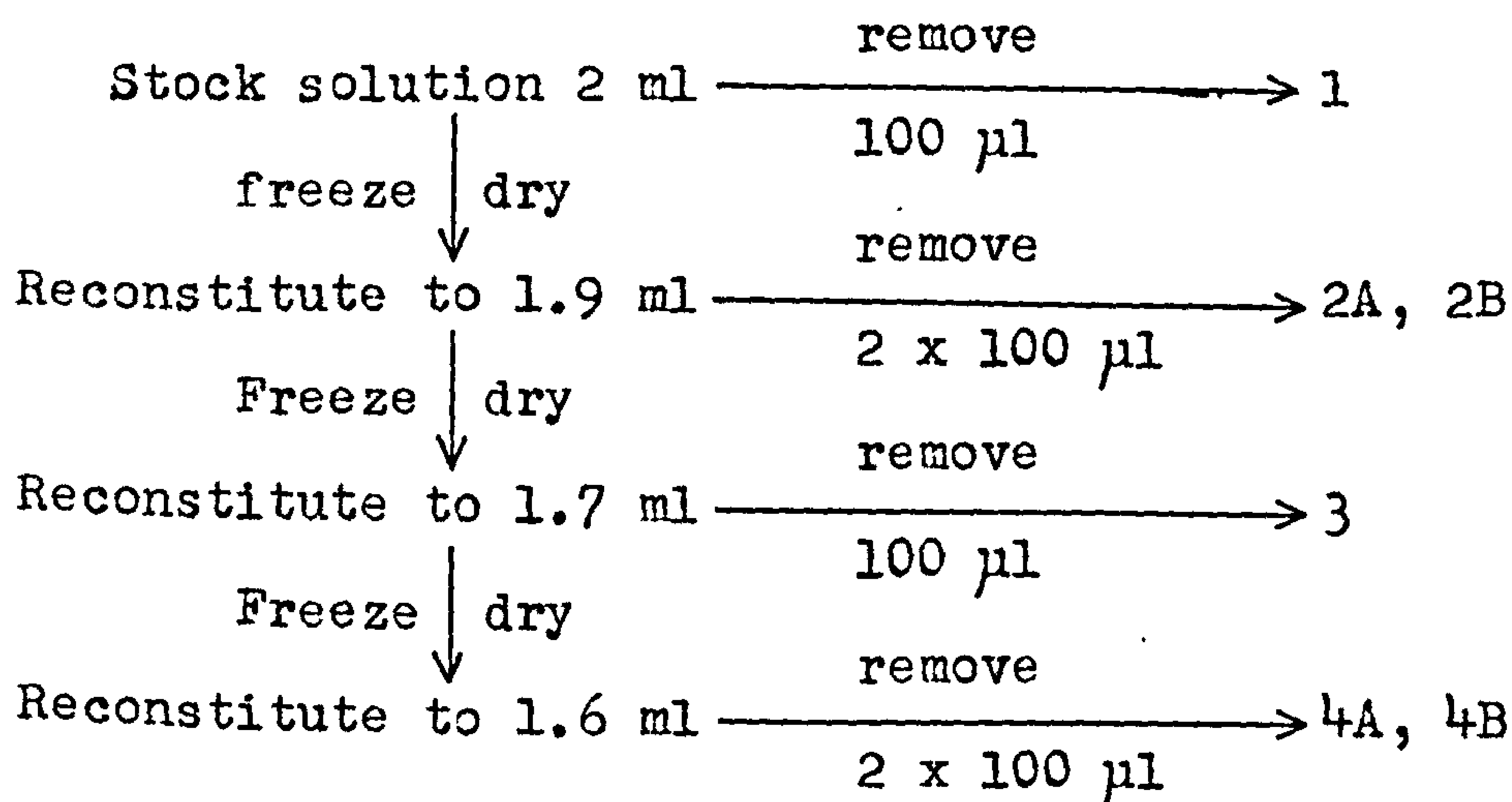
<u>Buffer containing 5-^{3}H-glucose</u>

The stock 5-^{3}H-glucose was always purified before use by the paper chromatographic method described above. It was

stored overnight dried on paper in an evacuated dessicator and eluted with Basic Buffer to give a working solution containing approximately 15 µCi/ml.

In the absence of insulin adipocytes use less than 5% of the available glucose in the system used in this work (2.4). However, lyophilisation of the stock 5-^{3}H-glucose solution (about 80 nCi, as described in 2.5.5) prepared in Basic Buffer as a cell blank (defined in 2.4), led to the transfer of almost 4% of the label initially present. Such a blank value was clearly unacceptable in view of the low glucose utilisations expected and the effect of 5-^{3}H-glucose purification on cell blanks was studied.

The first method of purification used was repeated lyophilisation and solution of the 5-^{3}H-glucose. 6 incubation vials were prepared containing 0.9 ml of Basic Buffer. 100 µl of stock 5-^{3}H-glucose solution was diluted to 2 ml with water. A 100 µl sample was withdrawn from this solution and the bulk was lyophilised. The dried 5-^{3}H-glucose was then redissolved in 1.9 ml of water, 2 x 100 µl samples were withdrawn and the process repeated according to the following scheme:



The samples removed were added to the vials containing buffer, 500 ul of 20% TCA was added and samples were lyophilised as described in 2.5.5. 3 x 100 µl aliquots of the final 5-^{3}H-glucose solution were incubated with 0.9 ml of Basic Buffer at 37° for 2 hours. These vials were then acidified and lyophilised. The results are shown in Table 2.1. The first column indicates the transfer of label during the lyophilisation of unpurified glucose. Successive purifications reduced this value from 3.7% to 0.2%, an acceptable value, though still considerably higher than the background count rate. Incubation of the 5-^{3}H-glucose for 2 hours at 37° (INC) slightly raised the blank value.

5-^{3}H-glucose was also purified by ascending paper chromatography in t-But-Form. The 5-^{3}H-glucose was applied to a 1 x 20 cm strip of Whatman No. 1 paper under a stream of air and developed in t-But-Form solvent. The solvent front was allowed to run 15 cm and the glucose, which runs with an Rf of 0.1, was eluted with Basic Buffer. 94% of the label applied ran as a compact spot with an Rf of 0.1, though there was also a minor spot with an Rf of 0.57 (Fig. 2.2). The eluted 5-^{3}H-glucose was diluted with Basic Buffer to give the same specific activity as the sugar purified by repeated lyophilisation. 1 ml of this solution was then acidified by addition of 500 µl of 20% TCA and the sample was lyophilised. The activity of the lyophilisate is described in Table 2.1. Clearly the purification by paper chromatography reduced the blank value below that obtained with 3 successive lyophilisations. This method was therefore used routinely for 5-^{3}H-glucose purification. Incubation at 37° for periods up to 2 hours has not been found to

Table 2.1 Effect of purification of 5-^{3}H-glucose on cell blank values

Purification method	Number of purification steps					
	0	1	2	3	INC	BG
Freeze drying						
cp10 min^{x} 250 µl sample	11,135	4,452	2,445	1,151	1,635	411
Proportion of total label transferred,%	3.7	1.4	0.8	0.2		
Paper chromatography						
cp10 min^{x} 250 µl sample		872				

^{x} 250 µl of the lyophilisates were counted in toluene-Triton X-100

Figure 2.2 Paper chromatography of 5-^{3}H-Glucose in t-but-form (for details see text).

Recovery, %

19

solvent front

0

15

Distance from origin, cm

seriously elevate blank values.

Buffer containing collagenase

The enzyme was dissolved in basic buffer to give a working solution of approximately 1 mg/ml. The exact concentration of the working solution was adjusted according to the specific activity of each batch of enzyme purchased (see Preparation of adipocytes).

2.3 Preparation of isolated adipocytes

Isolated adipocytes were prepared from the epididymal fat pads of male Wistar rats with a weight range of 100-120g. The animals were allowed unlimited access to water and commercial animal feed. The narrow weight range was specified since it has been shown that the sensitivity of adipose tissue to insulin decreases rapidly as the age and size of the animal increases (Stock and Beigelman, 1967).

The method used in the adipocyte preparation is a modification of the methods published by Rodbell (1964) and Gliemann (1967). Gliemann (1967) has shown that adipocytes are extremely sensitive to the vigorous mechanical procedures, particularly stirring, which are required in the isolation process. Since great care was found to be necessary in the handling of fat pads and free adipocytes if hormone sensitivity was to be preserved, the procedure used is described in full.

All apparatus to which fat cells were exposed was made of plastic, except the dispensing micropipette, since it has been shown that fat cells adhere to glass and rupture (Rodbell, 1964).

After the animals had been killed by a blow on the head

the epididymal fat pads were removed to a petri dish containing Basic Buffer and rinsed. Each pair of fat pads was then transferred to a new 30 ml polythene scintillation vial (G. D. Searle & Co.) containing 1.5 ml of buffer with 0.1 mg/ml glucose and approximately 0.5 mg/ml collagenase. A stream of 95% O_2/5% CO_2 was passed over the buffer and the vial sealed. In a typical experiment fat pads from four rats were incubated simultaneously in individual vials.

The vials were incubated at 37° on a rapidly shaking metabolic bath (140 cycles/min., stroke length 3 cm) for about an hour until disruption of the fat pads occurred.

The integrity and hormone sensitivity of the adipocytes released was found to depend on the concentration of collagenase used and the duration of the incubation period. The purer preparations of enzyme were found to be less effective in releasing cells from fat pads. Consequently the optimum concentration was determined for each new batch of enzyme purchased.

After an hour the vials were inspected and any containing large fat droplets, indicating substantial cell damage, were rejected. The contents of the remaining vials were filtered through a nylon gauze into a polypropylene test tube. 5 ml of buffer containing 0.1 mg/ml glucose was then poured into each vial and filtered in similar fashion, these washings being added to the test tube. A plastic cannula was passed through the cell layer and after the tube had been centrifuged (approximately 200 xg for 30 seconds), the infranatant buffer was removed with a syringe. The adipocytes were resuspended by gentle agitation in 10 ml of buffer containing 0.1 mg/ml glucose, and washed three times by the

same procedure. Finally the cells were suspended in the required volume of buffer containing 0.1 mg/ml glucose in a 30 ml vial containing a 2 cm Teflon-coated stirring bead. The vial was placed in a water bath (37°) on top of a magnetic stirrer, the stirring speed being adjusted so that the cells were just maintained in a homogeneous suspension, and a stream of 95% O_2/5% CO_2 was passed over them. This cell suspension contained 2-8 µmol triglyceride per ml which is approximately equivalent to 3.0×10^4 to 1.5×10^5 cells/ml.

The adipocyte suspension has been examined by light microscopy and found to consist of intact, single adipocytes essentially free of other cell types.

2.4 Adipocyte incubation

Incubations were carried out in 6 x 1.5 cm polythene specimen tubes (Xlon Ltd.) sealed with a Suba seal carrying a small hanging plastic well. New vials were used for each experiment as the use of washed vials was found frequently to result in decreased insulin sensitivity and reduced reproducibility.

Incubation vials were prepared by dispensing into each a total volume of 250 or 400 µl of buffer containing glucose, radioactive glucose and insulin. Radioactive glucose was dispensed in 50 µl volumes, the volumes of other constituents being determined by the required final concentration. All additions were made from a syringe microburette (calibrated 1 µl/division or 5 µl/division, Micrometric Instrument Co., Cleveland, Ohio). Weighing experiments indicated that the dispensing methods used were accurate and reproducible (Table 2.2).

Table 2.2 Weighing experiments performed to study the accuracy and reproducibility of the dispensing techniques used

Nominal volume dispensed, µl	Weight of water collected, mg Mean ± S. D.	Determinations
50[1]	49.56±1.1	5
100[2]	98.9±4.6	5
250[2]	250.4±0.4	5

[1] Dispensed by microburette

[2] Dispensed by micropipette

Adipocytes were dispensed at 30 second intervals from a micropipette, bringing the total incubation volume to 500 µl. 250 µl aliquots of cell suspension were usually dispensed, though when the glucose concentration was low and high rates of glucose utilisation were expected, 100 µl aliquots were used.

After the addition of cells vials were regassed, stoppered and placed on a metabolic shaker at 37° and 60 cycles/min. Incubations were routinely for 1.5 hours.

At the end of the incubation period the adipocytes were killed by injection through the Suba seal of 500 µl of 20% trichloroacetic acid. The methods used for the extraction and estimation of metabolites of glucose will be described in the next section.

Suitable control samples (termed cell blanks) to measure the blank values for incorporation of radioactivity into the various fractions examined were always prepared by adding adipocytes directly to buffer which had received 500 µl of 20% TCA.

Table 2.3 shows the good reproducibility achieved in dispensing adipocytes by micropipette, the variation between replicates being less than 5%. These results were obtained by pipetting cells labelled with ^{14}C in the triglyceride as follows. A suspension of adipocytes was prepared and divided equally between two 30 ml polythene vials, the final volumes being adjusted to 5 ml. Each vial contained 2 µCi/ml of U-^{14}C-glucose and 0.1 mg/ml of total glucose. One vial contained a maximally stimulating concentration of insulin (10^{-9}M). The vials were gassed thoroughly with 95% O_2/5% CO_2, sealed and incubated at 37° on a gently

Table 2.3 The reproducibility of cell dispensing, indicated by the transfer of ^{14}C-triglyceride

Volume of suspension, µl	Insulin	^{14}C triglyceride transferred, c.p. min., background subtracted (Mean $\pm$ S.D., 10 obs.)	Standard deviation as % of total
250	-	4,570 $\pm$ 240	5.25
	+	13,530 $\pm$ 630	4.65

shaking water bath (60 cpm) for 1 hour. Then, at 30 second intervals, 250 µl aliquots of the suspension were removed by micropipette to 5 ml vials containing 500 µl of 20% TCA and the triglyceride was immediately extracted by the method of Dole and Meinertz (1960) (see 2.5.2). The reproducibility of the transfer of ^{14}C-triglyceride, corrected for incubation time, gives a measure of the reproducibility of cell transfer.

2.5 Analysis

Scintillation solutions used

1. Bray's scintillator (1960)

Naphthalene	SLR	120 g
PPO		8 g
POPOP		0.4 g
Methanol	AR	40 ml
Ethanediol	AR	40 ml
1, 4-Dioxan	SLR	to 2 L

2. Toluene scintillator

PPO		2 g
POPOP		0.1 g
Toluene	AR	500 ml

3. Toluene Triton X-100 (Patterson & Green, 1965)

PPO		12 g
POPOP		0.6 g
Triton X-100		1 L
Toluene	AR	2 L

1M Ethanolic KOH: 2.8 g of potassium hydroxide (S.L.R.) dissolved in 5 ml of water and diluted to 100 ml with absolute ethanol (analytical grade).

Radioactivity in samples was counted in a Nuclear Chicago Model 6850 Liquid Scintillation Counter or a Packard Model 2002 Counter for 10 minutes or until 1,000 counts above the background had accumulated.

The Model 6850 counted ^{14}C-hexadecane (Radiochemical Centre, Amersham) disintegrations with an efficiency ranging from 52-88%, depending upon the scintillator used and the quench of the sample counted. ^{3}H-hexadecane in 6 ml of Toluene Triton X-100 scintillator counted with an efficiency in the range 22-25%.

The Model 2002 counted ^{14}C-toluene (Packard Co.) with an efficiency of 75-85% depending on the scintillator and the sample quenching. ^{3}H-toluene in 6 ml of Toluene Triton X-100 was counted with 40-45% efficiency, depending upon the sample preparation.

Internal standardisation was carried out by adding to each vial a sample of ^{14}C-triglyceride of known activity and calculating the ratio of the theoretical increment in count rate to the observed value. Sample counts were then increased in this ratio to correct for quenching.

The specific activity of the U-^{14}C-glucose used in these experiments was such that its contribution to the total glucose concentration in the incubation vial had to be taken into account when calculating results. For example, an incubation vial containing 500 µl of buffer with 0.1 mg/ml glucose and 125 nCi U-^{14}C-glucose would contain 318 nmol of glucose, of which 42 nmol was derived from the U-^{14}C-glucose.

2.5.1 Estimation of carbon dioxide production

Reagent used: Hyamine Hydroxide 1M in methanol, obtained from Nuclear Enterprises Ltd., Edinburgh.

100 µl of Hyamine Hydroxide was injected through the Suba seal into the hanging well by syringe after acidification of the medium. 1 hour was then allowed to elapse to ensure complete trapping, the vials being gently agitated at room temperature. The adequacy of this procedure has been demonstrated in this laboratory (Evans, 1973) and elsewhere (Rodbell, 1964). The well was removed from the seal, carefully wiped on the outside and transferred to a scintillation vial containing 6 ml of Bray's scintillator for counting.

Addition of 100 µl of Hyamine to 6 ml of Bray's quenched the ^{14}C counts by up to 10%. The exact quench was therefore determined either by internal standardisation (Model 6850) or by the use of a ^{226}Radium external standard (Model 2002) and the counts were corrected to the efficiency of a standard with no Hyamine.

At the same time a standard, consisting of the same amount of U-^{14}C-glucose as the incubation vials in 6 ml of Bray's, was counted.

The proportion of glucose converted to carbon dioxide was then calculated using the ^{14}C-glucose standard counts and the appropriate quench correction factor. The results were expressed as glucose incorporation into carbon dioxide in nmols/hour/µmol triglyceride. For triglyceride estimation see 2.6.1.

2.5.2 Estimation of triglyceride production

Reagents used: Propan-2-ol S.L.R.

n-Heptane S.L.R.

Triglycerides were extracted from the adipocytes by the method of Dole and Meinertz (1960). The extraction was performed on the incubation vial contents after CO_2 collection, the residue after lyophilisation or the TCA precipitate after decanting the supernatant for further analysis. The aqueous supernatant contained no significant heptane-soluble radioactivity.

5 ml of a mixture of propan-2-ol:n-heptane (4:1) was then added to each vial, the vials were sealed and shaken for 6 hours. The vial contents were transferred to stoppered test tubes, each vial being washed with 3 ml of heptane and the washings added to the tube. The heptane layer was washed with 3 ml of water and the tubes were centrifuged at about 2,000 xg for 20 minutes to remove traces of water from the heptane before removal of a 500 µl sample to 6 ml of toluene scintillator for counting. A 2 ml sample was also removed for saponification (2.5.3). Heptane does not quench ^{14}C counts in this scintillator.

Since some heptane was lost by evaporation during the sampling it was necessary to calculate the proportion of the total triglyceride contained in these samples. This was done as follows.

Triglyceride labelled with ^{14}C was prepared by incubating adipocytes with U-^{14}C-glucose and it was extracted by the Dole-Meinertz method. The product was concentrated on a rotary evaporator to give a heptane solution of ^{14}C-triglyceride of high activity. Aliquots (usually 10 µl) of this solution were added to the residual heptane in the stoppered

tubes after the necessary samples for counting and saponification had been removed. An aliquot of this volume introduced sufficient label to give a considerable excess over the sample counts. A sample of the heptane layer was then removed to 6 ml of toluene scintillator and counted together with a ^{14}C-triglyceride standard. The dilution of the ^{14}C-triglyceride in the heptane was calculated and used to estimate the residual solvent volume.

This procedure was normally applied to a random sample of 10 tubes in each experiment, a typical value being 3.73 ± 0.04 ml (mean $\pm$ S.D., 10 determinations) for the total volume of heptane.

The samples were counted together with a standard prepared from the U-^{14}C-glucose working solution. This standard consisted of 50 µl of the working solution in 6 ml of Bray's scintillator, which counted with an efficiency of $75.9 \pm 0.6\%$ (mean $\pm$ S.D., 3 determinations). The samples counted with an efficiency of $74.95 \pm 0.7\%$ (3 observations) in toluene scintillator.

The conversion of glucose to triglyceride was then estimated by relating sample counts to the standard (after correcting for the difference in counting efficiency) and applying the appropriate volume correction. Results were expressed as glucose incorporation into triglyceride in nmols/hour/µmol triglyceride.

2.5.3 Glyceride fatty acids and glycerol estimation

2 ml of the heptane layer of a Dole-Meinertz extraction was transferred to a stoppered tube and evaporated to dryness in a 70° water bath under a stream of air. The residue was redissolved in 1 ml of 1M ethanolic potassium hydroxide solution and the tubes were heated at 70° for 6 hours. Residual alcohol was removed under a stream of air and the solid residue was taken up in 1 ml of water. This solution was acidified by addition of 2 ml of 0.5 M hydrochloric acid and 2 ml of heptane was added to extract the fatty acids. The tubes were then carefully stoppered and shaken (100 cpm) overnight at room temperature.

After centrifugation at 2,000 xg for 20 minutes a 1 ml aliquot of the heptane was removed to 6 ml of toluene base scintillator for counting.

No quench correction was necessary, though the total volume of the heptane layer was determined by the method detailed in the previous Section.

The adequacy of the saponification and extraction procedures was determined by saponifying standard tripalmitin solutions and estimating the liberated palmitic acid by the colorimetric method of Duncombe (1963) (Section 2.6.3).

The effectiveness of the partitioning procedures used in this laboratory has been demonstrated (Evans, 1973).

Sample counts in fatty acids were related to the same standard used in the triglyceride estimation. They were corrected for the total heptane volume and the results expressed as glucose incorporation into fatty acids, nmol/hour/µmol triglyceride.

Conversion of glucose to glyceride glycerol was estimated as the difference between triglyceride and glyceride

fatty acid production, since counting the acidified aqueous phase after heptane extraction is complicated by severe and variable quenching. The results were expressed as glucose incorporation into glyceride glycerol, nmol/hour/µmol triglyceride.

2.5.4 Total anionic metabolite estimation

This analysis was performed by anion exchange chromatography in 0.8 x 35 cm glass columns containing 0.6 g of DEAE-Sephadex-A-25-120. Radioactive anions were estimated in samples from material obtained by decanting and pooling the acidified incubation medium from cells incubated under identical conditions.

A 500 µl sample of the medium was diluted into 10 ml of water and the whole was applied to a column. Unreacted glucose and uncharged metabolites were washed through with 50 ml of water. The anionic metabolites were eluted with 15 ml of 0.5 M hydrochloric acid, and 1 ml aliquots of this eluate were counted in 6 ml Bray's. The total volume of eluate collected was determined by collecting the fractions in preweighed test tubes and reweighing them when collection was completed. Severe quenching occurred in the counting of this material and the appropriate quench correction factor was determined for each vial by internal standardisation.

Sample counts were related to the standard after correction for quenching and volume of sample and the results expressed as glucose incorporation into anions, in nmols/hour/µmol triglyceride.

2.5.5 Tritiated water

Tritiated water derived from 5-^{3}H-glucose was measured by separating the water from unmetabolised glucose and counting an aliquot. This separation was achieved by lyophilisation. A simple manifold was constructed (Fig. 2.3) which allowed a series of sample vials to be connected to a vacuum pump simultaneously and the lyophilisates collected independently. In order to avoid thawing of samples during loading, with consequent violent boiling and transfer of 5-^{3}H-glucose into the collecting trap, it was necessary to incorporate into the system a large pressure vessel to act as a reservoir. This vessel, together with the ability to isolate each side-arm by means of a 3-way tap, allowed continuous operation of the apparatus.

No carryover of ^{14}C-glucose occurred during this lyophilisation procedure, showing that effective separation of tritiated water from unmetabolised glucose was possible. Use of purified 5-^{3}H-glucose (described in 2.2, Buffer containing 5-^{3}H-glucose) gave cell blank values less than double the background count rate. Lowest sample counts in experiments with 5-^{3}H-glucose were usually greater than the cell blanks by a factor of 3.

The specific activity of the lyophilisate was found always to be the same as the water from which it was derived, showing that isotope effects did not affect the lyophilisation process. 1 ml samples of tritiated water were taken and different volumes were removed by lyophilisation. Aliquots of the residue and of the lyophilised water were taken for counting. Any difference in the rate of lyophilisation of the heavier tritiated water should result in a higher count in the residue. Table 2.4 shows that this was not so.

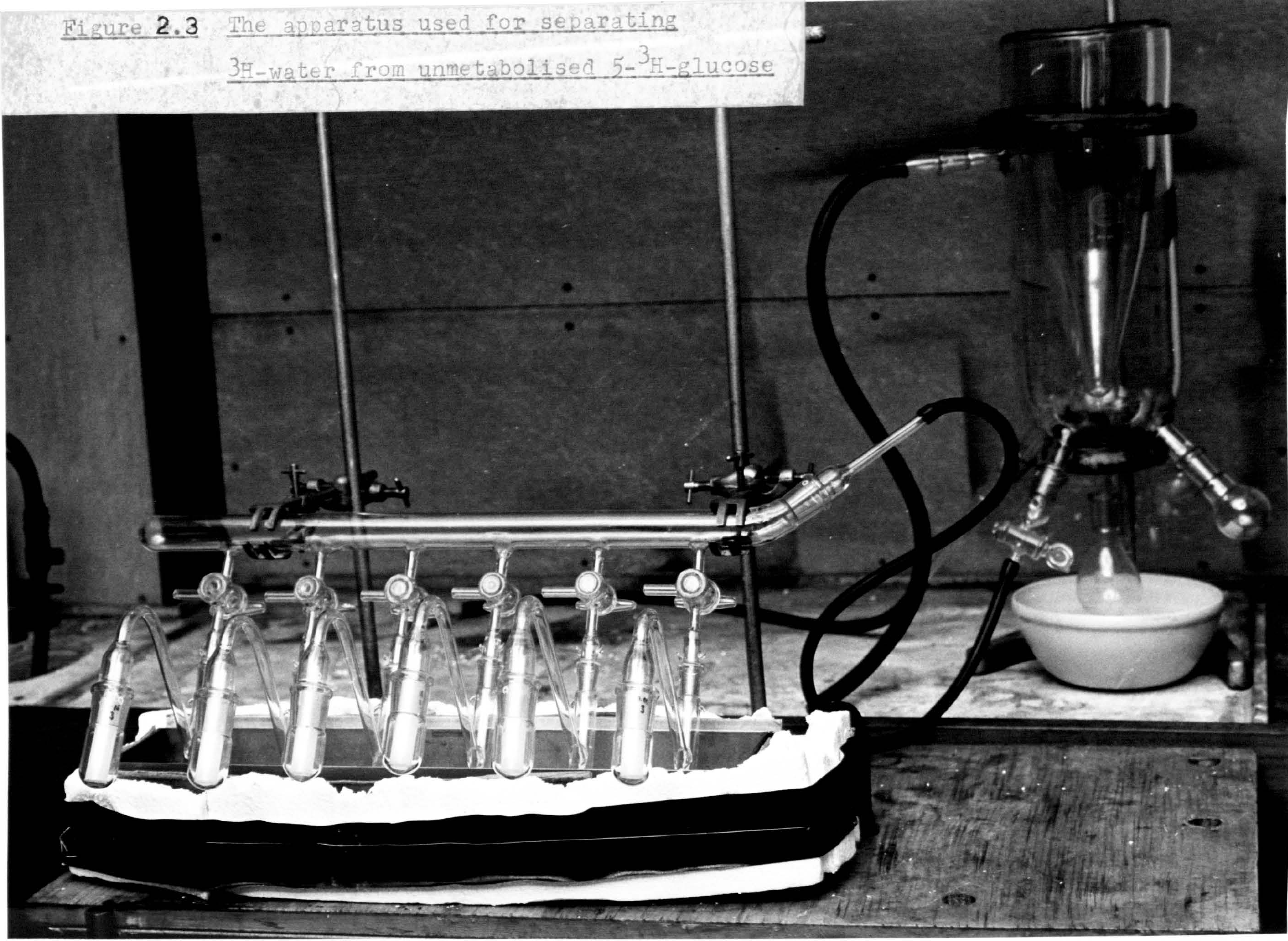


Figure 2.3 The apparatus used for separating ^{3}H-water from unmetabolised 5-^{3}H-glucose

Table 2.4 Comparison of the specific activity of lyophilised tritiated water with the specific activity of the residual tritiated water. 1 ml samples of tritiated water were lyophilised for different times and the amount collected was determined by weighing. The specific activity of aliquots of lyophilised and residual water was measured.

Wt. of water collected, gm	Residue (cpm/gm)	Lyophilisate (cpm/gm)	Specific activity ratio (residue/ lyophilisate)
0.17	4,946	5,275	0.94
0.20	4,888	5,250	0.93
0.26	5,288	5,106	1.04
0.27	5,171	5,097	1.01
0.30	4,916	5,051	0.97
0.34	4,833	4,987	0.97
0.35	4,788	4,807	1.00
0.35	4,865	4,858	1.00
0.40	4,943	4,922	1.00
0.45	4,724	4,812	0.98
0.46	4,763	4,817	0.99
0.50	4,827	4,847	1.00

When sufficient water had been trapped (usually about 1.5 hours for 12 samples) 500 µl aliquots were removed by micropipette to 6 ml of toluene Triton X-100 scintillator. A standard containing the same amount of 5-^{3}H-glucose as the experimental vials in a volume of 500 µl of water was prepared and counted with the experimental vials. Use of this reference standard in calculating glucose uptake obviates the need for determination of the quenching produced by the water in this system, which is of the order of 10%. Sample counts, corrected for volume of sample, were related to the standard in calculating glucose uptake, which was expressed in nmols/hour/µmol triglyceride.

2.6 Colorimetric assays

These were used to determine the concentration of triglyceride esters, glucose and fatty acids. In order to make possible direct comparison of different experiments it is necessary to make some estimate of the number of adipocytes present in each vial. The amount of triglyceride which was added to the vials was therefore determined by a modification of the method of Rapport and Alongo (1955). Evans (1973) has demonstrated the small size distribution of adipocytes produced from rats in the weight range specified. The glucose oxidase method was used to estimate the glucose concentration in buffer before and after incubation with adipocytes. This glucose disappearance measurement was then compared with the glucose utilisation measured with 5-^{3}H-glucose (2.7). Fatty acids were estimated by the method of Duncombe (1963). This technique was used to determine the efficiency of the saponification and fatty acid extraction procedures.

In each case the colour was developed in samples, a range of standards and reagent blanks. The absorbance was then measured against the reagent blank with a Unicam SP 500 Series II spectrophotometer with a 1 cm light path. A line of best fit was calculated for the standard curve data by unweighted linear regression and sample concentrations were computed from this.

2.6.1 Triglyceride ester determination

<u>Tripalmitin solutions</u>

Stock: a 6.67 mM solution prepared by dissolving 538.5 mg of tripalmitin (obtained from B.D.H., Poole) to 100 ml with chloroform (analytical grade). This solution was stored in the cold.

Working solution: 0.33 mM solution prepared by diluting the stock solution with chloroform.

<u>Basic hydroxylamine reagent</u> (Prepared immediately before use)

Solution A: 100 mg of hydroxylamine hydrochloride (B.D.H., Poole) was dissolved in 0.17 ml of water and mixed with 3.2 ml of absolute alcohol.

Solution B: 0.2 ml of 12.5 N NaOH (prepared by dissolving 50 g of solid in water and diluting to 100 ml) was mixed with 3.2 ml of absolute alcohol.

Equal volumes of Solutions A and B were carefully mixed and centrifuged briskly to spin down the NaCl formed. When properly prepared this reagent is clear.

<u>Ferric perchlorate reagent</u> (Prepared on the day of the assay)

A set of standards containing 0.2-3.0 microequivalents of tripalmitin was prepared from the 0.33 mM working solution. These standards, chloroform blanks and 500 µl samples of the heptane layers from the Dole-Meinertz extraction (1960) were

taken to dryness in a 70° water bath under a stream of air. Final traces of solvent were removed by leaving the tubes overnight in a vacuum dessicator.

3 ml of ether was added to each tube and samples were dissolved with a vortex mixer. 100 µl of a freshly prepared basic hydroxylamine solution were added, the contents mixed and the tubes returned to a 70° bath until the solvent had evaporated.

After hydroxylaminolysis of the triglyceride the tubes were placed in a vacuum oven at 60° for 1 hour to remove traces of solvent. The tubes were allowed to cool to room temperature before the colour was developed by adding to each tube 3 ml of freshly prepared ferric perchlorate solution. The tubes were allowed to stand for 30 minutes to develop the colour fully before the absorbance at 530 nm was read against the reagent blanks.

Successful use of this technique to give accurate, reproducible results requires very careful drying of the samples and careful preparation of the basic hydroxylamine reagent so that precipitation is complete and the reagent is clear.

2.6.2 Glucose determination

Standard glucose solution, 1.0 mg/ml: 10 mg of glucose dissolved in 5 ml of Basic Buffer and diluted to 10 ml with 20% TCA.

M Sodium dihydrogen phosphate (pH 7.0): 15.6 g of the salt dissolved in 100 ml of water and the pH adjusted to 7.0 with 10 M sodium hydroxide.

Glucose oxidase reagent

125 mg of glucose oxidase (crude preparation containing

catalase. Activity 0.6 E.U./mg, obtained from B.D.H., Poole).

0.5 mg of peroxidase (activity 820 E.U./mg, from B.D.H., Poole).

The enzymes were stirred in 100 ml of 0.2 M sodium dihydrogen phosphate for 2 to 3 hours. The solution was then adjusted to pH 7.0 and 1 ml of 0.5% o-dianisidine in 95% ethanol/5% 0.1 N hydrochloric acid was added. The reagent was filtered and used immediately.

A range of standards was prepared containing 50-100 µg of glucose. The volume in the standards was adjusted to 500 ul with solvent (prepared by mixing equal volumes of Basic Buffer and 20% TCA) and 500 µl solvent blanks were prepared. 500 µl aliquots were removed from incubation vials after centrifuging down TCA precipitated material (2,000 xg, 15 mins.).

500 µl of M sodium dihydrogen phosphate was added to buffer the TCA followed by 2 ml of glucose oxidase reagent and the stoppered tubes shaken in a water bath for one hour at 37°. The absorbance of these solutions was then determined by reading against a reagent blank at 450 nm.

In order to use this procedure on samples which had been lyophilised to remove tritiated water incubation vials were weighed before lyophilisation. After the sample of tritiated water had been removed vials were adjusted to their original weight by addition of water before a sample was removed for use in the glucose oxidase procedure.

2.6.3 Fatty acid determination

Fatty acid standard: 1.67 mM palmitic acid in heptane.

Copper reagent: 50 ml of a 6.45% (w/v) solution of copper nitrate trihydrate mixed with 5 ml of N acetic acid and 45 ml of M triethanolamine.

Sodium diethyl dithiocarbamate: 0.1% solution (w/v) in isobutanol.

1.67 mM palmitic acid in heptane was dispensed into stoppered tubes to give a set of standards containing 0.05 to 0.5 μmol of palmitic acid. Heptane was added to bring the volume to 300 μl. Standard tripalmitin solutions were saponified and extracted with heptane as described previously (2.5.3). 300 μl samples of the heptane layer were taken together with heptane blanks. To each tube 5 ml of chloroform was added and, after mixing, 2.5 ml of freshly prepared copper reagent was added. The tubes were vigorously shaken at room temperature for 2 minutes. The tubes were centrifuged briskly to separate the two layers and the upper, aqueous layer was removed and discarded. 3 ml of the chloroform phase was taken and mixed with 500 μl of sodium diethyl dithiocarbamate solution. The absorbance at 440 nm was measured against the reagent blank.

This experiment indicated that the saponification and extraction of the fatty acids was satisfactory, as shown below:

Theoretical yield of palmitic acid nmol	100	250
Actual palmitic acid yield nmol	97.3 ± 0.2 (5)	245.5 ± 1.5 (5)
Efficiency of saponification and extraction, %	97.3	98.2

Number of observations in parentheses

2.7 The use of 5-^{3}H-glucose in estimating total glucose utilisation by adipocytes

A simple method for determining total glucose utilisation by isolated adipocytes has recently been published (Brown and Garratt, 1974). This technique was developed because the accurate use of conventional methods of measuring glucose disappearance requires substantial changes in glucose concentration and this did not appear to be desirable in some of the studies described. It is possible to measure the incorporation of ^{14}C from glucose into all its metabolic products and so obtain a measure of glucose utilisation. ^{3}H incorporation from glucose has also been studied. For example, Katz and Rognstad (1969) found that when 2-^{3}H-glucose was metabolised by fat pad pieces the label was distributed between water, glyceride glycerol, fatty acids and lactate. However, with most labelled glucose molecules the label appears in so many products that this method is too cumbersome to be routinely used. Ashcroft et. al. (1972) suggested that the use of 5-^{3}H-glucose might avoid this problem since in some tissues water is almost the only radioactive metabolite of glucose.

Hydrogen from the 5C position of glucose exchanges with water during triose phosphate isomerisation (Ashcroft et.al., 1972), further exchange being possible at the enolase reaction if this step is not in complete equilibrium. Only glucose which is converted directly into glycogen does not pass through these steps and therefore retains its label. Since the synthesis of glycogen is normally negligible in the isolated adipocyte (Evans, 1973) virtually all of the metabolised 5-^{3}H-glucose should lose its ^{3}H to water and measurement of the specific activity of the tritiated water

after an experiment should provide a precise estimate of the total glucose utilisation.

This hypothesis was tested by studying the recovery of ^{3}H in the major glucose metabolites and by comparing the values obtained for total glucose utilisation with similar estimates obtained using U-^{14}C-glucose. In these experiments adipocytes (approximately 4×10^{5} cells/(vial) were incubated in a final volume of 500 ul of buffer containing 0.1 mg/ml glucose and 2% BSA and in addition either 75 nCi of 5-^{3}H-glucose or 125 nCi of U-^{14}C-glucose or both. The suba seals were fitted with cell vials bearing hanging wells for the trapping of CO_2. The utilisation estimates were also compared with estimates of glucose disappearance from the medium. This latter estimate was obtained by the glucose oxidase method (2.6.2).

The study of the metabolic distribution of label from 5-^{3}H-glucose showed that no ^{3}H was recovered in Hyamine under conditions normally used for trapping CO_2. Furthermore, no ^{3}H metabolites were retained on an anion exchange column.

Only 1/30 of the total radioactive anions in each sample was counted because of the volume of sample and the severe quenching of ^{14}C counts by the acid effluent. An estimate was therefore made of the minimum incorporation which could have been detected by the methods used. Triplicate cell blanks in this determination gave values of 765, 778 and 796 cp10 min. and only samples which gave counts of 880 (that is to say 100 counts above the mean blank) would be regarded as significant. Use of this limiting value allows calculation of the maximum incorporation of glucose into anions which could occur before it was detected. In the samples which gave the lowest count rate in anions (0.1

mg/ml glucose, no insulin) use of this value shows that the incorporation could not have been greater than 4% of the total uptake (Table 2.5) without being detected. In the other treatments the percentage is considerably lower. Loss of ^{3}H into anionic metabolites does not therefore impose a limitation on the use of 5-^{3}H-glucose in measuring total glucose utilisation.

Table 2.5 shows that less than 5% of the total 5-^{3}H-glucose metabolised was found in the triglyceride fraction. Thus tritiated water estimation gives at least 95% of the total glucose utilisation.

In a parallel experiment cells were incubated with U-^{14}C-glucose and the metabolic distribution of the glucose carbon was followed. When these samples were lyophilised no radioactivity could be detected in the water collected. The distribution of glucose into its various metabolites is shown in Table 2.5. The glucose utilisation determined with 5-^{3}H-glucose was rather higher than that determined with ^{14}C-glucose.

The observation that none of the ^{14}C-metabolites were detected in the lyophilised water means that cells can be incubated with ^{14}C-glucose and 5-^{3}H-glucose simultaneously without affecting the total glucose utilisation estimates obtained from tritiated water. Furthermore the finding that no ^{3}H was recovered in Hyamine indicates that the presence of 5-^{3}H-glucose and its metabolites would not preclude the simultaneous use of ^{14}C-glucose for studying CO_2 production. The small rate of incorporation of 5-^{3}H-glucose label into triglyceride does not affect the determination of ^{14}C-glucose incorporation since it is possible to count ^{14}C independently of ^{3}H. Thus the distribution of glucose into major metabo-

Table 2.5 Estimation of glucose utilisation by adipocytes incubated under various conditions with either 5-^{3}H-glucose or U-^{14}C-glucose. Total glucose utilisation is taken to be the sum of the radioactivity incorporated into the metabolic products shown. No incorporation could be detected in metabolites not shown. The figures are the means of 3 estimates (with S.D.) from fat cells incubated under identical conditions. Data are expressed as n.mols.glucose/hr./μmol of triglyceride

Glucose concentration (μg/ml)	Insulin ($10^{-8}M$)	^{3}H incorporation into			^{14}C incorporation into				Incorporation ratio $^{14}C/^{3}H$
		H_2O	triglyceride	total	CO_2	triglyceride	anions	total	
100	-	8.88±1.26	0.29±0.04	9.27±1.33	2.37±0.34	3.75±0.46	2.77±0.05	8.29±0.79	0.89
100	+	47.47±1.02	1.32±0.20	48.79±1.77	14.83±0.68	25.83±0.18	11.17±1.51	48.75±0.52	1.00
250	-	15.96±1.22	0.71±0.34	16.68±2.14	3.28±0.21	5.04±0.15	4.21±0.55	11.77±0.19	0.71
250	+	83.49±1.11	1.53±0.62	85.02±1.43	21.35±0.91	39.35±0.78	21.33±2.07	80.84±0.89	0.95

lites can be readily studied at precisely determined levels of glucose uptake by the use of U-^{14}C- and 5-^{3}H-glucose in the same vial. Furthermore glucose uptake studies in the adipocyte, such as those described in Chapter 5 of this thesis, can be performed with an accuracy not previously available by using 5-^{3}H-glucose.

Table 2.6 shows the results of an experiment in which the double label procedure was used. Although glucose utilisation estimated from 5-^{3}H-glucose will be slightly under-estimated because of the incorporation of tritium into triglycerides, the utilisation calculated from ^{14}C data was consistently lower. Since it is difficult to see how 5-^{3}H-glucose could give an over-estimate of glucose uptake these results suggest a systematic under-estimation in glucose utilisation determined from U-^{14}C-glucose metabolism. The estimate of glucose uptake obtained by adding CO_2, triglyceride and anion production estimated with U-^{14}C-glucose, was similar to the ^{3}H incorporation from 5-^{3}H-glucose into water, though the procedures involved were far more cumbersome.

The variation between replicates was similar for the two analyses, although one might expect the U-^{14}C-glucose estimates to contain larger errors since they involved the summation of several extraction procedures, each with its attendant variability. However, since incorporation of ^{14}C into anions was measured in pooled medium samples an average value for anion production was used in determining the glucose utilisation for each treatment. This could give rise to an artificially low estimate of the variation between the U-^{14}C-glucose utilisation measurements.

Finally, the glucose utilisation estimate obtained with

Table 2.6 Comparison of estimates of glucose utilisation obtained from measurements of the metabolism of 5-^{3}H-glucose and U-^{14}C-glucose. In this experiment both kinds of radioactive glucose were present in the same incubation medium. Data are expressed as in Table 2

Glucose concentration (μg/ml)	Insulin (10^{-8}M)	^{3}H in water	^{14}C incorporation into				$\frac{CO_2+TG}{total}$	Incorporation ratio $^{14}C/^{3}H$
			CO_2	triglyceride	anions	total		
100	-	8.20±0.88	2.05±0.19	2.53±0.30	2.77±0.05	7.35±0.40	0.62	0.90
100	+	57.11±2.90	15.15±2.99	22.70±0.80	11.17±1.57	49.06±1.43	0.77	0.96
250	-	16.72±2.73	3.74±0.71	4.68±0.86	4.21±0.55	12.63±1.55	0.67	0.76
250	+	90.01±2.48	22.19±0.98	35.01±1.23	21.33±2.07	78.54±1.57	0.73	0.87

5-^{3}H-glucose was compared with the loss of glucose from the medium, estimated by the glucose oxidase method. The results of this experiment are shown in Table 2.7. In the absence of insulin the glucose utilisation was so low that a reliable estimate could not be obtained by the glucose oxidase method. This is apparent from the reproducible values obtained for residual glucose which nevertheless gave huge variations in values of glucose utilisation calculated for these samples. However, in the presence of insulin there was close agreement between the values obtained for glucose utilisation by the two methods.

It is evident from Table 2.6 that CO_2 and triglyceride do not form a constant proportion of the total glucose utilisation, although this has been used to obtain an estimate of glucose uptake by several workers (for example, Gliemann, 1968). The proportion of the total metabolised glucose flowing into these products is shown in Table 2.6, from which it is clear that use of this simplified measurement may give rise to grave errors with certain treatments.

The glucose oxidase method gave similar results to the 5-^{3}H-glucose method, although the isotopic method gave far more reproducible results.

In conclusion, the incorporation of ^{3}H from 5-^{3}H-glucose into water provides a simple and satisfactory method for determining glucose utilisation by isolated adipocytes. Furthermore this method may be used in conjunction with ^{14}C-glucose to study the metabolic fate of the glucose taken up by adipocytes.

Table 2.7 Comparison of estimates of glucose utilisation obtained from measurements of the metabolism of 5-^{3}H-glucose and of residual glucose determined by glucose oxidase. The data are expressed as in Table 2.5 except that the number of determinations in each group is shown in brackets

Glucose concentration (μg/ml)	Insulin (10^{-8}M)	No. of replicate incubations	Residual glucose concentration	Glucose disappearance	^{3}H in water	Utilisation ratio disappearance/^{3}H
100	-	3	96.9±2.7	5.80±5.03	5.63±0.70	1.03
100	+	4	71.3±2.0	53.15±3.71	52.21±5.76	1.02
200	-	4	192.6±5.1	14.56±10.27	10.35±1.04	1.40
200	+	4	150.4±9.5	91.78±17.68	89.15±7.09	1.02

2.8 Exploratory experiments with isolated adipocytes

Several exploratory experiments were performed with the isolated adipocyte preparation to test the metabolic characteristics of these cells before the studies contained in this thesis were performed.

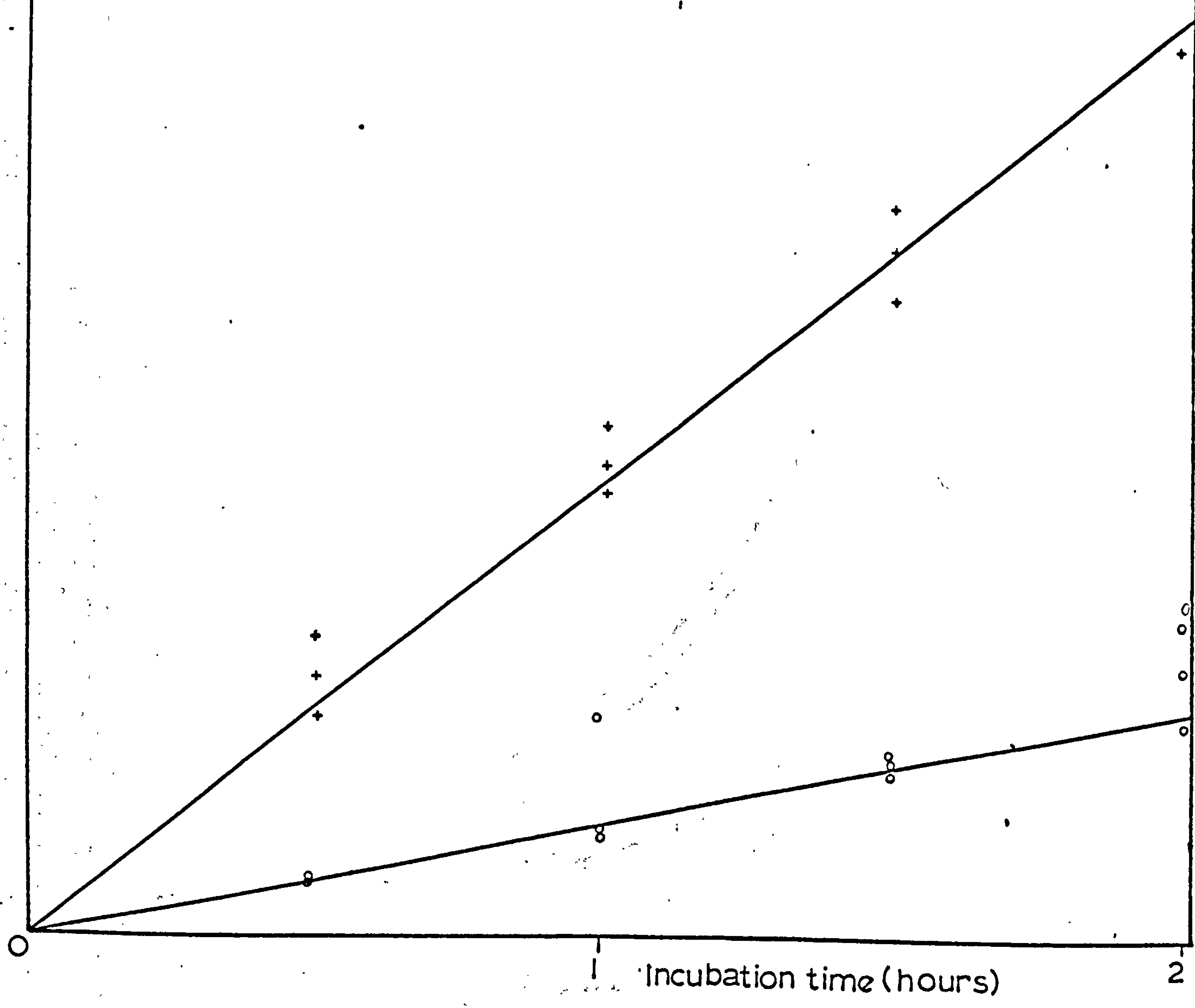
2.8.1 Time course

The effect of incubation time on glucose metabolism in the presence and absence of insulin was studied. Adipocytes were incubated in a total glucose concentration of 0.1 mg/ml, containing U-^{14}C-glucose and to some of the vials was added a maximally stimulating concentration of insulin (10^{-9}M). After selected periods of time the cells were killed by injection of 500 µl of 20% TCA and the CO_2 was collected and counted (see 2.5.1). The results of this experiment (Fig. 2.4) show that glucose utilisation was linear for two hours in the presence and absence of insulin. Therefore incubations were terminated within this time.

2.8.2 Effect of glucose concentration on adipocyte sensitivity to insulin

Adipocytes were incubated with a range of insulin concentrations and 3 different glucose concentrations. Using U-^{14}C-glucose, the conversion of glucose to carbon dioxide and triglyceride was measured, giving an estimate of glucose utilisation. These results are expressed as a series of dose-response curves (Fig. 2.5) and show that increasing medium glucose concentration leads to loss of insulin sensitivity. The greatest insulin response was obtained with a glucose concentration in the medium of 0.1 mg/ml. These results agree with the work of Gliemann (1967). Consequently,

Figure 2.4 The production of $^{14}CO_2$ from U-^{14}C-Glucose as a function of incubation time.



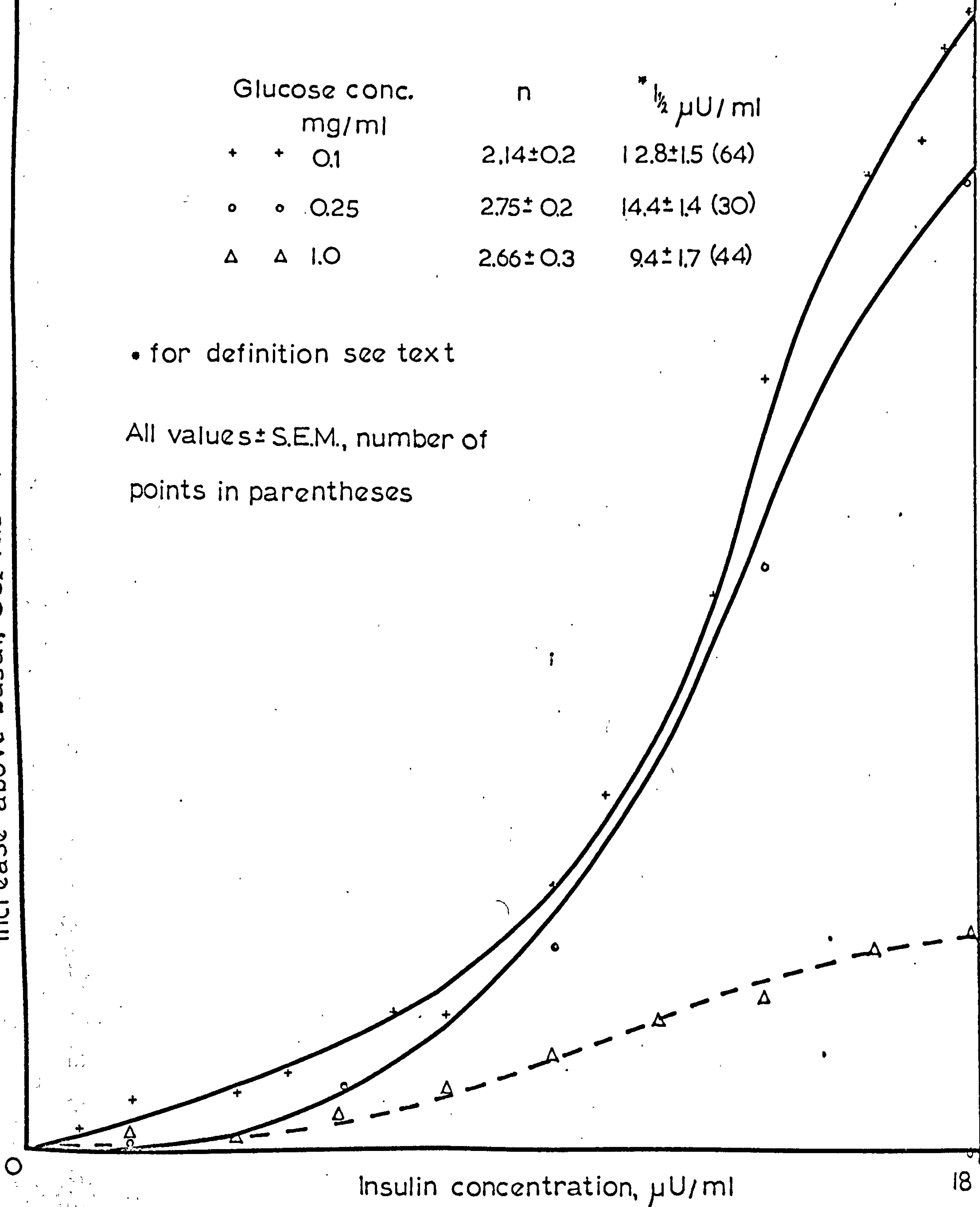


Figure 2.5 The effect of glucose concentration on the sensitivity of adipocytes to insulin.
1200
Glucose conc. mg/ml
n
* I½ µU/ml
+ + 0.1
2.14±0.2
12.8±1.5 (64)
o o 0.25
2.75±0.2
14.4±1.4 (30)
Δ Δ 1.0
2.66±0.3
9.4±1.7 (44)
• for definition see text
All values± S.E.M., number of points in parentheses
Increase above basal, 14CO2 (as %)
0
Insulin concentration, µU/ml
18

in experiments performed at a single glucose concentration (see Chapters 3 and 5) a level of 0.1 mg/ml was used.

2.8.3 Determination of the rate of glucose utilisation

It was necessary in the studies described in this thesis to prevent gross changes in the glucose concentration during adipocyte incubation. Exploratory experiments were therefore performed to study the rate of glucose utilisation with the adipocyte preparation described. Adipocytes were incubated as described, with a glucose concentration of 0.1 mg/ml for 2 hours in the presence and absence of insulin.

Insulin	-	+
Glucose utilisation Nmol/2 hr/μmol TG	5.60±0.7 (4)	52.2±5.8 (4)
Glucose utilised %	2.02±0.25 (4)	18.8±2.1 (4)

Number of observations in parentheses.

In the presence of insulin cells represented by 1 μmol of triglyceride used about 20% of the available glucose during a 2 hour incubation. Cell preparations used in this work contained up to 3 μmol of triglyceride per vial. It was therefore necessary to ensure that the glucose utilisation in each experiment was kept within acceptable limits. This was done as follows. With each experiment a parallel incubation was performed, using the same adipocyte suspension as the experimental vials. This incubation was performed with a high specific activity of U-^{14}C-glucose in the presence and absence of insulin. After 1 hour the incubation was terminated and CO_2 collected with only 10 minutes shaking. A standard of U-^{14}C-glucose in 6 ml of Bray's was then counted with these

samples and used to estimate glucose conversion to CO_2. Glucose utilisation was estimated as 3x glucose incorporation into CO_2. This estimation allowed the incubation to be terminated after a suitable period.

2.9 Radio-immunoassay of insulin in incubation medium

Reagents used

^{125}I-pork insulin, 20-30 mCi/mg

Anti-pork insulin guinea-pig serum

Beef insulin, 10 times recrystallised

These materials were a gift from Novo Industri, AS.

FAM: (0.04 M phosphate buffer, pH 7.4)

$Na_2HPO_4.2H_2O$	5.77 g
$Na\ H_2PO_4.H_2O$	1.05 g
BSA	1.00 g
sodium merthiolate (BDH)	0.24 g
distilled water to	1 L

Washing buffer

FAM	18 ml
Water	162 ml
96% Ethanol	960 ml

^{125}I-insulin preparation

The freeze-dried material, containing approximately 20 mU of total insulin and 10 mg of human albumin, was dissolved in 1.4 ml of distilled water. 1 ml of this solution was diluted with 59 ml of FAM and the solution stored at -15° in 10 screw-cap plastic bottles.

Anti-insulin serum preparation

The freeze-dried material was dissolved in 1.4 ml of distilled water. 1 ml of this solution was diluted with 59 ml of FAM to give a working solution which was divided into

6 ml portions and stored at -15° in plastic, screw-cap vials.

Immunoassay procedure

100 µl aliquots of buffer from beef insulin standards ($0-10^{-9}$M) or unknowns were dispensed into 6 x 1.5 cm plastic tubes (Xlon Ltd.) by Hamilton syringe. 100 µl of antiserum was added from a repeating pipette (Repette, Chance), the tubes were carefully mixed on a vortex mixer and incubated for 20 hours at 4°. 100 µl of ^{125}I-insulin was then added by repeating pipette, the contents carefully mixed and incubated at 4° for 4 hours. 1.6 ml of 96% ethanol was added, the contents of the tube mixed on a vortex mixer and the precipitate centrifuged down for 10 minutes at 2000 xg. The supernatant was then decanted and discarded.

The precipitate was washed with 2.0 ml of washing buffer, centrifuged down (2000 xg for 20 minutes) and the washings discarded. The tubes were inverted and allowed to drain for 20 minutes. To check the efficiency of the washing procedures a set of tubes was always prepared which received ^{125}I-insulin and Basic Buffer but no antiserum.

The dry precipitates were counted in a well-type counter with a sodium iodide crystal (Panax). A standard curve was constructed using the beef insulin standards and the concentration of insulin in unknown samples was obtained from this curve.

2.10 Isolation and purification of guinea-pig insulin

Reagents used

Absolute ethanol Analytical grade

Anhydrous ether Analytical grade

Ammonium hydroxide (spec. gr. 0.88) Standard laboratory reagent

Hydrochloric acid 6N Analytical grade

2M Ammonium acetate buffer, pH 5.3

Acetic acid 1M Analytical grade

Sephadex G50 washed until free of fines by repeated suspension in 1M acetic acid

Isolation and purification

Guinea-pig insulin (see Chapter 3, Section 4) was prepared essentially by the method of Davoren (1962). Since experiments using large amounts of beef insulin were being performed in the laboratory where the isolation was carried out it was thought to be important to check that any biological activity could only be attributed to material extracted from the guinea-pig. Consequently a blank extraction was carried out with the same apparatus and solutions immediately before the preparation of the guinea-pig insulin. This was done to demonstrate that the activity detected in the extract was not due to contaminating beef insulin.

The pancreases of 12 guinea-pigs were removed into liquid nitrogen and homogenised in 40 ml of chilled, acidified ethanol. The tissue was allowed to extract overnight at 4°. The extract was adjusted to pH 8.2 by addition of ammonium hydroxide and the precipitate was removed by centrifugation at 0° and discarded. The pH of the supernatant was adjusted to approximately 5.5 with 6N hydrochloric acid and 1 ml of 2.0 m ammonium acetate buffer (pH 5.3) was

added. The pH of the solution was adjusted to 5.3. Two volumes of absolute ethanol and 4 volumes of anhydrous ether were then added and the solution was allowed to stand at -10° overnight (18 hours). The precipitate was collected by centrifuging the solution at -5°. This precipitate was suspended in 20 ml of distilled water and 2N hydrochloric acid was added to give a clear solution of pH 3 (indicator paper). 20 ml of aqueous sodium chloride solution (30%, w/v) was added with constant stirring and the crude insulin precipitate was collected by centrifugation for 20 mins. at 10,000 xg.

The precipitate was redissolved in 2 ml of 1.0 M acetic acid and purified by gel filtration through a 2 cm x 1 m column of Sephadex G-50 (with a void volume of 88 ml) eluted with 1.0 M acetic acid at a flow rate of 3 ml/hour. The column eluate was monitored continuously at 280 nm and collected in 2.5 ml fractions. One significant protein peak was observed (fig. 2.6). Aliquots of the fractions were bioassayed for insulin activity using the isolated adipocyte preparation. When column eluates were bioassayed the acetic acid was first neutralised with 1.8 M sodium hydroxide.

Adipocytes were incubated in a final volume of 1 ml of Buffer containing 0.1 mg/ml glucose, 20 mg/ml BSA and 0.25 μCi of U-^{14}C-glucose. 100 μl aliquots of neutralised column effluent fractions were used in the bioassay. The conversion of glucose to CO_2 and triglyceride in the course of a one hour incubation was followed and related to a standard of the U-^{14}C-glucose working solution as described previously (2.5).

Although the biologically active material did not exactly coincide with the protein peak (Fig. 2.6) the fact

Figure 2.6 Elution profile of an extract of guinea pig pancreas

that it eluted as a sharp peak after the greater part of the protein showed that substantial purification was achieved. The fractions containing biological activity (Fractions 74 to 90) were pooled, lyophilised and redissolved in 2 ml of 10 mM hydrochloric acid. Fractions eluted in a corresponding position from the blank extraction were treated similarly. The biological activity of this concentrated extract was then studied with essentially the same procedure as described in 2.4 except that a final incubation volume of 1 ml was used. Serial dilutions of the extract were made in 10 mM hydrochloric acid, diluting by a factor of ten each time. 10 µl samples of these solutions were then added to the appropriate incubation vials and incubated with adipocytes for 1 hour. The addition of 10 µl of 10 mM acid to the incubation medium did not change the pH. After 1 hour the incubation was terminated and the glucose incorporation into CO_2 and triglycerides determined by the procedures described in Section 2.5.

10 µl of the extract diluted 1:100 in the 1 ml used for the insulin bioassay not only stimulated glucose metabolism but did so to the same extent as a maximally stimulating concentration of beef insulin:

Glucose utilisation Nmol/Hr/µmol triglyceride

(Mean of 3 observations ± S.D.)

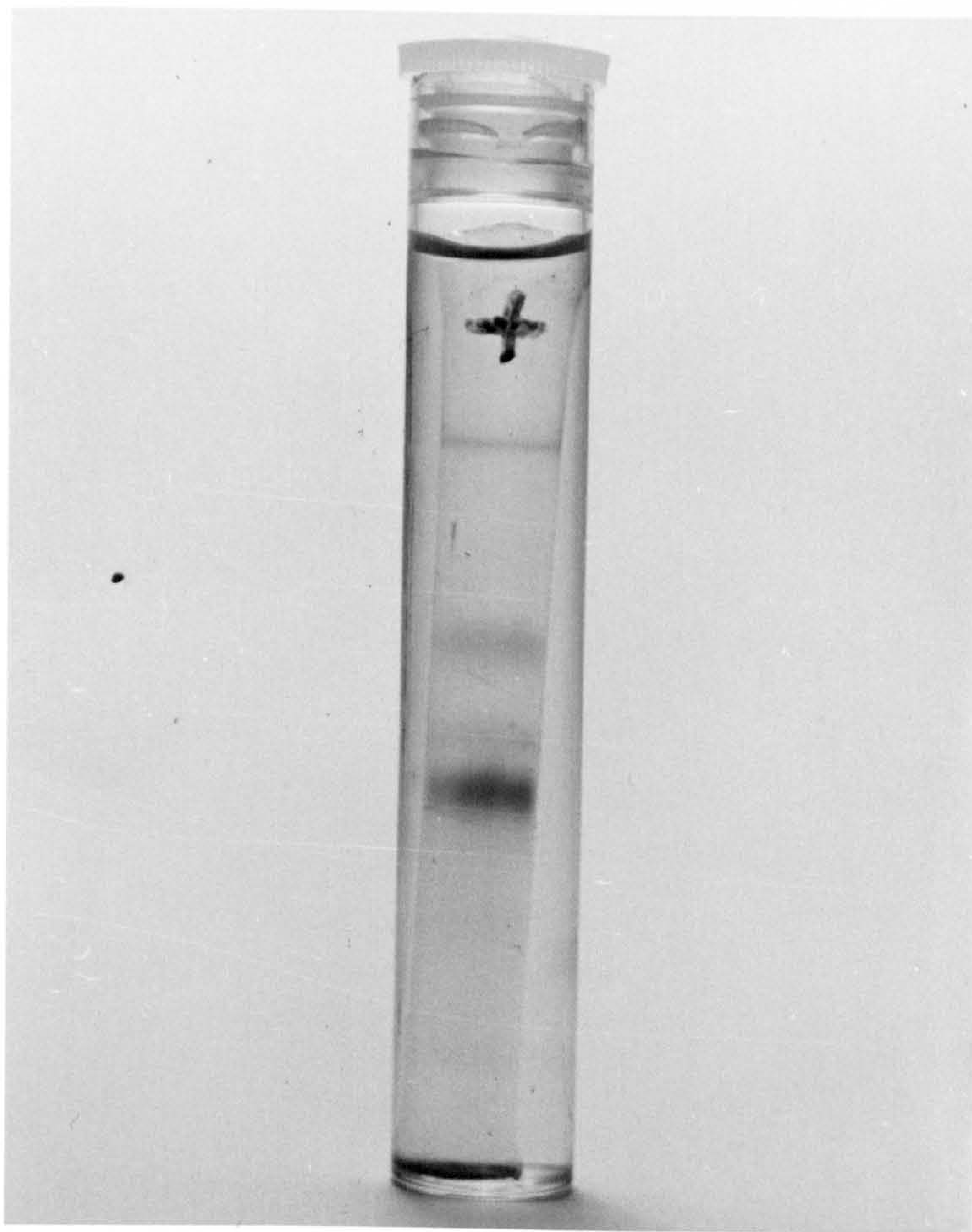
	Basal	Beef insulin (10^{-9}M)	Guinea pig insulin (1:100 dilution)
Carbon dioxide	2.38±0.31	24.79±1.1	24.91±0.99
Triglyceride	4.36±0.57	36.43±2.0	35.84±1.77

No biological activity was found in the blank extract. This experiment also showed that a half-maximal stimulation was produced by diluting the original extract approximately 1: 2000. The concentration of beef insulin required to give a half-maximal stimulation is typically 4×10^{-11}M (see Chapter 3, Table 4). If the biological activity of the extract was due to contaminating beef insulin, therefore, the original solution would have contained 1×10^{-7}M insulin. This concentration is readily detected by the radioimmunoassay technique described in 2.9.

Using this single antibody radioimmunoassay technique it was possible to distinguish 5×10^{-11}M beef insulin from blanks in a 100 µl sample. However, 10 µl aliquots of the original extract diluted 1:10 with Basic Buffer were found to contain no immunoreactive insulin (using anti-pork insulin guinea-pig serum). The blank extract was also found to contain no immunoreactive insulin. This shows that the biological activity of the guinea-pig extract cannot be attributed to contaminating beef insulin.

The total protein concentration in the guinea-pig extract was estimated by a modified Lowry method (Lowry et. al., 1951). This is an extremely sensitive technique, being able to detect 5-10 µg of protein/ml. Duplicate samples of the guinea-pig insulin were taken for this determination together with identical samples of the blank extract. 10-250 µl volumes of a working solution of beef insulin (prepared from the stock solution described in 2.2) were dispensed to give a range of standards from 10-250 µg of insulin/ml. All volumes were adjusted to 250 µl with 10 mM hydrochloric acid before analysis. The guinea-pig insulin concentration was found to be 260 µg/ml, no protein being

Figure 2.7 Gel electrophoresis of an acid-ethanol extract of guinea pig pancreas



detected in the blank extract. This assay was performed by G. L. Igloi.

Finally, four protein components were discernible after electrophoresis at pH 8.5 in 7.5% acrylamide gel (Ornstein and Davis, 1964), showing that although considerable purification of the extract had been achieved, the preparation was not homogeneous (Fig. 2.7).

Chapter 3

POSSIBLE ORIGINS AND IMPLICATIONS OF A SIGMOID DOSE-RESPONSE RELATIONSHIP

3.1 Introduction

A primary step in the action of insulin is believed to be a readily reversible interaction with a specific receptor in the cell membrane.

Evidence for this hypothesis has been presented by Cuatrecasas (1969), showing that insulin could exert its characteristic effects on glucose metabolism in the adipocyte without penetrating the cell membrane. Furthermore, adipocytes activated by low concentrations of insulin can be returned immediately to the basal state by washing with insulin-free buffer or by adding insulin antiserum during the incubation (Crofford, 1968).

The metabolic effects of insulin have been shown to follow closely the binding of hormone to the cell. For example, the time required for a given concentration of insulin to stimulate glucose utilisation fully in the adipocyte (Gliemann, 1970 a, b) has recently been shown to correlate well with the time required for the same concentration of hormone to equilibrate with the plasma membrane of intact adipocytes (Gammeltoft and Gliemann, 1973). The close relationship between the binding of insulin and the consequent metabolic stimulation was also demonstrated in studies with trypsin-treated adipocytes. In such cells the loss of sensitivity of glucose metabolism to insulin was found to follow closely the decreased affinity of the membrane for insulin (Cuatrecasas, 1971b, Kono and Barham, 1971).

The biological response of isolated adipocytes to insulin can be measured by observing the increased incorporation of glucose into carbon dioxide and triglycerides or the increased total glucose utilisation. It is generally assumed that incorporation of glucose into carbon dioxide and triglyceride represents such a large proportion of total glucose utilisation that it can be used to represent total utilisation (Gliemann, 1968). Moreover, it is assumed that glucose utilisation reflects the rate of inward transport of glucose since the transport process appears to be the major rate-limiting step governing the utilisation of glucose in adipocytes (Crofford and Renold, 1965).

In this chapter the relationship between insulin concentration and insulin effect will be described. Possible interpretations in terms of insulin binding to the membrane will be made, assuming that there is a linear relationship between insulin association with its receptor and its subsequent effect on glucose metabolism.

A typical response curve from this laboratory is presented in Fig. 3.1. This sigmoid dose-response relationship cannot be interpreted in terms of the simplest binding model

$$I+R \rightleftarrows IR \rightarrow \text{effect}$$

since this would give a hyperbolic relationship between free insulin and its effect and, providing R is very much smaller than I, between total insulin concentration and its effect.

Sigmoid progress curves are often associated with allosteric enzyme systems in which the activity of the protein is regulated by co-operative subunit interactions (see, for example, Changeux et. al. 1968). The origin of the sigmoid biological response at the level of co-operative insulin

Figure 3.1 The relationship between insulin concentration and glucose utilisation in the adipocyte.

binding to the membrane was postulated in the model of Garratt et. al. (1970) described in Chapter 1. Such a binding curve could also arise from co-operative subunit interactions within the membrane (Changeux, 1968) or from the binding of only polymeric species of a self-associating ligand such as insulin, providing the polymerisation is an equilibrium process (Nichol et. al., 1969).

The contribution of the major sources of experimental artefact to the form of the relationship will be assessed before proceeding to a consideration of the possible mechanistic implications of a sigmoid binding curve.

3.2 Studies of some artefacts which may generate a sigmoid response curve

The sigmoid dose response does not arise through the failure of insulin, at the lower concentrations used, to equilibrate fully with its receptor in the time course of the experiment (Gliemann, 1970). This study did show, however, that preincubation of adipocytes with insulin reduced the sigmoidicity of the response without completely abolishing it. The smooth dose response curve might reflect a graded response of individual adipocytes to variations in the concentration of insulin. Alternatively, each cell might respond maximally above a threshold insulin concentration and not at all below this concentration. The sigmoid dose response curve could then arise if these threshold concentrations were normally distributed through the population of cells used in these experiments. Gliemann (1970) however, has shown that the adipocyte response to insulin is graded and threshold concentration variations do not contribute to the form of the

dose response curve.

Two possible experimental artefacts which could give rise to a sigmoid response curve from a hyperbolic binding curve were not conclusively excluded by Gliemann's experiments. These were the possible loss of insulin from solution by non-productive binding or degradation and the possibility that insulin has a specific directive effect on metabolism so that the response measured does not adequately represent the primary effect of insulin.

Loss of insulin from solution through degradation or non-specific binding was therefore studied over an appropriate concentration range to assess the effect of possible changes in free insulin concentration on the dose-response relationship.

The possible implication of a metabolic directive effect at low concentrations of hormone was also studied by means of a technique specially developed for this purpose (see Chapter 2).

3.2.1 Estimation of the loss of insulin from solutions incubated with adipocytes

Several workers have shown that incubation of adipocytes with insulin can under certain conditions lead to a loss of hormone through binding or enzymic degradation (Crofford, 1968; Cuatrecasas, 1971; Kono and Barham, 1971 and Gammeltoft and Gliemann, 1973). Clearly if such processes appreciably reduced the concentration of free insulin available to bind to its receptor, potentially grave errors would be introduced into the values used for the insulin concentration when determining the dose-response relationship.

There is no direct evidence available on insulin loss and the concentration dependence of non-productive insulin binding to cells or apparatus (Newerly and Berson, 1957) is not known. If such sites of insulin loss were of high affinity and low capacity one would expect insulin loss to be proportionately most severe at the very low concentrations close to the origin in Fig. 3.1, becoming less severe with increasing insulin concentration as these sites are progressively saturated. That is to say one might expect insulin loss to be proportionately greatest over the range of concentrations describing the first inflexion on the dose-response curve. Reduction of the amount of free insulin available at these low concentrations to or below the lowest concentration giving a measurable stimulation could thus produce an apparently sigmoid dose response if the concentration terms were not corrected for this loss.

The loss of insulin which would be required to produce such a response from a hyperbolic binding curve can be calculated in the following way. Data from the dose response curve, when plotted in the form

$\log i$ versus $\log \left(\frac{e}{E-e}\right)$ where i is insulin concentration

e, observed stimulation

E, the maximal obtainable stimulation

give a line with a slope greater than one, the slope indicating the degree of co-operativity, or Hill coefficient (see Chapter 1). By comparison a hyperbolic binding curve gives a line of unit slope when plotted in this form. It is therefore possible, using such a line, to obtain pairs of values for insulin concentration and effect which would fit

Figure 3.2 The insulin loss required to produce a sigmoid response curve from a hyperbolic binding curve: the method used in the calculation.

a hyperbolic binding curve. The insulin concentration needed to produce a given effect on a hyperbolic curve can then be compared with the insulin concentration actually used to produce that effect. The difference between the experimental and calculated insulin concentrations gives a measurement of the quantity of insulin which would have to be lost from the experimental vials in order to generate a sigmoid response from a hyperbolic binding curve.

The difficulty with drawing the line of unit slope lies in choosing its intersection with the line of higher slope which best fits the experimental data. However, the logarithmic scale on which insulin concentration is plotted means that at the highest insulin concentrations a large change in total insulin concentration makes a comparatively small change to the reading used. For the purposes of the comparison outlined above the line of slope one was therefore drawn through the highest insulin concentration used to give a submaximal response.

The typical curve presented in Fig. 3.1 was used to perform this calculation (Table 3.1). It is apparent that at the very low concentrations where sigmoidicity is manifest, a substantial proportion of the insulin present would have to be lost to produce the observed response from a hypothetical hyperbola. For example, the observed insulin stimulation in the presence of $1x10^{-11}M$ insulin would require a concentration of $2.5x10^{-13}M$ in the absence of insulin loss, if the true binding curve were hyperbolic. An insulin concentration of $1x10^{-11}M$ is distinguishable from an insulin blank in both adipocyte bioassay and radioimmunoassay systems under suitable conditions, whereas $2.5x10^{-13}M$ is not.

Table 3.1 Insulin loss required to produce a sigmoid response from a hyperbolic binding curve (Fig. 3.2). All concentrations are 10^{10}M.

Insulin concentration from sigmoid response	Insulin concentration from the hyperbola	Difference as %
0.05	0.0005	99.0
0.1	0.0025	97.5
0.3	0.0282	90.6
0.5	0.0912	81.75
1.0	0.407	59.30

It seemed, therefore, that if the postulated non-specific insulin loss was of the order indicated by this calculation it would be measurable.

Adipocyte bioassay and radioimmunoassay (Chapter 2) were then used to estimate the true loss of insulin from KRB solutions which had been incubated with adipocytes. The cell suspensions used in these experiments were denser than those normally used by a factor of four, in order to enhance any loss of insulin compared with the typical case shown. This seemed advisable since Crofford (1968) was unable to measure a loss of insulin from solutions incubated with dilute cell suspensions.

In the first type of experiment the exquisite sensitivity of the adipocyte preparation to insulin, clearly shown in Fig. 3.1, was utilised to bioassay the residual insulin in experimental vials which had been incubated for one hour with adipocytes. The effect of a wide range of insulin concentrations on glucose incorporation into triglycerides was measured and at the same time a set of identical standards was prepared for incubation without adipocytes. At the end of the incubation period the incubation vials were centrifuged briskly to spin up the adipocytes, the triglycerides were then extracted from the cells and 250 µl aliquots were removed from the infranatant buffer by Hamilton syringe for incubation with a fresh adipocyte suspension. The sensitivity of this second suspension was simultaneously determined with the range of standards prepared with the first standard curve solutions and so any differences observed should be due to the exposure to adipocytes rather than to dilution errors. Loss of glucose

during the first incubation period was less than 5% in the vials containing a maximally stimulating concentration of insulin so the subsequent bioassay was not performed under conditions of glucose deficiency.

Fig. 3.3 shows the results of this assay in the form of a series of Hill plots. Line 1 ($n=1.37 \pm 0.12$) is the standard curve of the first cell preparation, Line 2 (broken line) being the line of unit slope used to indicate the insulin loss required to generate this sigmoid response from a hyperbolic binding curve, as described above. The hypothetical loss of hormone was calculated at the three lowest insulin concentrations (see table accompanying Fig. 3.3). Line 3 ($n=1.89 \pm 0.17$) is the standard response of the second adipocyte preparation, Line 4 ($n=1.33 \pm 0.13$) being the bioassay of buffer derived from vials of Line 1 with these cells. The difference between these lines (3 and 4) indicates the magnitude of the insulin loss during the initial incubation with adipocytes. If the difference between the theoretical hyperbolic binding curve (Line 2) and the observed sigmoid response (Line 1) was due to insulin loss upon incubation with adipocytes one would expect the slope of the bioassay data (Line 4) to be steeper than its corresponding standard curve (Line 3). This would indicate that a higher insulin concentration was required to produce a given effect. The separation between the two lines would then indicate the true insulin loss. The slope of Line 4 is clearly less than that of its standard curve.

The sigmoidicity of the response, indicated by the n value, was less in medium which had previously been exposed

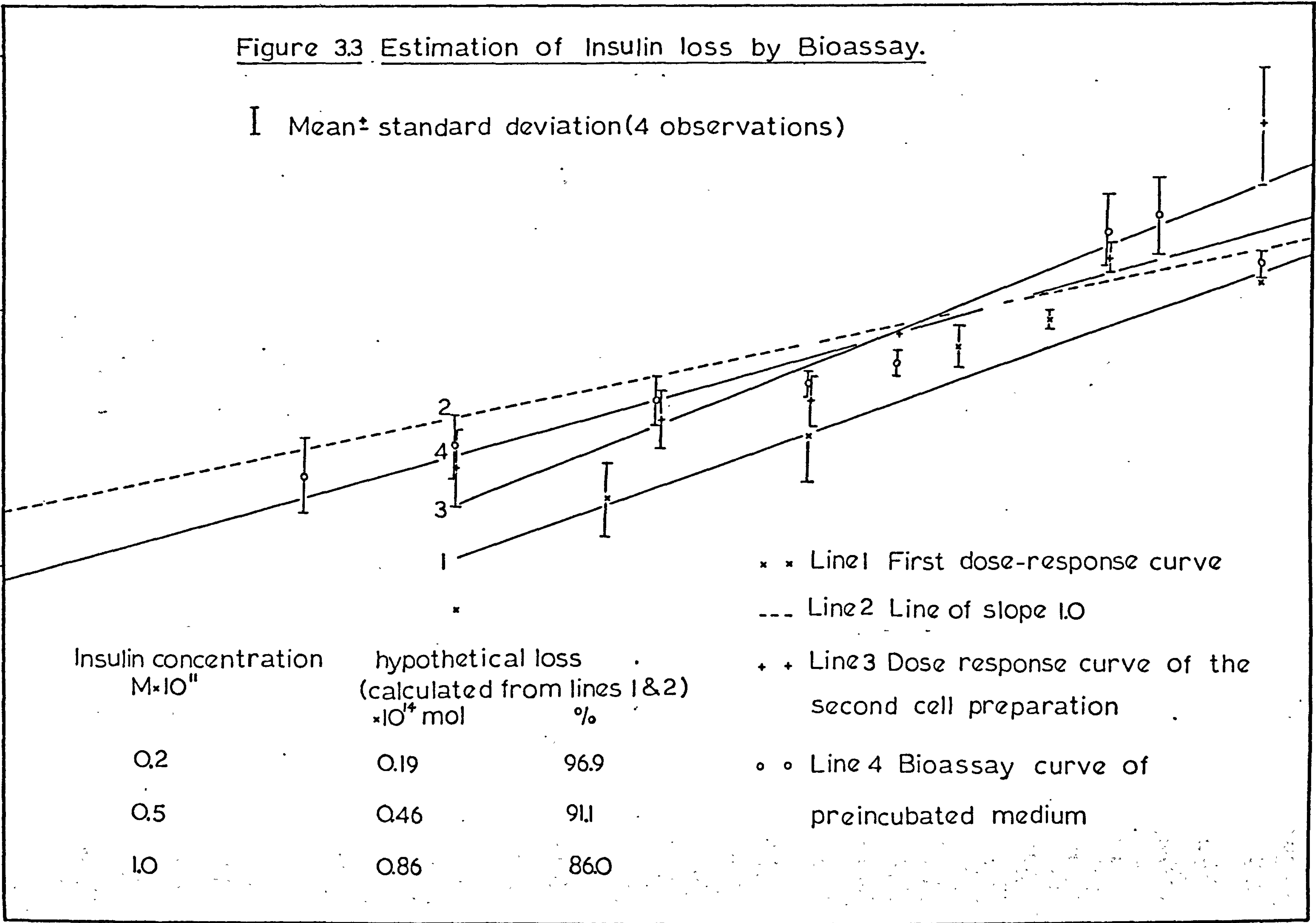


Figure 3.3 Estimation of Insulin loss by Bioassay.
I Mean± standard deviation(4 observations)
2.5
0.0
-2.5
$\log(\frac{e}{E-e})$
-1.0
0.0
0.9
log(insulin concentration)
1
2
3
4
Line1 First dose-response curve
Line2 Line of slope 1.0
Line3 Dose response curve of the second cell preparation
Line 4 Bioassay curve of preincubated medium
Insulin concentration M×10¹¹
hypothetical loss (calculated from lines 1&2)
×10¹⁴ mol
%
0.2
0.19
96.9
0.5
0.46
91.1
1.0
0.86
86.0

to cells than in fresh medium. If the sigmoidicity was due to loss of free insulin one would have expected the bioassay of preincubated buffer to indicate an enhanced sigmoidicity and a larger n value, since the loss of insulin should be greatest in these vials, which were incubated with two preparations of adipocytes. A comparison of the insulin concentrations giving half the maximal stimulation ($I\frac{1}{2}$) suggests that the preliminary incubation removed small amounts. The $I\frac{1}{2}$ value obtained with preincubated medium was 14% higher than that obtained with fresh buffer, though the difference was not significant at any probability level tested, using Student's t-test. This shows that insulin loss of the order calculated to be necessary did not occur on incubation with adipocytes.

The extent of insulin loss during incubation with adipocytes was also estimated by radioimmunoassay. A range of insulin concentrations which covered the full adipocyte dose-response curve was incubated with adipocytes for one hour and aliquots of buffer free from cells were obtained for the assay as described above. In order to preclude concentration changes arising as a result of the incubation procedure as distinct from the exposure to cells, a parallel incubation of standards was performed as follows (for further details see Chapter 2). A large volume of insulin-free buffer was incubated with adipocytes for one hour, after which time the cells were removed as described previously. Aliquots of this buffer were then used to complete the dilution of the standards in place of the aliquots of adipocyte suspension which were added to sample vials. Any difference between

Table 3.2 Loss of immunoreactive insulin following exposure of KRB to adipocytes

Initial insulin concentration $x10^{11}M$	Residual concentration $x10^{11}M$		Concentration change %	
	Immunoassay	Calculated[1]	Immunoassay	Calculated[1]
0.5	0.25	0.048	50	90.5
1.0	0.69	0.095	31	90.2
2.0	2.00	0.778	0	61.1
4.0	4.00	3.979	0	0.5
8.0	8.00	8.00	0	0.0

[1] This was calculated as the difference between the Hill plot of the experimental data and a line of unit slope (see Fig. 3.4 and text for details).

samples and standards is therefore attributable to the presence of adipocytes during the incubation period with hormone, since other sources of insulin loss should affect both samples and standards equally.

This procedure indicated a finite loss of immunoreactive insulin (Table 3.2) over a range of concentrations which covered the first inflexion on the dose response curve obtained. At the lowest concentrations used the proportion of insulin lost appeared to be inversely related to the hormone concentration. No loss could be detected from a solution of 2×10^{-11}M insulin owing to the shallowness of the standard curve used, although a loss of the order of 25% would have been measurable.

The response curve obtained from the adipocytes used in this experiment (measuring the stimulation of incorporation of glucose into triglycerides) was sigmoid. The hypothetical loss of insulin required to derive this response from a hyperbolic binding curve has been calculated in the manner set out previously and this loss is compared with the actual loss in Table 3.2. These data show that the loss of immunoreactive insulin, although substantial, was not sufficient to account entirely for the observed sigmoidicity.

This point is illustrated in Fig. 3.4 where the dose-response is presented in the form of a Hill plot ($n=1.86 \pm 0.26$, $I_{\frac{1}{2}}=2.83 \pm 1.24$), with a line of unit slope drawn for comparison. The true insulin concentrations obtained from the immunoassay were then used to calculated revised Hill plot parameters ($n=1.80 \pm 0.20$, $I_{\frac{1}{2}}=2.66 \pm 1.29$). Although the slope was in fact slightly reduced by this procedure, indicating a reduction of sigmoidicity, the difference was

Figure 3.4 Comparison of a line of unit slope with dose response lines drawn from the initial and corrected insulin concentrations.
the line of slope>1.0 describes the initial insulin concentrations
I values as mean±S.D.
1.0
0.0 log(e/(E-e))
-1.0
-2.8
∘ initial concentrations
+ residual concentrations (by immunoassay)
log(insulin concentration)M
-1.0
0.0
1.0

not a significant one (t-test, $p>0.05$). The magnitude of insulin loss estimated by this procedure was insufficient to account for the observed sigmoid response and is consistent with the estimate of loss obtained by bioassay (Fig. 3.3). The immunoassay results suggest that the lost insulin was bound to or degraded in the adipocyte plasma membrane and was not destroyed by proteolytic enzymes released into the buffer from the adipocytes. Had this been the case then the controls, which were incubated with medium which had previously been exposed to adipocytes, would have been indistinguishable from the samples, which were incubated with adipocytes.

Recent published work using ^{125}I-insulin (Gammeltoft and Gliemann, 1973) suggests that the degradation of insulin would not be significant with the density of cell suspension used in these experiments. Furthermore, studies with ^{125}I-insulin have recently shown that only a very small proportion of the total insulin present is bound to the cell over the concentration range used to study the adipocyte response (Cuatrecasas, 1971; Kono and Barham, 1971; Hammond et. al., 1972 and Gammeltoft and Gliemann, 1973). This is consistent with the work of Crofford (1968), who was unable to detect a loss of insulin from solutions exposed to cell suspensions approximately twice as dense as those used in the experiments described in this Section. It is clear, therefore, that the sigmoid dose response of the adipocyte does not arise from severe loss of insulin at low concentrations in these experiments and since the adipocyte suspension was far denser than that normally used, it appears unlikely

that insulin loss contributed significantly to the sigmoid response normally obtained.

3.2.2 The possible contribution to sigmoidicity of a metabolic directive effect

The measurement of insulin response made in these experiments and in the literature examined was the increase in conversion of glucose into its major metabolites (carbon dioxide and triglycerides). This method is dependent on the assumption that the metabolites measured form a constant proportion of the total glucose metabolites under the varying conditions of glucose uptake used. A directive metabolic effect of low concentrations of insulin, favouring the production of a metabolite not studied, could generate a sigmoid response from a hyperbolic binding curve by changing the pattern of glucose distribution. Moody and Gliemann (1968) have reported such an effect, low concentrations of insulin favouring glucose conversion to glycogen in adipocytes.

In order to examine the contribution of such an effect to the form of the dose response curve total glucose uptake and its relationship with insulin concentration were studied by means of a specially developed technique using 5-^{3}H-glucose. This technique is described in Chapter 2. 5-^{3}H-glucose uptake from the medium, even in the presence of insulin, was found to agree well with glucose loss from the medium. This shows that in the adipocyte preparation used conversion of glucose to glycogen was insignificant and that 5-^{3}H-glucose was a valid tracer for estimating total glucose uptake.

Two experiments were performed to study the insulin

concentration dependence of glucose uptake and in both of them the relationship was clearly sigmoid ($n>1$, Table 3.3). Glucose uptake was sensitive to the same range of insulin concentrations as the production of carbon dioxide, glyceride fatty acids and glyceride glycerol in both experiments. The estimates of the Hill coefficient obtained within each experiment were essentially similar. In Experiment 1 the values of n obtained from Hill plots of glucose uptake and fatty acid production against insulin concentration were significantly different (t-test, $p<0.05$). In Experiment 2 a significant difference at the same probability level was observed between the n values obtained from fatty acid and glycerol production data. A consistent difference between n values was not observed. There was no significant difference between the $I_{\frac{1}{2}}$ values obtained within an experiment (t-test was used for this comparison).

The sigmoid response observed with a measurement of total glucose uptake clearly excludes the possibility that the sigmoidicity arises from a directive metabolic effect operating at low insulin concentrations.

3.3 Implications of the sigmoid dose response

The chief sources of artefact in these experiments have been examined and found to be insufficient to account for the observed sigmoidicity. This means that either the binding of insulin to the cell membrane is sigmoidally related to concentration or that if hormone binding is hyperbolic binding and biological activation are not linearly related. The Hill coefficients obtained were examined in the light of the

Table 3.3 The relationship between insulin concentration, glucose utilisation and its distribution between the major metabolites[x]

	Hill coefficient n	$I_{\frac{1}{2}}$
Experiment 1		
Glucose utilisation	1.84±0.16	6.77±1.11
CO_2	2.18±0.15	4.31±1.05
Glyceride fatty acids	2.24±0.13	6.58±1.08
Experiment 2		
Glucose utilisation	1.756±0.14	2.62±1.20
CO_2	1.776±0.17	3.18±1.26
Glyceride fatty acids	1.710±0.19	3.89±1.33
Glyceride glycerol	2.338±0.32	1.62±1.27

x Values are expressed as Mean ± S.EM. Curves in Experiment 1 were fitted to 25 points and in Experiment 2 to 31 points.

three sigmoid binding models proposed (Chapter 1) in order to consider the mechanistic implications of a sigmoid binding curve.

Table 3.4 contains the results of all the experiments which were performed for this purpose. A finite lag period has been observed between the addition of insulin to adipocytes and the production of the full biological response which was inversely related to the concentration of hormone used (Gliemann, 1970). Since this study also indicated that preincubation of adipocytes with insulin decreased the sigmoidicity of the response it seemed appropriate to measure the contribution of this phenomenon to the sigmoid response obtained. In Experiment C2 and Y2 the adipocytes were preincubated with insulin for one hour before the addition of labelled glucose. This procedure did not abolish the sigmoidicity of the response and did not, in fact, give a lower estimate of the Hill coefficient than a comparable experiment performed on the same adipocyte preparation without preincubation (compare C2 with C1, Table 3.4). This supports the conclusion of Gliemann (1972) that sigmoidicity does not arise through the failure of insulin at the lowest concentrations to equilibrate with its receptor in the time course of the experiment. This being so, preincubation was not used in the subsequent experiments.

Considering Tables 3.3 and 3.4 together one may see that glucose uptake and its conversion to carbon dioxide, triglyceride and its constituent fatty acids and glycerol were all sigmoidally related to insulin concentration ($n>1$). Furthermore the Hill coefficient was observed in several

Table 3.4 Studies of the relationship between insulin concentration and glucose incorporation into CO_2 and triglyceride

Metabolite	Experiment	n	$I_{\frac{1}{2}}$ $\times 10^{11}$M	N[2]
CO_2				
	[1]C.1	2.54±0.082	4.64±0.778	77
	C.2[x]	2.79±0.062	3.89±0.749	64
	Y.1	1.23±0.118	4.49±1.31	30
	Y.2[x]	1.53±0.213	4.42±1.53	26
	Y.3	1.67±0.085	4.96±1.18	35
	Y.4	1.68±0.330	7.03±2.01	30
	Y.5	1.68±0.180	3.61±1.33	24
	Y.6	1.84±0.224	3.48±1.40	24
	Y.7	1.36±0.112	3.96±1.23	30
Triglycerides				
	Y.5	1.596±0.110	2.70±1.18	24
	Y.8	2.160±0.210	2.83±1.24	26
	Y.9	1.250±0.110	3.26±1.26	24

1 Experimental prefix denotes laboratory in which the experiment was performed - C indicates the laboratory of J. Gliemann in Copenhagen and Y indicates experiments at York.

2 Number of points used to fit the curve.

x Experiments in which adipocytes were preincubated with insulin.

All values are expressed as Mean ± S.E.M.

instances to be greater than 2 (Experiments C1, C2 and Y8 of Table 3.4; CO_2 and fatty acid production in Experiment 1 and glycerol production in Experiment 2, Fig. 3.3).

An n value greater than two suggests that in the model systems described, there must be a degree of co-operativity greater than two. If the binding occurs according to the self-interacting ligand hypothesis of Nichol and his co-workers (1969), the active, binding species must be greater than the dimer. Similarly if binding is of the form described by the co-operative insulin binding model (Garratt et. al., 1970) then more than two insulin molecules must bind to activate each receptor. Finally the Changeux model (1968) is consistent with these results if more than two insulin receptors interact co-operatively in the binding of hormone. Since insulin in solution forms a self-associating system (for example, see Jeffrey and Coates, 1966) these models may be related to the well characterised physical properties of the hormone. It is widely accepted that under suitable conditions, in the presence of zinc, most insulins form an equilibrium mixture according to the following scheme

6 Monomers ⇄ 3 Dimers ⇄ Hexamer

The self-associating ligand hypothesis of Nichol et. al. (1969) is consistent with this scheme and with a Hill coefficient greater than two if the hexamer is the active species. The model of Garratt et. al. (1970) allows the attractive hypothesis that three insulin dimers bind co-operatively to form an activated receptor-hexamer complex, the structure of this hexamer being essentially that of the repeating unit in the insulin crystal (Blundell et. al. 1971) and known to exist in solution (Sjogren and Svedberg, 1931;

Cunningham et. al., 1955 and Fredericq, 1956).

It has recently been stated (Blundell et. al., 1972) that there is uncertainty over the active species of insulin, though there is evidence that at physiological pH and concentration little or no hexamer exists. The absence of appreciable amounts of the binding polymer (hexamer) indicated by the response curve analysis precludes the model of Nichol et. al. (1969) as a viable model for the binding of insulin to its cellular receptor.

In order to elucidate the possible role of higher insulin aggregates in cell activation, the response of adipocytes to guinea pig insulin was studied. Sedimentation equilibrium studies have shown that in neutral solution at a concentration of approximately 50 μM (0.3 mg/ml) guinea pig insulin exists as a monomer even in the presence of zinc ion (Zimmerman et. al., 1972). The sequence of guinea pig insulin differs from that of the bovine hormone in 18 positions (Smith, 1966), the effect of these substitutions being to destabilise the hexamer without, apparently, excluding the possibility of dimerisation (Blundell et. al., 1971).

3.4 The activity of guinea pig insulin in the rat adipocyte bioassay system

Since guinea pig insulin appears not to form hexamers and dimerisation has not been observed even at high concentration one would not expect it to be active in a system binding only higher polymers of insulin (Nichol et. al., 1969). Similarly if the sigmoidicity were related to the ability of insulin to form hexamers, as proposed by Garratt et. al. (1970), a different dose response relationship would be predicted for guinea pig insulin.

A sample of guinea pig insulin for use in this study was isolated and purified by a method only slightly different from that of Davoren (1962). Full details are included in Chapter 2.

The preparation of guinea pig insulin used in these experiments was not homogeneous on polyacrylamide gel electrophoresis, although substantial purification was achieved (Chapter 2). It did not appear to be extensively contaminated with glucagon since it increased carbon dioxide and triglyceride production from glucose in a proportion characteristic of insulin. It was concluded therefore that the biological activity in the extract was due to insulin.

In the first experiment performed with this insulin a clearly sigmoid response was obtained, following carbon dioxide production from glucose, which gave a Hill coefficient of 1.96 ± 0.1 (Table 3.5). This value is typical of those obtained in this laboratory with beef insulin (Table 3.4). The maximum stimulation of carbon dioxide and triglyceride synthesis in this experiment was not different from that obtained with a saturating concentration of beef insulin.

Table 3.5 Analysis of the response curves obtained with guinea pig insulin. The curves presented were calculated from CO_2 production. Values are Mean ± S.E.M.

Experiment	n	$I\frac{1}{2}$ ng/ml	$I\frac{1}{2}$ $x10^{11}M$	N
1	1.96±0.09	13.10±0.06		32
2	1.61±0.12	19.41±0.09		38
Beef insulin	0.96±0.08		2.71±1.23	38
3	1.12±0.18	45.5±0.36		38
Beef insulin	0.90±0.11		4.21	38

Table 3.5 also shows a comparison of Hill coefficients obtained with guinea pig and beef insulins in parallel determinations using the same adipocyte preparation. Clearly the values obtained with guinea pig insulin are not less than the beef insulin values, as one would expect if hexamerisation were required for the production of the sigmoid response.

These results show that the binding of beef and guinea pig insulins are essentially similar and if beef insulin requires the binding of three molecules to each receptor to produce a stimulus then so does the guinea pig hormone. The sigmoidicity of the guinea pig insulin dose response clearly excludes the binding of hexamers or the binding of three dimers into a receptor-bound hexamer related to crystal structure as possible mechanisms for the primary action of insulin.

Despite the presence of some impurities in the guinea pig insulin preparation the $I_{\frac{1}{2}}$ values obtained in these experiments do give an indication of the maximum amount of insulin which could have been present. Estimates of the $I_{\frac{1}{2}}$ obtained (13.1, 19.4 and 45.5 ng of protein per ml) were several orders of magnitude smaller than the concentration at which guinea pig insulin was shown to be monomeric (300 µg/ml, Zimmerman et. al., 1972). It therefore seems improbable that dimerisation of guinea pig insulin occurred under these conditions and that aggregation of the insulin monomer plays any role in the biological activity of the hormone.

3.5 Discussion

The adipocytes used in this work displayed the same sensitivity to insulin that was noted by other workers (for example Gliemann, 1969). Gammeltoft and Gliemann (1973) have recently suggested that the half maximal stimulation of carbon dioxide and triglyceride production requires an insulin concentration of 5×10^{-11}M, a value which compares well with the figures presented (Table 3.4).

The sigmoidicity of the biological response curve was shown not to be due to experimental artefact. Preincubation of the adipocytes with insulin, without eradicating the sigmoidicity reduced it and slightly lowered the $I_{\frac{1}{2}}$ value. This is consistent with the need for insulin to equilibrate with its receptor before the full effect of hormone is produced (Gliemann, 1970 and Gammeltoft and Gliemann, 1973). The loss of insulin during incubation with adipocytes was only measurable at the lowest concentrations used and did not contribute significantly to the sigmoidicity of the response. This is consistent with the work of Crofford (1968) and the recent studies using ^{125}I-insulin (Cuatrecasas, 1971; Kono and Barham, 1971; Hammond et. al., 1972 and Gammeltoft and Gliemann, 1973).

The guinea pig insulin studies presented demonstrate that the sigmoid response requires neither the presence of insulin hexamers nor the ability of the insulin to hexamerise. It appears unlikely that at physiological concentrations guinea pig insulin can exist as a dimer and these studies suggest that the active insulin species is the monomer. Further support for this hypothesis may be derived from chemical modification studies of insulin. For example,

Maloney et. al. (1964) produced sulphated insulins which were monomeric yet biologically active. The considerable biological activity retained by insulin covalently attached to bulky Sepharose beads (Cuatrecasas, 1969) also strongly suggests that the active insulin species is the monomer. Two recent physical studies (Grant et. al., 1972, Izzo et. al., 1973) have suggested that under physiological conditions of pH and concentration insulin is probably monomeric. This is consistent with the full biological activity obtained in the guinea pig insulin studies (3.4) presented here, under conditions where the hormone was probably monomeric.

If the biological response is linearly related to the hormone binding, as postulated, then the binding curve must also be sigmoid. Of the models considered in this chapter only the co-operative subunit model membrane can still be sustained as a viable model of a sigmoid binding phenomenon. Evidence has recently been presented of negatively co-operative interactions between the insulin receptors of cultured lymphocytes and of liver plasma membranes (De Meyts et. al., 1973) which is not consistent with the positive co-operativity postulated in this chapter to describe the sigmoid response of the adipocyte to insulin. Furthermore, recent studies with ^{125}I-insulin (Cuatrecasas, 1971, Kono and Barham, 1971) appear to have demonstrated a hyperbolic binding of insulin to the adipocyte membrane though House (1971), noting a complex time course of binding, suggested that the physiologically significant binding displayed sigmoid saturation kinetics. This report has not yet been

confirmed. The dissociation constants (Kdiss) measured in these studies (for example $3x10^{-9}$, Gammeltoft and Gliemann, 1973) were considerably higher than the $I_{\frac{1}{2}}$ of the biological response, although Cuatrecasas (1971) has published a dissociation constant of $8x10^{-11}$M. The higher Kdiss values suggest that the maximal biological response may be obtained with only 2.4% of the receptors occupied (Kono and Barham, 1971), indicating a complex relationship between insulin binding and biological response.

Clearly there is still considerable doubt about the mechanism of association of insulin with the cell membrane. The consensus of opinion, however, is that formation of the insulin-receptor complex displays Michaelis-Menten saturation kinetics. It now seems probable, therefore, that the initial hypothesis that there is a linear relationship between binding of insulin and cell stimulation is no longer tenable. Should this in fact be the case then the sigmoid response must arise subsequent to the hyperbolic binding of insulin to its receptor.

<u>Summary</u>

1. The sigmoid dose-response curve represents the true response of adipocytes to variations in the concentration of free insulin.
2. Sigmoidicity does not depend on the ability of insulin to form aggregates.
3. Since the binding of insulin to the cell membrane appears to be hyperbolic, sigmoidicity must arise subsequent to the hormone binding.

Chapter 4

STUDIES OF A DIRECT EFFECT OF INSULIN ON GLUCOSE METABOLISM IN THE ADIPOCYTE

4.1 Introduction

In the previous chapter the glucose utilisation of isolated adipocytes was shown to be sigmoidally related to the concentration of insulin in the medium. There is no evidence for a sigmoid relationship between insulin concentration and its binding (Cuatrecasas, 1971; Kono and Barham, 1971) and in the experiments described in Chapter 3 none could be found favouring the hypothesis that the sigmoid dose-response relationship resulted from the ability of insulin to self-associate. It therefore seemed likely that the sigmoid dose response curve arose from a sigmoid relationship between insulin bound to its receptor and the effect of the bound insulin. In an attempt to trace the origin of the dose response curve it was decided to examine the dose-response relationship not between insulin concentration and its effect on net glucose utilisation but between insulin concentration and its effects on metabolism.

The insulin stimulation of fatty acid synthesis in the two experiments presented in Table 3.3 was much greater than the stimulation of glucose utilisation or its incorporation into other metabolites (see Table 4.1). This is consistent with the known effects of insulin on lipogenesis and lipolysis in the adipocyte. Winegrad and Renold (1958) showed that insulin preferentially augmented fatty acid synthesis in adipose tissue. Subsequently Leonards and Landau (1960) were able to demonstrate that insulin plus low concentrations of glucose did not give the same pattern of incorporation

Table 4.1 Preferential effect of insulin on glucose incorporation into fatty acids

Experiment[x]	Parameter studied	Insulin stimulation[1]
1	Glucose utilisation	13
	CO_2 production	14.6
	Fatty acid synthesis	28
2	Glucose utilisation	6.6
	CO_2 production	9.6
	Fatty acid synthesis	14
	Glyceride glycerol synthesis	7.6

x Experiments are those described in Table 3.3

1 Insulin stimulation is defined as

$$\frac{\text{Production of metabolite with insulin}}{\text{Production of metabolite without insulin}}$$

of glucose into its major metabolites as high concentrations of glucose without hormone. However these experiments did not allow a distinction to be drawn between a direct effect of insulin on lipogenesis or an indirect effect mediated through increased glucose utilisation.

In order to distinguish between these two mechanisms of stimulation of lipogenesis it is important to study the pattern of glucose metabolism in the presence and absence of insulin over the same range of glucose utilisation values. However, such an overlap of utilisation values is not readily achieved by manipulation of the glucose concentration in the medium, as is apparent from Fig. 4.1. In this example the addition of insulin increased the glucose utilisation by a factor of 10 at 0.05 mg/ml glucose. The rate of glucose utilisation observed at this glucose concentration in the presence of insulin was greater than the rate obtained at 0.7 mg/ml in the absence of insulin by a factor of 2.6.

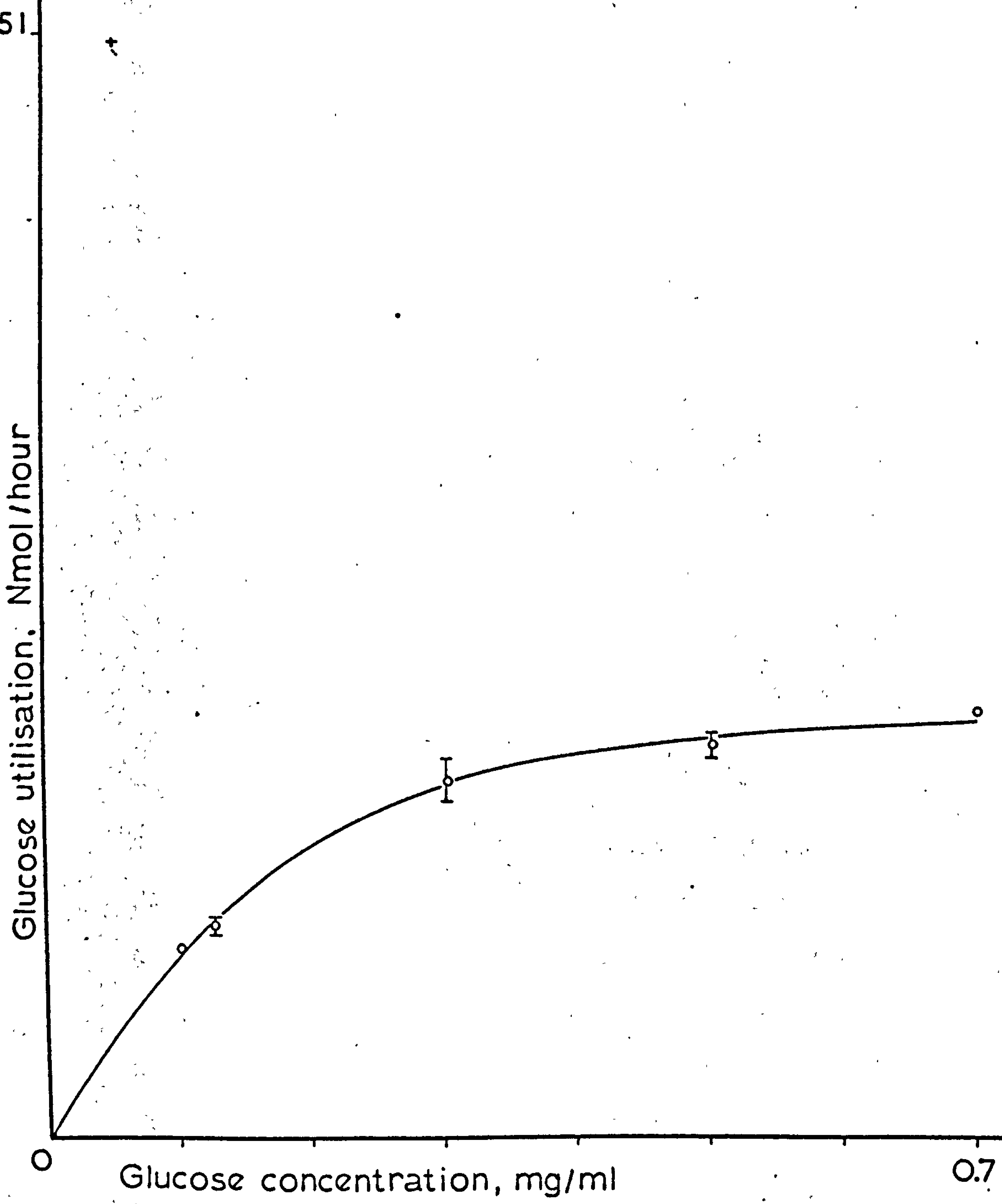
Glucose uptake in the adipocyte is thought to involve a facilitated diffusion process mediated through a mobile carrier mechanism (see Chapter 5, Section 3), so that uptake is described by Michaelis-Menten kinetics (Crofford and Renold, 1965). Several workers have used linear transformations of the Michaelis Menten equation to calculate a theoretical maximal rate of glucose uptake by the adipocyte (see, for example, Glieman, 1970). A theoretical maximal uptake rate of 21.8 Nmol/hour was calculated for the basal data shown in Fig. 4.1 using the Eadie transformation fitted by unweighted linear regression. Thus the rate of glucose uptake observed at 0.05 mg/ml glucose in the presence of insulin was greater than the theoretical maximal rate which could have been obtained without insulin by a factor of 2.3.

Figure 4.1 The effect of insulin on glucose utilisation in the adipocyte.

∘ ∘ Adipocytes without insulin

+ Adipocytes with insulin (10^{-9} M)

Values are Mean ± S.D. (4 observations)



In order to bring the glucose utilisation in the presence of insulin within the basal range a lower glucose concentration than that indicated in Fig. 4.1 might be used. However at such low glucose concentrations the adipocytes would use a considerable proportion of the available glucose and this would lead to substantial changes in the medium glucose concentration. Since the effect of glucose concentration on glucose utilisation was being studied it was important that the concentration did not change significantly during the incubation. It was therefore necessary to increase glucose utilisation in the absence of insulin or to reduce the utilisation values obtained with insulin or both to obtain an overlap.

Figs. 4.2 and 4.3 illustrate how the action of insulin on glucose transport complicates an analysis of the effects of the hormone on fatty acid synthesis in the adipocyte. These Figs. show the relationship between glucose utilisation and fatty acid synthesis in unstimulated cells and in cells incubated with submaximally and maximally stimulating concentrations of insulin. Fig. 4.2a shows that in a typical experiment a linear relationship exists between glucose utilisation and fatty acid synthesis over the whole range of basal utilisation values. The line was fitted to the data using an unweighted linear regression analysis. Fig. 4.2b shows the relationship obtained by incubating cells with varying concentrations of glucose in the presence of a single, submaximally stimulating concentration of insulin (2.5×10^{-11}M). The fitted basal line is included for comparison. The arrow indicates the calculated Vmax for glucose utilisation. It is apparent from this Fig. that the basal observations could not have been extended to cover

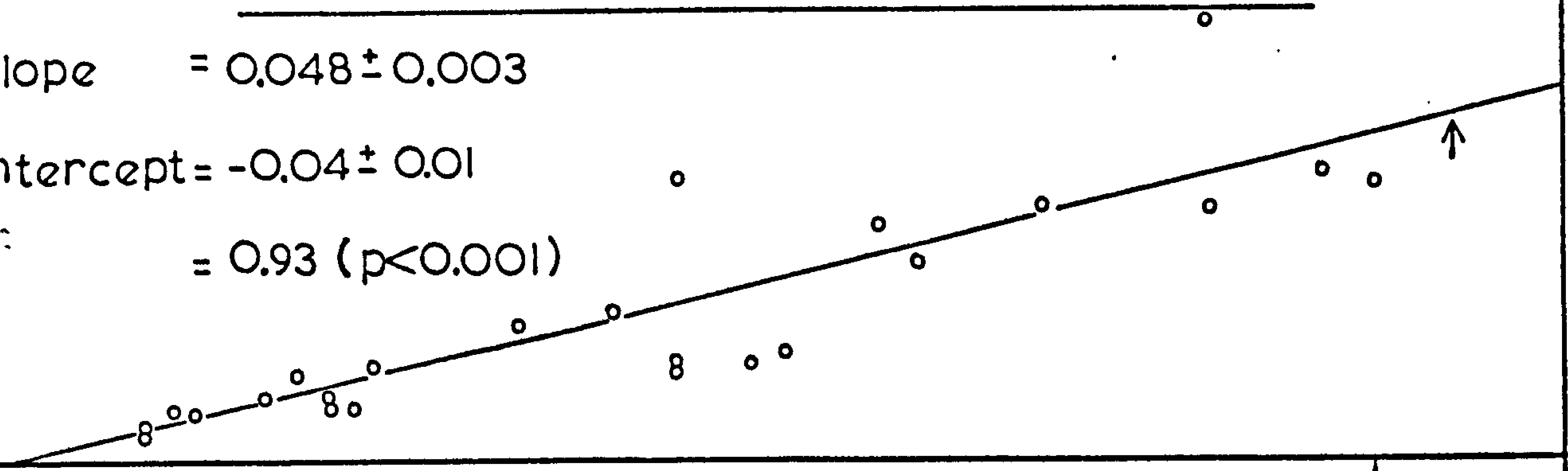
0.7

Figure 4.2a The relationship between glucose utilisation and incorporation into fatty acids (unstimulated).

Slope = 0.048 ± 0.003

Intercept = -0.04 ± 0.01

r = 0.93 ($p<0.001$)



0

Figure 4.2 b Comparison of the relationship between glucose utilisation and incorporation into fatty acids in basal and submaximally stimulated cells (+).

	Basal	Submaximal
↑ V_{max}	8.43±2.2	23.7 ± 4.1

2.6

submax

basal

Incorporation into fatty acids, Nmol/hour

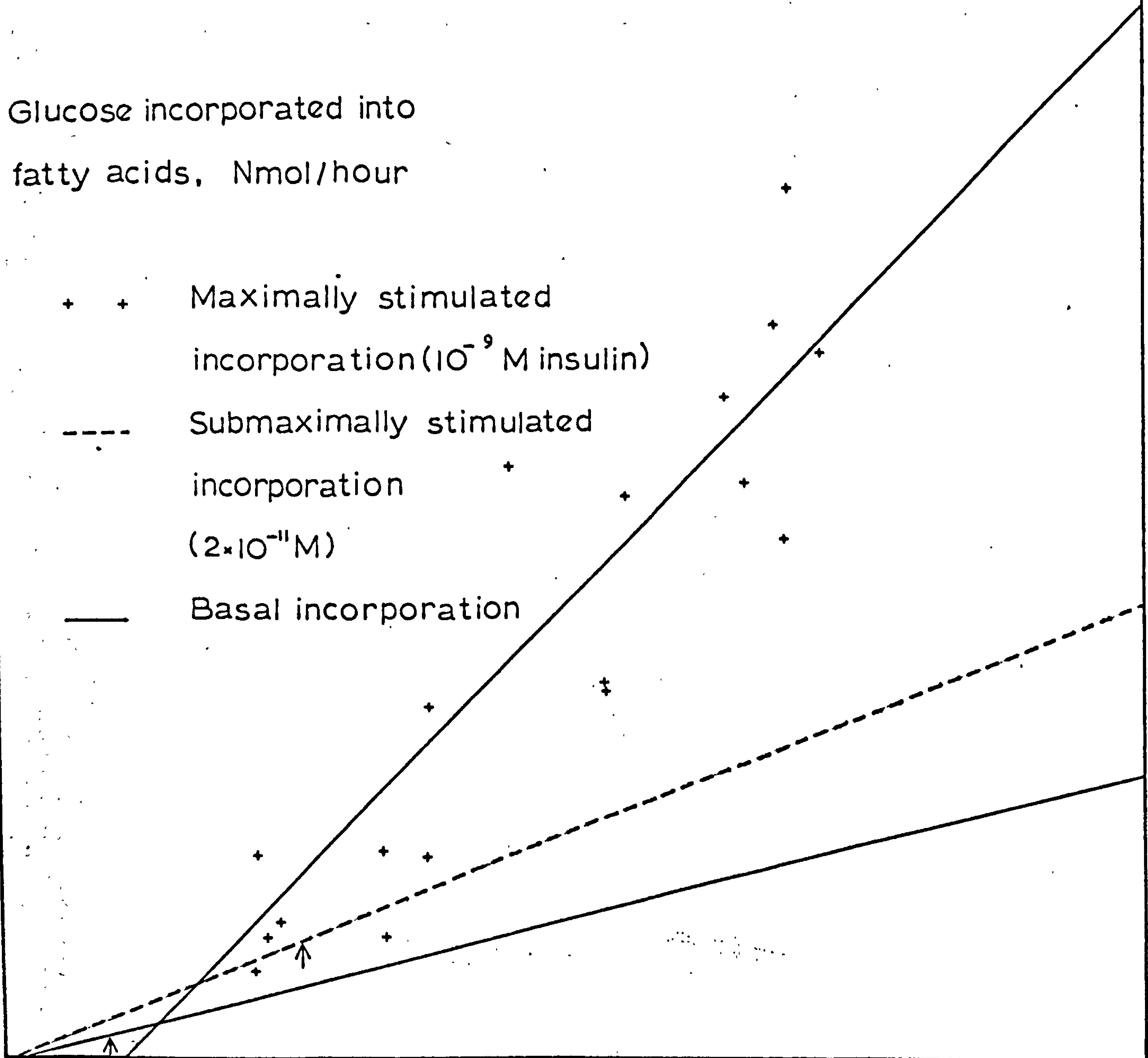
0

Glucose utilisation, Nmol/hour

32

Figure 4.3 The relationship between glucose utilisation and incorporation into fatty acids in basal, submaximally and maximally stimulated. adipocytes.

↑ Calculated V_{max}, Nmol / hour



the submaximally stimulated utilisation range merely by increasing the medium glucose concentration. However this Fig. shows that the cells treated with insulin converted a greater proportion of the available glucose into fatty acids than the extrapolated basal line would predict. This shows either that insulin increases the flow of glucose into the fatty acid synthesising pathway or that the extrapolated line does not truly describe the relationship between glucose utilisation and fatty acid synthesis.

In Fig. 4.3 this comparison is extended to include cells incubated with a maximally stimulating concentration of insulin (10^{-9}M). Once again the best fitting lines to the experimental points (by unweighted linear regression) have been drawn for basal and submaximum insulin data. Individual points are omitted for clarity. A small degree of overlap was obtained between the points with a submaximal insulin concentration and high glucose (0.5 mg/ml) and those with a maximal insulin concentration and low glucose (0.025 mg/ml). However it was impossible to compare fatty acid synthesis in the absence and presence of a maximally stimulating insulin concentration because of the great difference in glucose utilisation values observed.

Figs. 2 and 3 show that insulin increased the rate of glucose utilisation and the proportion of glucose incorporated into fatty acids. However they do not distinguish between a direct action of insulin at some point on the fatty acid synthetic pathway or an action mediated through increased glucose utilisation.

Glucose utilisation was increased in the absence of insulin by the addition of mM Zn^{2+} to the incubation medium. This method was chosen because an independent study in this

laboratory had shown Zn^{2+} to be a powerful stimulator of glucose utilisation in isolated adipocytes:

	Basal	Zinc ($10^{-3}M$)	Insulin ($10^{-9}M$)
Glucose utilisation[x] (Nmol/Hr)	1.87±0.54 (4)	37.64±7.53 (4)	20.84±0.36 (4)

[x]Figures are mean ± S.D., with the number of observations in parentheses.

Use of Zn^{2+} at this concentration allowed the basal glucose utilisation to attain values typical of insulin-treated cells. Quarterman (1969) has reported that Zn^{2+} stimulates glucose uptake in adipose tissue and that this stimulation is independent of insulin. It therefore seemed probable that the mechanism of stimulation observed with Zn^{2+} was different from that observed with insulin.

Glucose utilisation in the presence of insulin was reduced by incubating adipocytes with 3-o-methylglucose (3-OMG). 3-OMG is a competitive inhibitor of the glucose transport system and should only inhibit an effect of insulin mediated through increased glucose utilisation.

An exploratory experiment was performed to check that 3-OMG did not alter the proportions of glucose flowing into the major metabolites. Adipocytes were incubated with 3-OMG in the presence and absence of insulin in the incubation system previously described (see Chapter 2, Section 4). The inhibitor was added in a 10:1 ratio with glucose (3.0 mg/ml and 0.3 mg/ml respectively) and the effect on glucose

Figure 4.4 The distribution of glucose between its major metabolites as a function of glucose utilisation. Data obtained with 3-OMG (+).

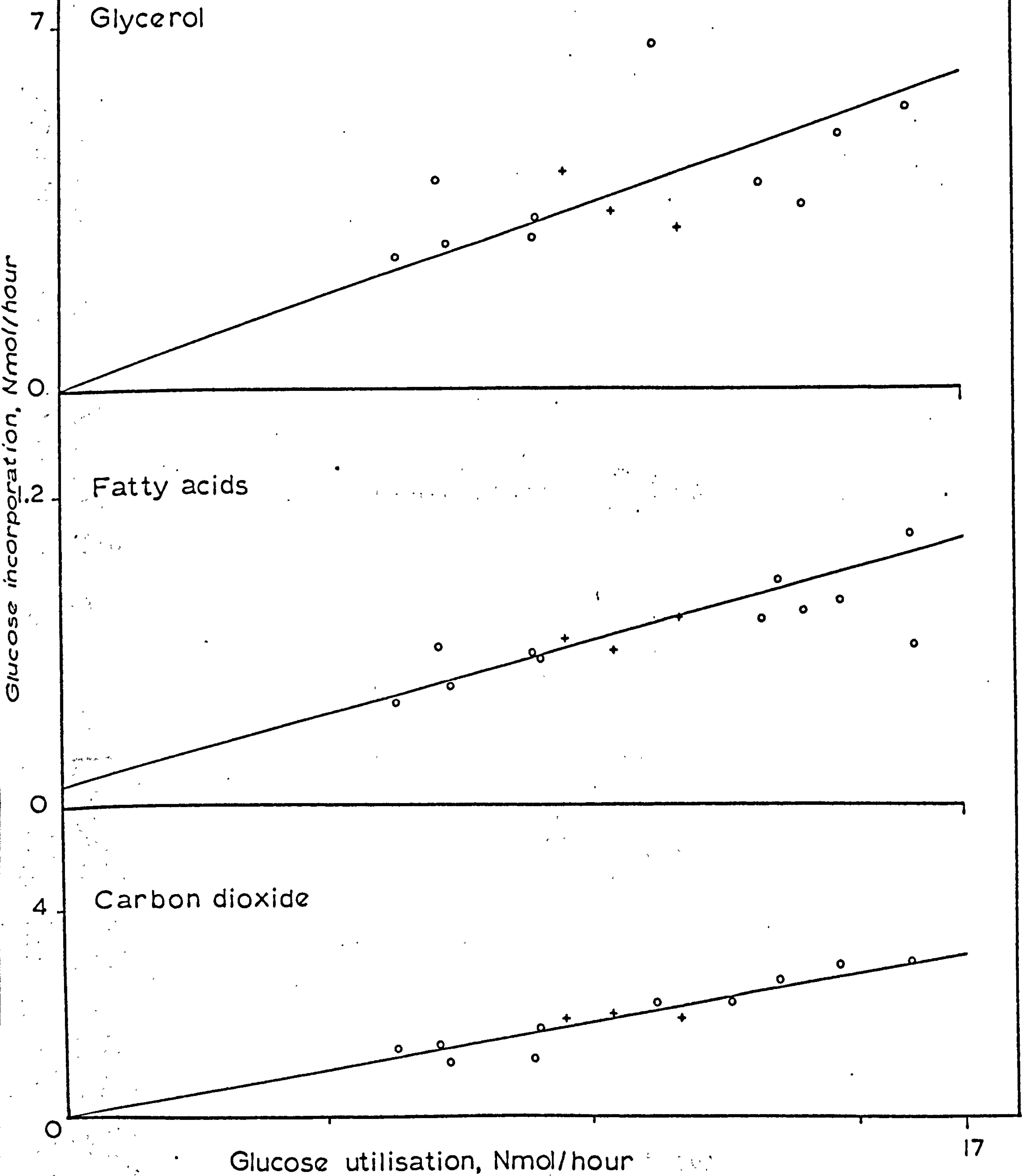
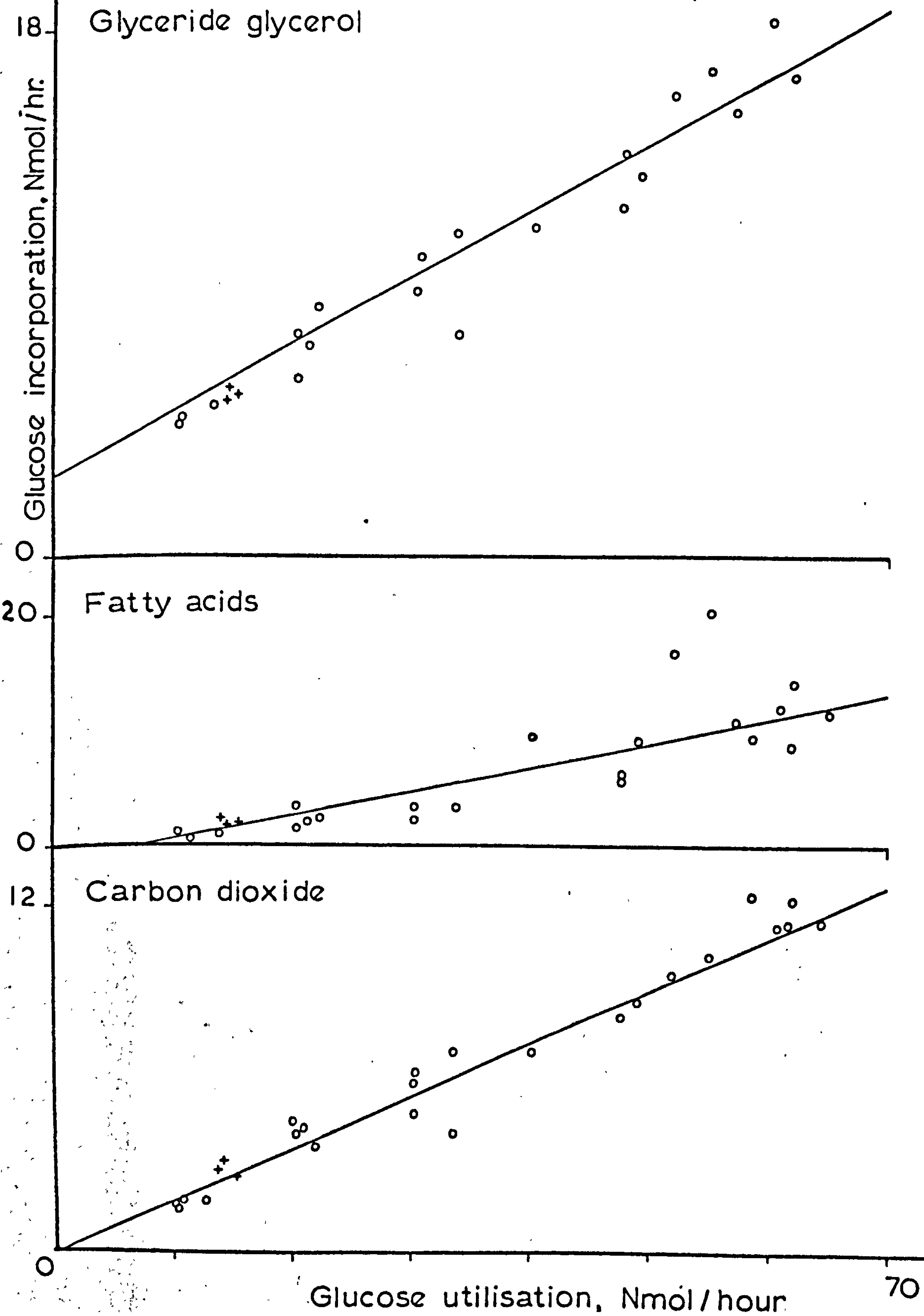


Figure 4.5 The distribution of glucose between its major metabolites in insulin-stimulated cells with and without 3-OMG.

+ indicates 3-OMG data



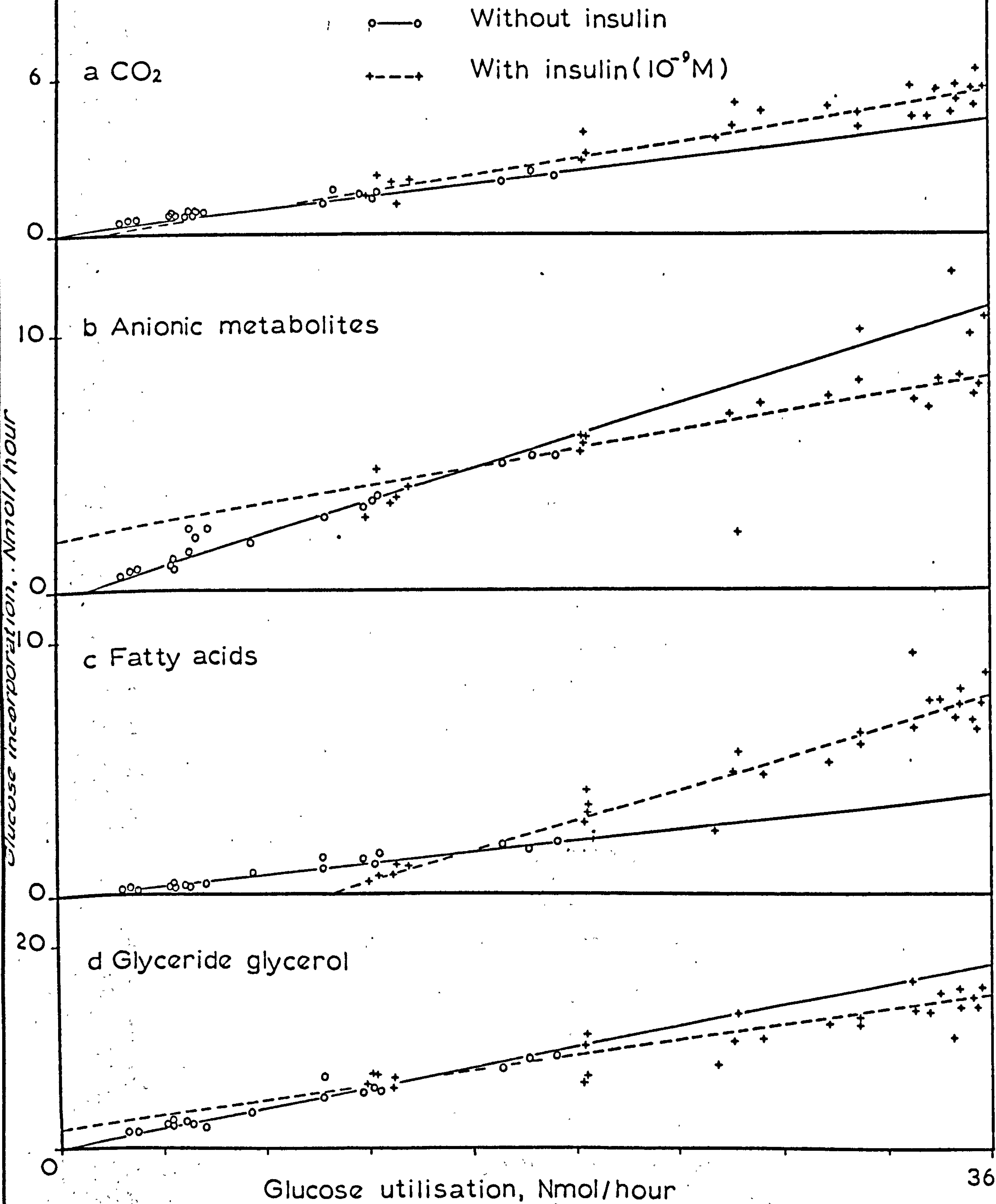
utilisation and the pattern of glucose metabolism was followed in a one hour incubation. The results of this experiment are shown in Figs. 4.4 and 4.5. Although the glucose utilisation was inhibited by about 35% there was no apparent change in the proportions of glucose entering the major metabolites in the absence or presence of insulin. It was therefore concluded that 3-OMG was a suitable inhibitor for reducing glucose utilisation in the presence of maximally stimulating insulin concentrations.

4.2 Studies of a directive effect of insulin on glucose metabolism in the adipocyte

Using the methods described above to control glucose utilisation a series of experiments was performed to study the effect of glucose utilisation on the distribution of glucose between its major metabolites and the effect of insulin on this distribution. By using 3-OMG to reduce the availability of glucose to the maximally stimulated adipocytes it was sometimes possible to bring glucose utilisation with insulin and low glucose levels into the range of glucose utilisations observed in the absence of insulin. Thus any effect of insulin not mediated through increased glucose utilisation could be detected. Glucose incorporation into CO_2, anionic metabolites (pyruvate and lactate), fatty acids and glyceride glycerol was studied.

The results of a typical experiment are shown in Fig. 4.6. A linear relationship was found to exist between glucose incorporation into each of the metabolites studied and glucose utilisation in the absence of insulin. The lines drawn through the experimental points were fitted to the data by unweighted linear regression analysis. A degree

Figure 4.6 The influence of insulin on the distribution of glucose between its major metabolites as a function of glucose utilisation.



of overlap was obtained between basal and insulin data in the range of glucose utilisations from 12 Nmol/hour to approximately 20 Nmol/hour. It is apparent that for each of the metabolites studied a pair of distinct lines was obtained in the presence and absence of insulin. With the exception of the CO_2 data an intersection of these lines was observed at a glucose utilisation of about 15 Nmol/hour.

Some evidence was obtained that in the presence of insulin a greater proportion of the available glucose was oxidised to CO_2 than in the absence of hormone when an overlap of utilisation values was obtained (Fig. 4.6a). However, numerous metabolic pathways produce CO_2 from glucose and so interpretation of this result in terms of the activation of specific pathways was not possible. The intersection of the fitted lines suggests that at very low values of glucose utilisation insulin would act to reduce the incorporation of glucose carbon into CO_2. No insulin points were obtained at the appropriate low enough values of glucose utilisation to permit this expectation to be checked. This reduction in glucose oxidation was not observed in any of the experiments performed.

At the lowest values of glucose utilisation observed with stimulated cells glucose incorporation into anions was not influenced by the addition of insulin (Fig. 4.6b). The majority of the insulin points in this range fell on the basal line. Examination of the insulin points at high glucose utilisation shows that at these levels of utilisation insulin reduced the proportion of glucose incorporated into anions below the level which would be predicted from the basal relationship between glucose utilisation and anion production. In some experiments a reduction in the flow of

glucose into anions in response to insulin was observed when stimulated and unstimulated utilisation values were directly comparable.

Fig. 4.6c shows that the best fitting lines to the basal and insulin stimulated fatty acid synthesis data intersect at a glucose utilisation of approximately 15 Nmol/hour. Glucose incorporation into fatty acids in the presence and absence of insulin could be directly compared at values of glucose utilisation below this intersection. It is apparent that glucose incorporation into fatty acids was reduced below the level observed in unstimulated cells by the addition of a maximally stimulating concentration of insulin when the availability of glucose to the cell was reduced by the presence of 3-OMG.

At values of glucose utilisation above 15 Nmol/hour the insulin points were with only one exception above the extrapolated basal line, and in three of the series of experiments performed an overlap of basal and insulin values above the intersection was obtained. In each case the insulin points lay above the basal line. Thus when glucose incorporation into fatty acids was studied at comparable values of glucose utilisation in the presence and absence of insulin, the hormone was shown to influence glucose incorporation into fatty acids independently of its action on glucose utilisation.

Fig. 4.6d shows the effect of insulin on the incorporation of glucose into glyceride glycerol. Again the lines fitted to the experimental data intersect at a glucose utilisation of 15 Nmol/hour. At high values of glucose utilisation insulin decreased the incorporation of glucose into glyceride glycerol below the values predicted from the

extrapolated basal line. In three of the experiments performed a substantial degree of overlap was obtained in this region and the majority of the insulin points were found to lie below the comparable basal values. At low values of glucose utilisation the addition of insulin appeared to lead to a slight increase in the proportion of glucose incoporated into glyceride glycerol (see Fig. 4.6d).

These results showed that insulin affected the pattern of glucose metabolism in the adipocyte independently of its widely recognised ability to increase glucose uptake. Thus insulin controls the proportion of glucose incorporated into, say, fatty acids by a mechanism which is distinct from the hormone's action in stimulating glucose uptake. However, the level of substrate availability does appear to determine the nature of the effect of insulin on fatty acid synthesis. That is to say at high values of glucose utilisation insulin increased fatty acid synthesis above the basal rate at comparable values of utilisation whereas at low rates of utilisation fatty acid synthesis was reduced. The latter effect of insulin, while probably not normally of physiological importance, does permit inferences to be made about the action of insulin on some metabolic processes within the cell (see Section 4 of this Chapter).

It has been shown that addition of insulin to adipocytes will increase or decrease the proportion of glucose flowing into fatty acids relative to the proportion observed in unstimulated cells. These effects of insulin may be regarded as metabolic directive effects.

In some experiments only a small degree of overlap was obtained between basal and insulin data owing to the

magnitude of the insulin stimulation of glucose utilisation. In order to permit inferences to be made about the way in which insulin was regulating glucose metabolism it was necessary to study the relationship between glucose utilisation and the pattern of metabolism over the full range of values of utilisation in the region of overlap. The validity of combining the data so as to extend the observations over the whole range of utilisations obtained was therefore tested. Fig. 4.7 shows the relationship between glucose utilisation and fatty acid synthesis in the combined data. It will be recalled that a linear relationship was found to exist between glucose utilisation and fatty acid synthesis in individual experiments (for example see Fig. 4.6). In order to test whether the line y=a + bx was a good fit to the combined experimental data an analysis of variance of the regression coefficient, b was carried out. The type of calculation performed is illustrated below (Clarke, 1969).

The first column lists the components into which the total sum of squares, S, can be divided. The first component, Sr, indicates how much of the variation in the individual y values (yi) was explained by the fitted regression line (that is to say to what extent variation among the yi was due to a linear relationship with x). The second component, Sd, indicates the size of the deviations of the yi from this line. The size of this component is an indicator of the fit of the points to the line, since when it is small relative to Sr, the data will closely fit the line.

Figure 4.7 Combined data from studies of the influence of insulin and Zn^{2+} on the relationship between glucose utilisation and incorporation into fatty acids.

	Combined basal data	Combined insulin data
Correlation coefficient (r^2)	0.89	0.94
$intercept_{x=0}$	-0.29±0.07	-1.57±0.05
slope	0.12±0.01	0.25±0.02

The quantities calculated are

$$\text{Total sum of squares, } S = \frac{1}{N}\left[N\sum_{i=1}^{N} yi^2 - Gy^2\right]$$

$$\text{Sum of squares of regression, } Sr = \frac{\left[\left(\frac{1}{N}\right)\left(N\sum_{i=1}^{N} xiyi - GxGy\right)\right]^2}{\left(\frac{1}{N}\right) N\sum_{i=1}^{N} xi^2 - Gx^2)}$$

Sum of squares of deviations, $Sd = S - Sr$

$Gx, Gy = \sum_{i=1}^{N} xi$ and $\sum_{i=1}^{N} yi$ respectively

The Null Hypothesis set up to test the fit of the data was that the regression coefficient b=0.0. The Mean Squares for regression and deviation were then calculated.

Source of variation	Degrees of Freedom	S.S.	M.S.	Test
Regression	1	Sr	$Mr = \frac{Sr}{1}$	$\frac{Mr}{Md} = F(1, N-2)$
Deviation	N-2	Sd	$Md = \frac{Sd}{N-2}$	
Total	N-1	S		

Using the combined basal data values for the Sums of Squares were $Sd = 26.20$

$Sr = 98.97$, $N = 114$

and the value of F was considerably greater than that required for significance at the 0.001 probability level. Thus the Null Hypothesis that b = 0.0 was rejected ($p < 0.001$). This shows that the variation in the yi values can be adequately explained by a linear dependence of y on x. Furthermore the relative sizes of the sums of squares, Sd, Sr

shows that there was a small dispersion of values about the fitted line. A further indication of the fit of the points to the line may be obtained from the magnitude of the standard error associated with the slope of the combined data compared with the individual experiments (Table 4.2). The standard error of the slope of combined data, as a proportion of the calculated slope, was no greater than the error of the individual experiments, suggesting a good fit of the combined data.

Since the correlation coefficient, r^2, gives the proportion of the variance in y which is due to the regression of y on x, it may be used to indicate the dispersion of the data about the line (Yamane, 1970). For example, when $r^2 = 1$, all the data would lie on the fitted line. A correlation coefficient of 0.89 was obtained for regression of the combined fatty acid synthesis data on glucose utilisation, indicating that an 89% reduction in the total sum of squares was obtained by fitting the line (Yamane, 1970).

Thus a linear relationship was consistent with the dependence of fatty acid synthesis on glucose utilisation in the combined data obtained in the absence of insulin. Two estimates of the dispersion of the data showed a good fit of points to the line. When this procedure was extended to the insulin data a linear relationship was also found ($p < 0.001$ that the Null Hypothesis $b = 0.0$ was true). The correlation coefficient of 0.94 shows the small dispersion of the points. These procedures indicate that the combined experimental data were satisfactorily fitted by the regression lines described in Fig. 4.7 and that in each hormonal condition the individual experiments could be regarded as

Fig. 4.2 Studies of a directive effect of insulin on fatty acid synthesis

(All values are ±S.E.M.)

Experiment	Range of values of glucose utilisation Nmol/hour	*Slope	*Intercept x=0	1 E_{UT}	2 E_{DE}
1	6.0-32	0.12±0.03	-0.39±0.57	6	1.6
2	4.0-15.6	0.09±0.01	-0.14±0.11	5	None
3	4.3-10.9	0.06±0.004	0.033±0.031	11	3.1
4	8.3-20	0.14±0.02	-0.21±0.24	9.2	1.3
5	7.5-19.3	0.06±0.01	0.04±0.08	12.1	2.7
6	8.8-27.4	0.14±0.01	-0.57±0.19	13.3	1.5
7	4.9-8.9	0.11±0.02	0.08±0.07	13.8	4.95
8	1.9-6.2	0.05±0.01	-0.09±0.06	23.2	5.4
9	2.7-10.1	0.12±0.01	-0.26±0.17	10.2	1.5
10	7.7-20.6	0.13±0.03	-0.27±0.09	5	1.2

* Of plot of fatty acid production versus total glucose utilisation

1 Insulin stimulation of glucose utilisation

2 Directive effect of insulin (for calculations see text)

samples of a single population.

The effect of Zn^{2+} on the incorporation of glucose into fatty acids is also shown in Fig. 4.7. Although Zn^{2+} increased glucose utilisation considerably in these experiments, in some instances giving a greater stimulation than a maximally stimulating insulin concentration, the fatty acid points were distributed about the basal line. Thus these results show that Zn^{2+} does not exert a characteristic insulin-like effect on glucose metabolism in the adipocyte, in contrast with the reports of Quarterman (1967, 1969). This clearly shows that increasing the glucose utilisation in the absence of insulin did not give the same distribution of glucose between the various metabolites studied as addition of insulin. This finding justifies the extrapolation of the basal line in Fig. 4.6 and supports the conclusion that the directive effect of insulin on the pattern of glucose metabolism is not mediated through increased glucose utilisation.

Furthermore the Zn^{2+} study demonstrates the importance of relating any change in metabolic pattern to the changed uptake of glucose observed with an effector of utilisation in an analysis of the effector's action on the cell. Thus the effect of Zn^{2+} on glucose utilisation was typically insulin-like, though the effect on the pattern of glucose metabolism was not.

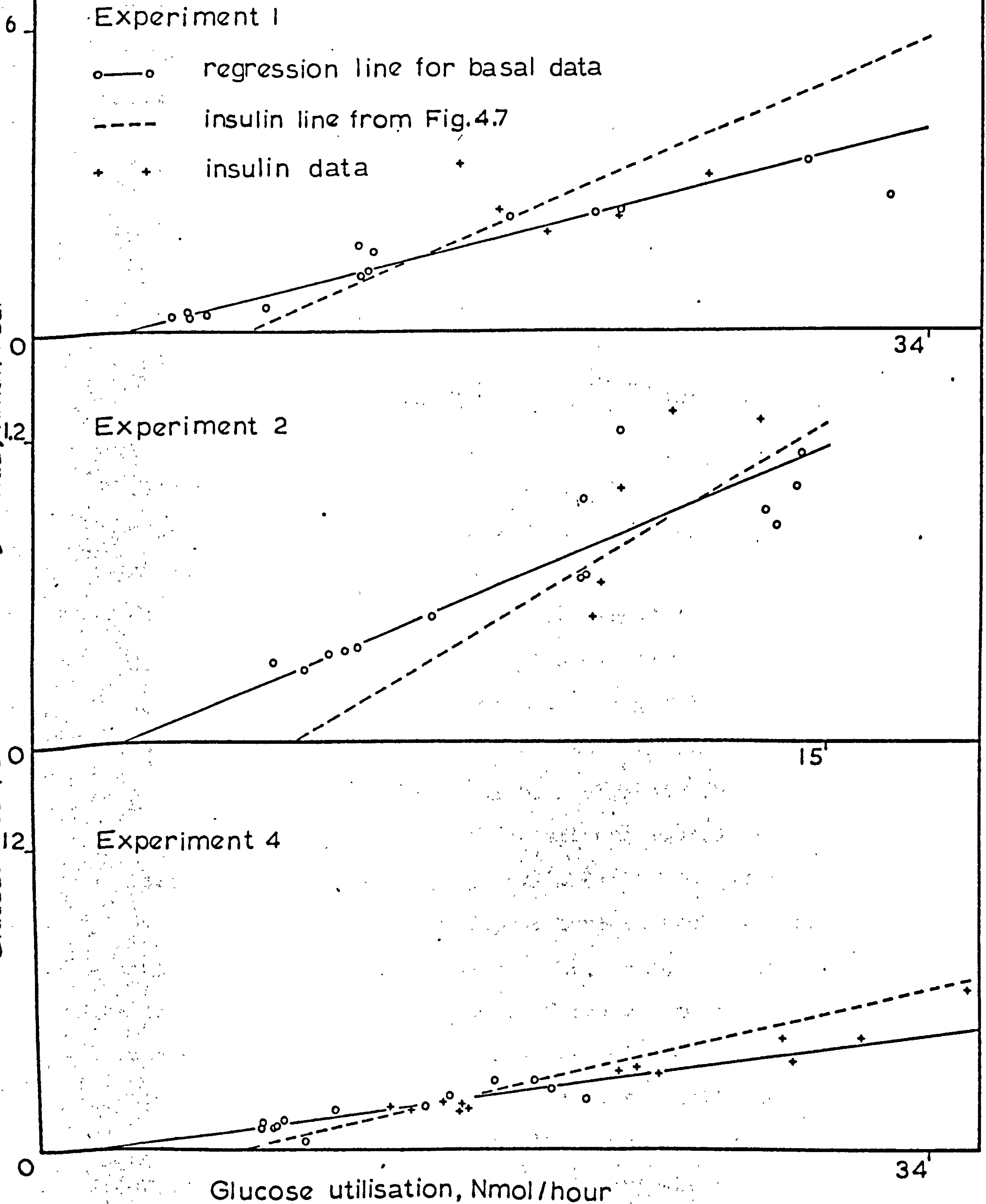
Fig. 4.7 shows an intersection between the basal and insulin lines for fatty acid synthesis versus glucose utilisation. There is, therefore, a limiting value of utilisation for the observation of a stimulation of fatty acid synthesis. In the absence of insulin glucose utilisation varied between 1.5 and 30 Nmol/hour, whereas in the presence of insulin a

range of 6 to 76 Nmol/hour was observed, giving a considerable overlap of values. Seven insulin points were obtained at glucose utilisation values below the intersection of the fitted lines and all were below the fitted basal line. This shows that at low values of glucose utilisation insulin-stimulated adipocytes converted a smaller proportion of the available glucose into fatty acids than unstimulated cells, confirming the observation made in Fig. 4.6.

It is also evident from Fig. 7 that an activation of fatty acid synthesis could only be detected experimentally when the insulin points exceeded the limiting value for glucose utilisation. This provides an explanation of the small enhancements of fatty acid synthesis observed in some experiments. Fig. 4.8 shows three such experiments. The basal data of the Fig. are those obtained in the experiment indicated. The fitted insulin line is the plot of combined insulin data given in Fig. 4.7, with the insulin points for the individual experiment indicated. In Experiment 2 (for experimental notation see Table 4.2) few insulin points exceeded the limiting rate of glucose utilisation and no clear directive effect could be detected. In Experiment 1 the preponderance of the insulin points lay in close proximity to the intersection of the basal and insulin lines. Consequently the scatter of the experimental points does not allow the observation of a directive effect at this level of glucose utilisation. This difficulty was also encountered in Experiment 4. Furthermore in this experiment the slopes of the basal and insulin lines were very similar.

These examples may be contrasted with an experiment in

Figure 4.8 The relationship between glucose utilisation and its incorporation into fatty acids in some experiments showing a small directive effect of insulin.



which a large enhancement of fatty acid synthesis was observed. Fig. 4.9 shows such an experiment. In this experiment the glucose utilisation in the presence of insulin was considerably greater than the limiting value predicted from the intersection of the fitted lines.

Fig. 4.7 shows that at low values of glucose utilisation fatty acid synthesis was lower in the presence than in the absence of insulin. This is consistent with reports that insulin reduces the rate of lipolysis of endogenous triglyceride in adipose tissue preincubated with lipolytic hormones (Jungas and Ball, 1962, 1963). In the presence of insulin the availability of endogenous fatty acids for oxidation would be reduced and therefore the supply of AcCoA and energy from endogenous stores would be reduced. Under conditions of low glucose utilisation in the presence of insulin a greater proportion of the available glucose and therefore of the AcCoA might be expected to be oxidised through Krebs' cycle to furnish the cell's basic energy requirements. This would reduce the proportion of the AcCoA available for incorporation into fatty acids.

Furthermore, evidence has been obtained for an effect of insulin on the activity of an adipose tissue lipase. Rizack (1961) and Hollenberg et.al. (1961) demonstrated that homogenates prepared from adipose tissue which had been preincubated with epinephrine contained a higher lipase activity than untreated tissue. There was no change in lipase activity when epinephrine was added directly to the homogenate. The stimulation of the lipase activity could be reduced by adding insulin to the incubation medium. Jungas and Ball (1963) showed that in the absence of glucose insulin suppressed the release of free fatty acids and

Figure 4.9 Comparison of basal and stimulated glucose incorporation into fatty acids as a function of glucose utilisation with the insulin line of Fig.4.7.

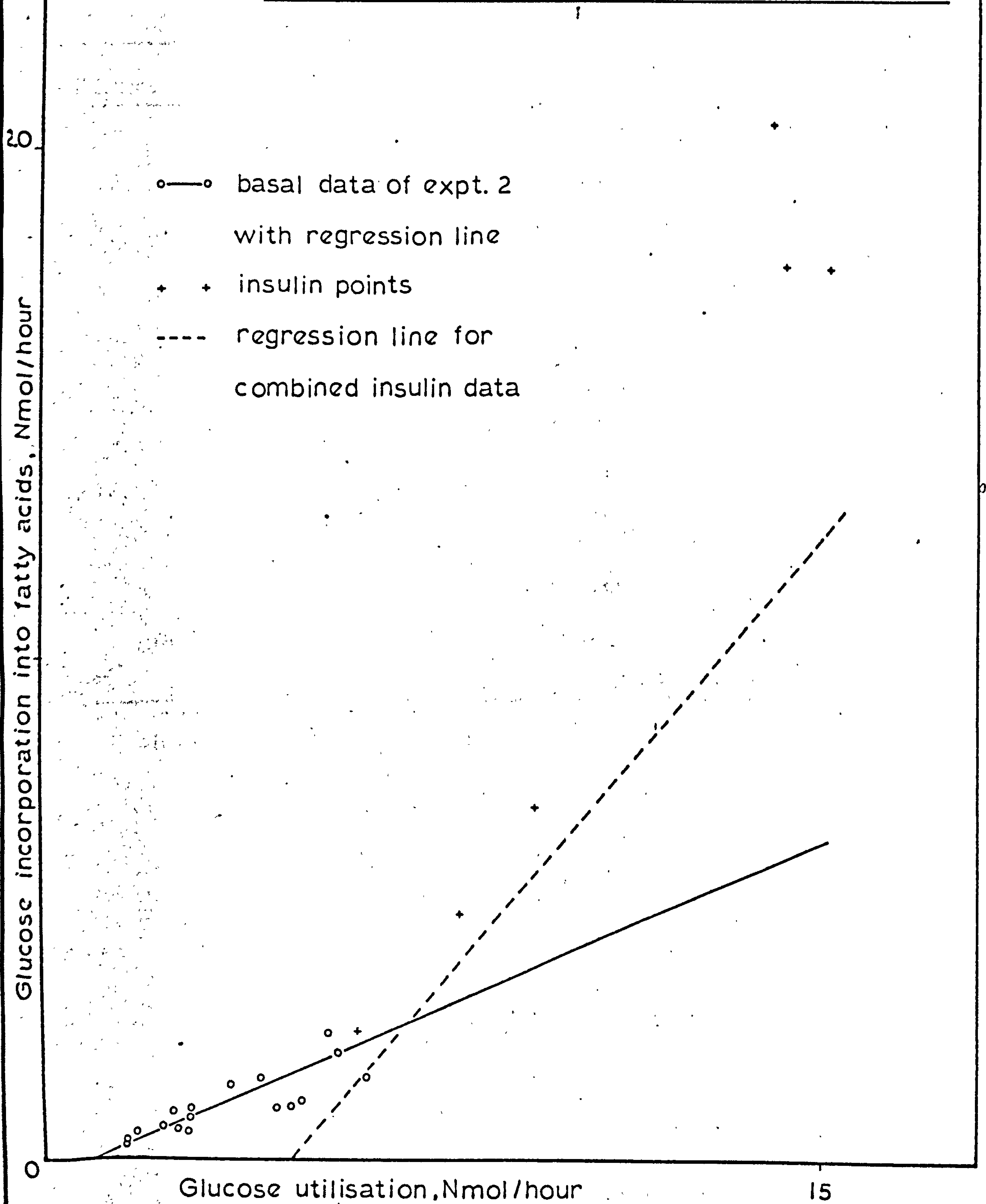


Figure 4.10 Combined data from studies of the influence of insulin on the relationship between glucose utilisation & incorporation into CO_2.

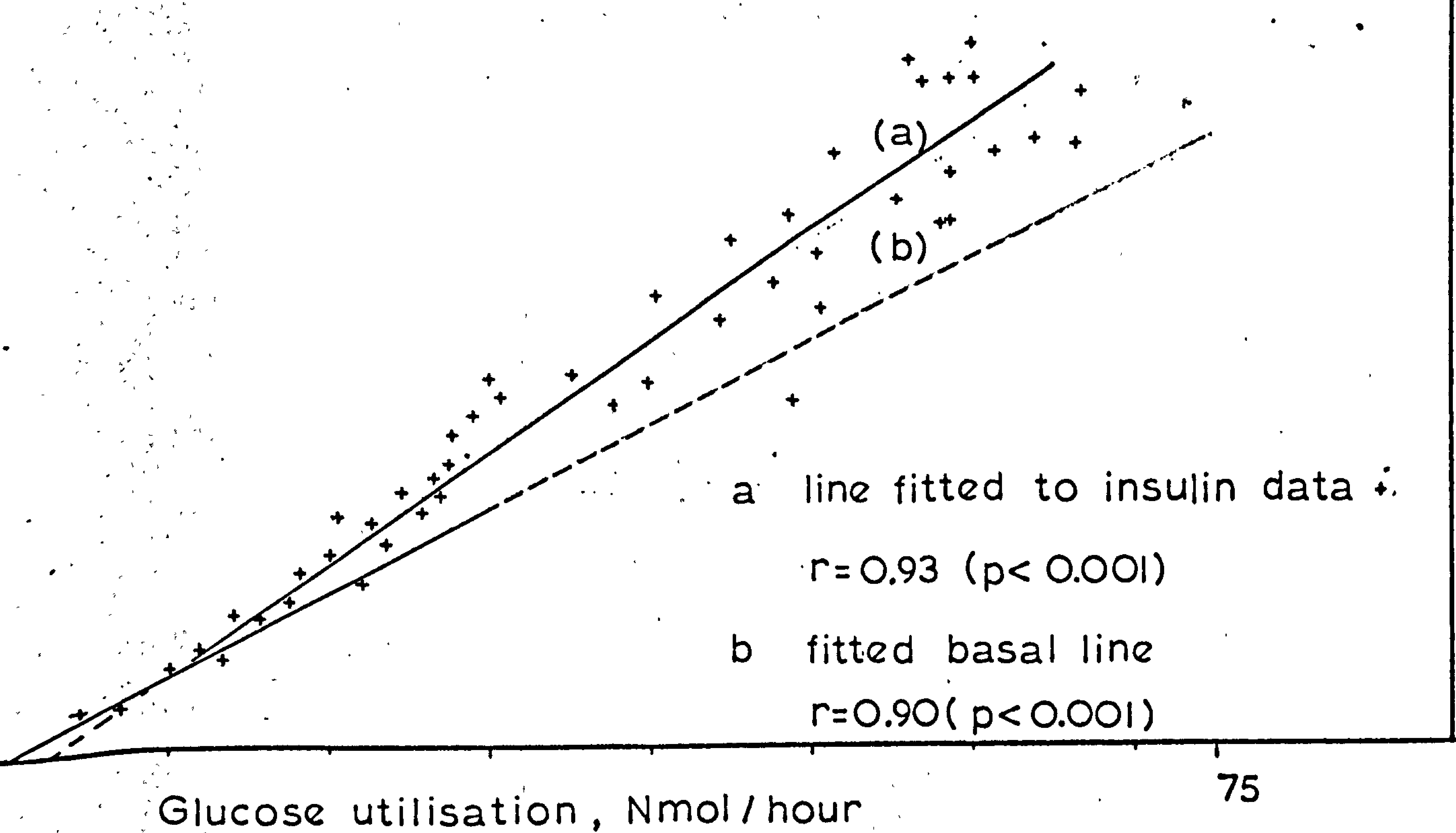
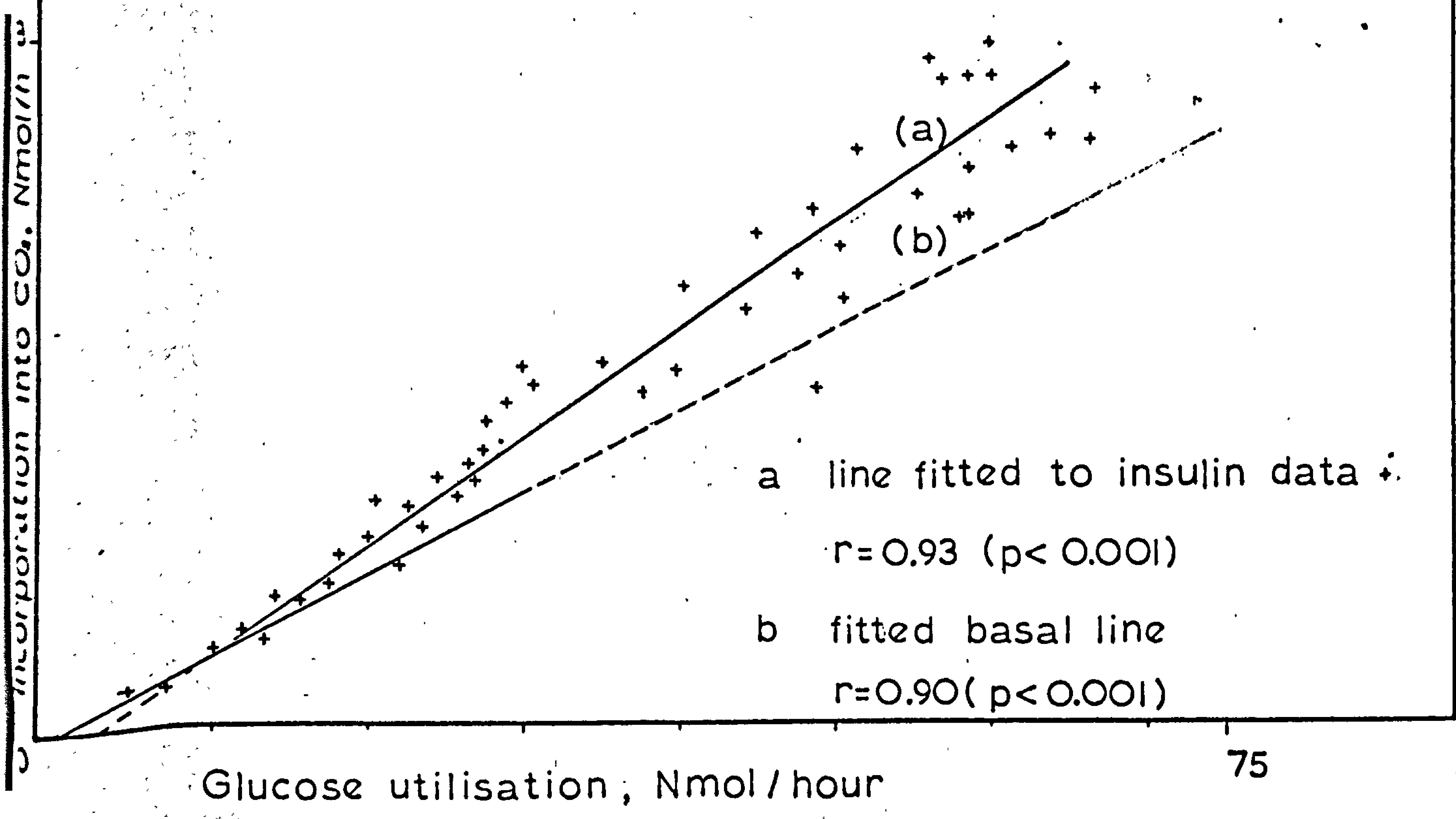


Figure 4.10 Combined data from studies of the influence of insulin on the relationship between glucose utilisation & incorporation into CO_2.



glycerol from fat pads treated with epinephrine. They interpreted these findings as evidence for an inhibitory action of insulin on a lipase.

The combined carbon dioxide data were examined for evidence of an increased oxidation of glucose at low values of utilisation in the presence of insulin. Fig. 4.10 shows a plot of carbon dioxide production versus glucose utilisation derived from the combined data of the experiments shown in Table 4.2. It is therefore directly comparable with the fatty acid synthesis versus glucose utilisation plots shown in the previous section of this chapter. This Fig. provides no evidence for the increased oxidation of glucose to CO_2 postulated above. However, since fatty acid production from glucose was reduced by insulin at very low values of glucose utilisation the energy requirement for fatty acid esterification would also be reduced. Thus more of the energy produced by a given rate of glucose oxidation would be available to supply the cell's fundamental requirements. The glucose directed away from fatty acid synthesis by insulin at low values of glucose utilisation was therefore not apparently oxidised to carbon dioxide.

The other major glucose metabolites were therefore studied to determine the metabolic fate of the extra glucose carbon. Figs. 4.11 and 4.12 are plots of glucose incorporation into anions and glyceride glycerol drawn from the combined data of the experiments in Table 4.2. Glucose incorporation into anions was linearly related to glucose utilisation in the absence of insulin. The hormone data appear to lie on a curve which meets the basal line at a point close to the limiting value described for the enhance-

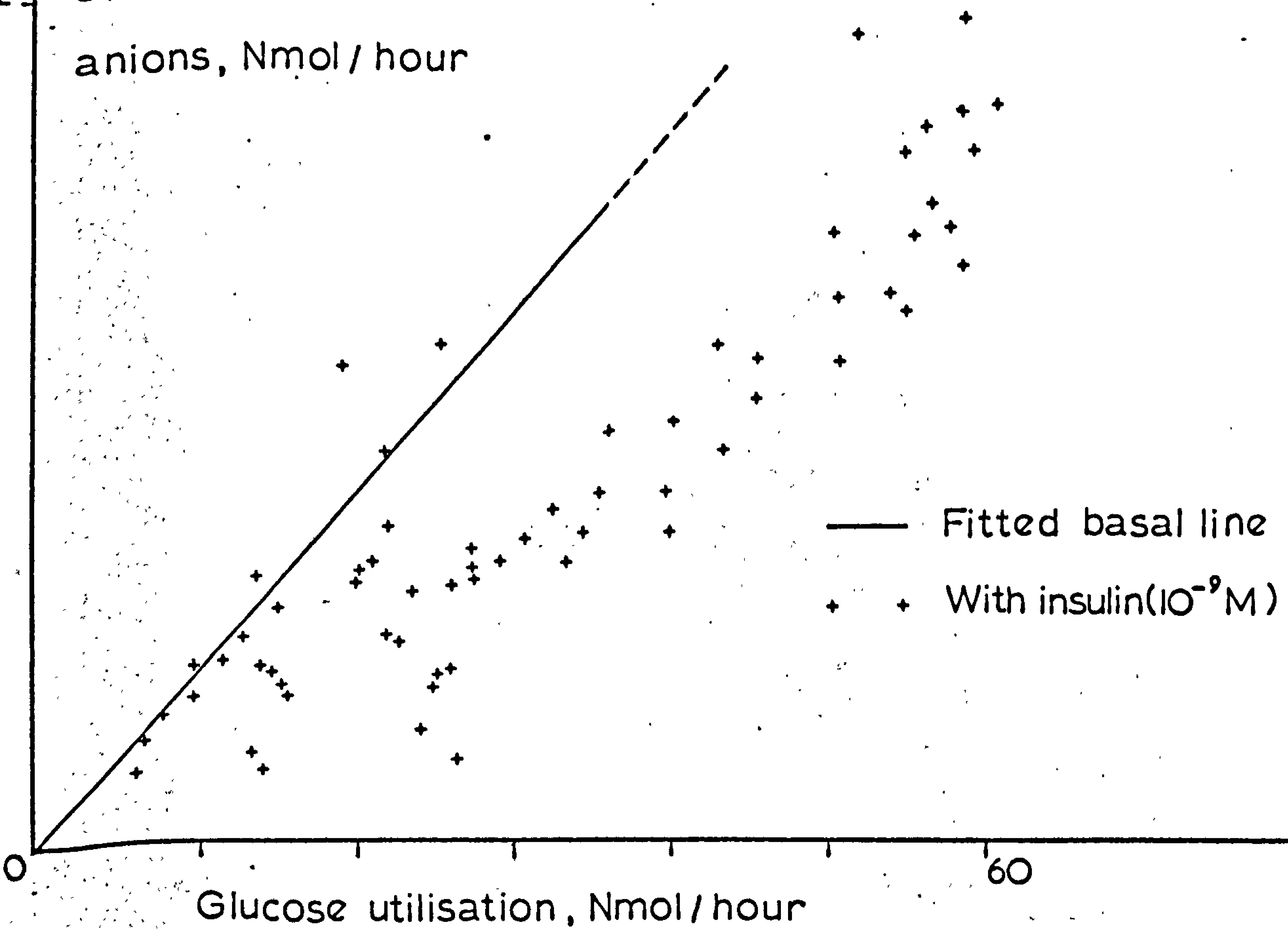
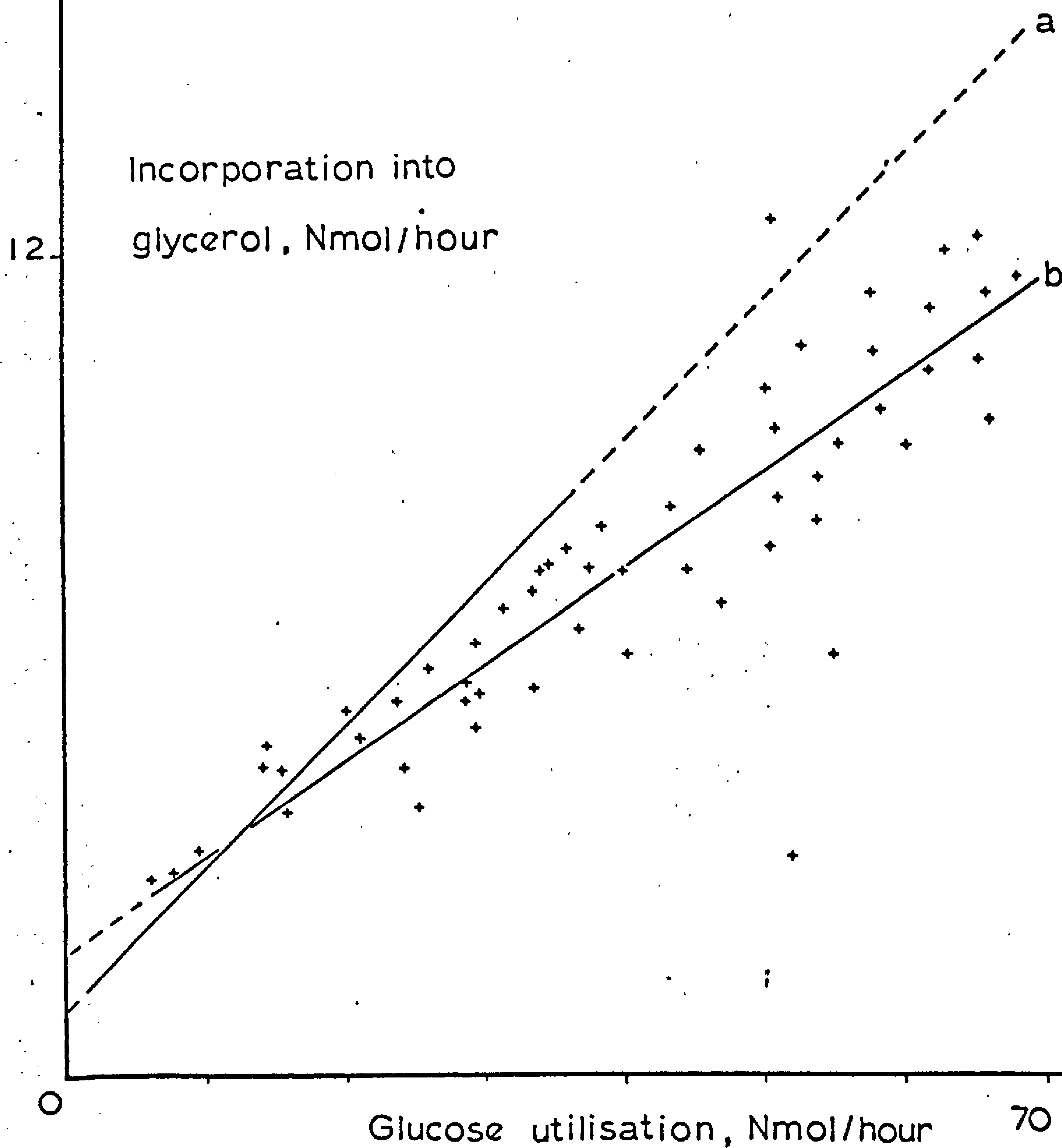


Figure 4.11 Combined data from studies of the influence of insulin on the relationship between glucose utilisation and incorporation into anions.

Figure 4.12 Combined data showing the effect of insulin on the relationship between glucose utilisation and its incorporation into glyceride glycerol.

a line fitted to basal data

b line fitted to insulin data (+)



ment of fatty acid synthesis. At values of glucose utilisation below this point the proportion of glucose incorporated into anions was the same in the presence and absence of insulin.

There is some evidence in Fig. 4.12 that at low levels of glucose utilisation insulin increased the flow of glucose into glyceride glycerol. However the magnitude of this change in distribution is such that it cannot be adequately distinguished from the dispersion of the basal points.

4.3 The relationship between insulin concentration and the directive effect on fatty acid synthesis

Varying the glucose concentration in the presence and absence of insulin has been shown to give lines of different slope for plots of fatty acid synthesis versus glucose utilisation. This effect has been attributed to a direct action of insulin on the metabolism of glucose within the adipocyte. The insulin dose response relationship of this directive effect was studied by incubating adipocytes with a wide range of insulin concentrations (2×10^{-12} to 10^{-9}M) at the lowest glucose concentration used to fit the basal line (0.05 mg/ml). This should allow the observation of a progressive increase in the directive effect up to a limiting, maximal value. The basal and insulin data of each experiment were compared with the combined maximum insulin data used in Fig. 4.7. The ratio of the stimulation of fatty acid synthesis observed with each submaximal insulin concentration to the maximal stimulation at the same glucose utilisation was measured. In order to obtain a true origin for the dose response relationship this procedure was repeated with the comparable glucose utilisation values

obtained in the absence of insulin (that is to say, the values obtained at 0.05 mg/ml glucose). Insulin points lying on the opposite side of the basal line to the fitted insulin line show negative directive effects and points showing a greater response than predicted from the fitted insulin line have values greater than unity.

Plots of the directive effect against the insulin concentration were constructed from data obtained in three experiments (Fig. 4.13). Interpretation of these plots was complicated by the small range of values obtained and the scatter of the experimental points. In each plot the lowest insulin concentrations gave directive effects below the origin. These points were associated with very small stimulations of glucose utilisation, the actual values of utilisation being close to the intersection of the basal and insulin lines used to estimate the directive effect. Small deviations from the fitted lines at these points on the graph would therefore give large variations in the directive effect. Furthermore the error in the calculated directive effect would be greatest in this range of utilisation values owing to the difficulty in accurately measuring the distance of the submaximum insulin points from the fitted basal line and the separation of the two fitted lines.

Reference to Fig. 4.7 will serve to illustrate this point. Consider the directive effect of a point ($y = 0.4$, $x = 7.0$). This point is below the basal line by a distance of 0.18 ± 0.01 Nmol/hr. Note that the error estimate used was not calculated from the error in the line fitting constants. It indicates the confidence with which the

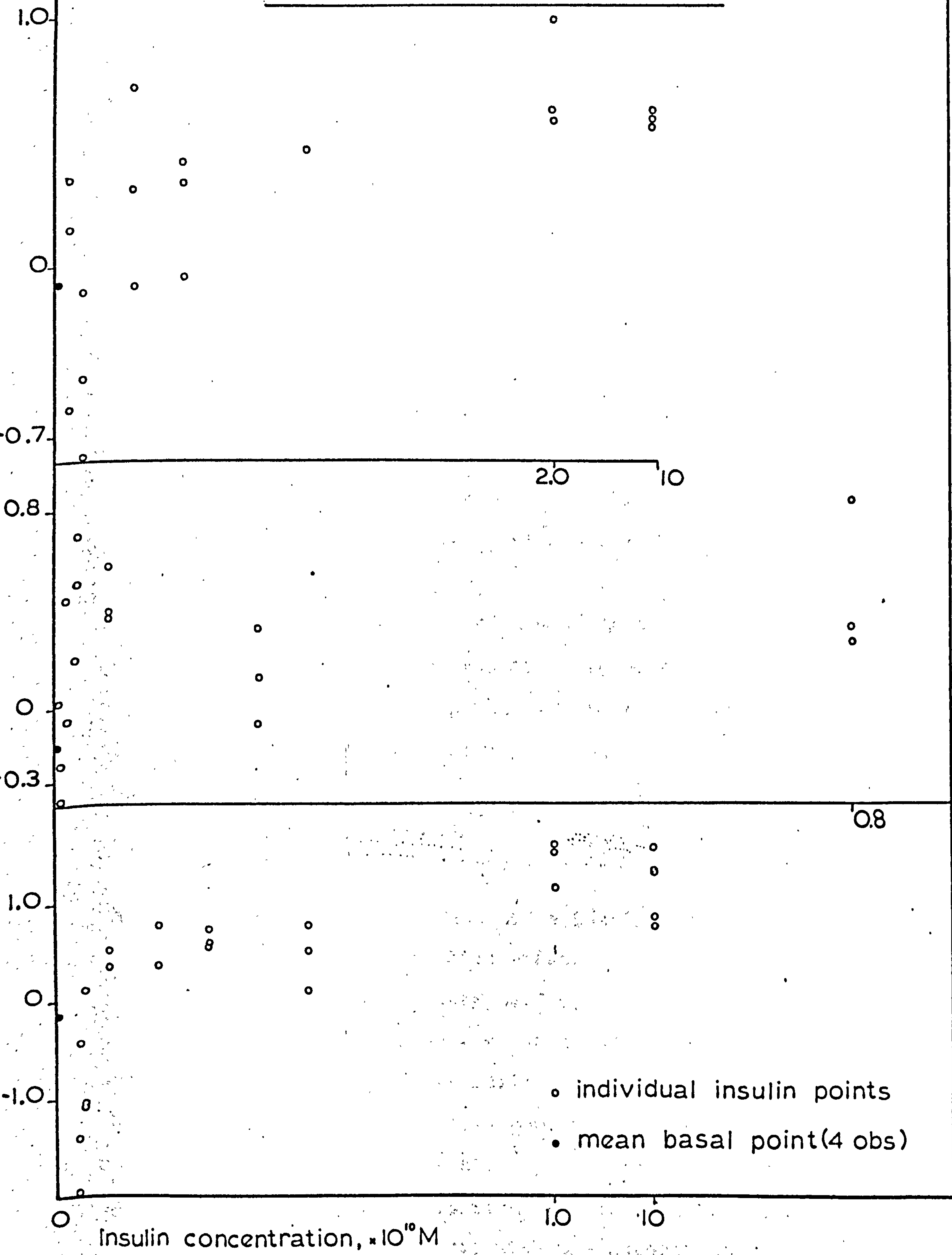


Figure 4.13 The relationship between insulin concentration and a metabolic directive effect.

value of 0.18 may be read from the graph. The separation between the fitted lines at the selected glucose utilisation value was 0.43±0.02 Nmol/hr. These error estimates, expressed as percentages of the estimated value, are 5.5 and 5.0 respectively. The combined errors in the calculated directive effect can be calculated from

$$S = \sqrt{S_1^{\ 2} + S_2^{\ 2}}$$

as 7.45%.

This procedure was repeated for a point (y = 1.3, x = 12), close to the intersection of the lines. The increase above the basal line was 0.09±0.01 (approximately 11%). The separation of the lines was estimated as 0.19±0.02 (10.5%). The total error in the directive effect estimate was therefore approximately 15%.

However, the possibility that a sigmoid curve was consistent with the observed relationship between insulin concentration and the development of a metabolic directive effect was tested by examining the fit of the data to the equation

$$e = \frac{Ei^n}{Km + i^n}$$

where e is the directive effect at an insulin concentration i

E is the maximal directive effect

The values of n which were used were 1, which describes a hyperbolic relationship between concentration and effect, and the appropriate Hill coefficient values calculated from the relationship between insulin concentration and glucose utilisation for each experiment. In each case the data fitted the equation better (the criterion used in this test was the value of the mean square error) when the experimentally

determined Hill coefficients were used. This suggests that a sigmoid relationship is consistent with the experimental observations. However, the error values obtained with these Hill coefficients were not significantly smaller than those obtained with a value of 1.

4.4 Discussion

A directive effect of insulin on the pattern of glucose metabolism in the adipocyte was observed when the glucose utilisation in the presence and absence of hormone was studied over the same range of values. This is the first direct demonstration that insulin exerts a physiologically significant directive effect on fatty acid synthesis from glucose which is not mediated through an increase in glucose utilisation. This shows that in addition to its widely accepted ability to facilitate glucose entry into the adipocyte (see, for example, Crofford and Renold, 1965) insulin directly influences the metabolic fate of the sugar. This is in contrast with the results of Kather et.al. (1972), who concluded that fatty acid synthesis was regulated by the rate of glucose uptake. These workers observed a linear relationship between glucose uptake and fatty acid synthesis and noted that the insulin points fell on this basal line. However, these studies were performed with tissues of different nutritional status and most of the points at high values of glucose utilisation were obtained using tissue from fasted-refed rats. The validity of this approach is open to question since it has been shown that the level of pyruvate dehydrogenase activity is elevated in tissue from fasted-refed rats (Jungas and Taylor, 1972; see Discussion below).

The stimulation of fatty acid synthesis by insulin described in this Chapter suggests that one or more of the enzymes of the fatty acid synthetic pathway is increased in activity in response to insulin. These studies permit some inferences to be made about the identity of the enzymes affected by insulin. Insulin reduced the incorporation of glucose into anions in sensitive preparations of adipocytes when compared with unstimulated cells at similar values of glucose utilisation. The results of a typical experiment are shown in Fig. 4.6b. The anions measured in this procedure are chiefly pyruvate and lactate (Evans, 1973). This suggests that in the presence of insulin the intracellular pyruvate concentration was reduced. One possible explanation of this phenomenon would be the activation by insulin of an enzyme involved in the conversion of pyruvate into fatty acids. This is consistent with the work of Fain (1964) who showed that insulin increased the incorporation of pyruvate into fatty acids in the absence of glucose. Studies with tissue from fasted-refed rats incubated in the absence of exogenous substrate (Halperin, 1970) support this conclusion. Addition of insulin to these adipocytes doubled the rate of fatty acid synthesis from endogenous substrate and halved the rate of output of pyruvate into the medium. There was no detectable glucose in the medium during these experiments.

The stimulation of fatty acid synthesis described in this Chapter is in accord with reports that pyruvate dehydrogenase (PDH) activity in adipose tissue was increased in response to insulin (Jungas, 1970; Jungas, 1971; Halperin, 1970 and Coore et.al., 1971 a,b). The decreased anion production observed in insulin stimulated adipocytes in these

studies is also consistent with an increased activity of PDH in the presence of insulin. However PDH activity was not affected when insulin was added directly to various cell fractions (Coore et.al., 1971a). This suggests that the activation requires an intact cell membrane or that a chain of events relaying the hormonal signal from a membrane-bound receptor (Cuatrecasas, 1969) to the enzyme was disrupted by the fractionation procedures. The increased enzyme activity was not due to de novo protein synthesis (Denton et.al., 1971).

The results presented in this Chapter show that the postulated enzyme activation was not brought about through increased glucose utilisation. Thus the metabolic fate of glucose is not determined solely by the rate at which the glucose transport system operates. Similarly Hall and Ball (1970) demonstrated that glucose metabolism was necessary for the glucose mediated reversal of the insulin inhibition of lipolysis in the adipocyte, increased transport of the non-metabolised sugar 3-OMG being without effect. Since insulin has been shown to change the pattern of glucose metabolism in the adipocyte it is possible that the hormonal signal is transferred in the form of increased levels of a particular metabolite. In this context it is of interest to note that Jungas and Taylor (1972) were only able to obtain fully activated PDH from tissue incubated with substrate. A consistent but small activation was observed in the absence of substrate. However, Coore et.al. (1971b) were able to observe a full stimulation of PDH by insulin in adipose tissue incubated with no substrate. The Zn^{2+} studies described in this Chapter show that increased glucose utilisation and therefore increased substrate availability does not per se

lead to a stimulation of a fatty acid synthesis.

The mechanisms of the regulation of the lipase and PDH activities by insulin is not known. However, an intact cell membrane is required for the observation of a regulatory effect of insulin on both enzymes and the increased PDH activity does not result from de novo protein synthesis. The postulated increase in PDH activity observed in these studies was not mediated through increased glucose utilisation, though the ATP level within the cell does appear to be important (Jungas and Taylor, 1972). A phosphorylation-dephosphorylation mechanism has been proposed for the regulation of PDH activity, the phospho-enzyme being inactive (Linn et.al., 1969a,b). Phospho-PDH was found to be reactivated by PDH phosphate phosphatase, which was itself only active in the presence of high concentrations of Mg^{2+} (Linn et.al., 1969a). Recent studies (Severson et.al., 1974) have revealed a requirement for both Ca^{2+} and Mg^{2+} in the activation of PDH phosphatase. Severson and his colleagues concluded that an increased mitochondrial Ca^{2+} uptake might contribute to the stimulation of PDH by insulin. PDH activity was not affected by conditions which produced large variations in the level of cAMP within the cell (Jungas, 1971).

However, there is some evidence that cAMP is involved in the regulation of an adipose tissue lipase (Rizack, 1964; Butcher et.al., 1965 and Tsai and Vaughan, 1970). Since insulin has been shown to decrease the level of cAMP within the adipocyte under suitable conditions (Butcher, 1970; Manganiello et.al., 1971 and Rizack, 1964) the hormonal regulation of the lipase activity may be mediated through its action on the level of the cyclic nucleotide within the cell.

Summary

1. A physiologically significant enhancement of fatty acid synthesis by insulin has been demonstrated.

2. The postulated enzyme activation was not mediated through increased glucose utilisation.

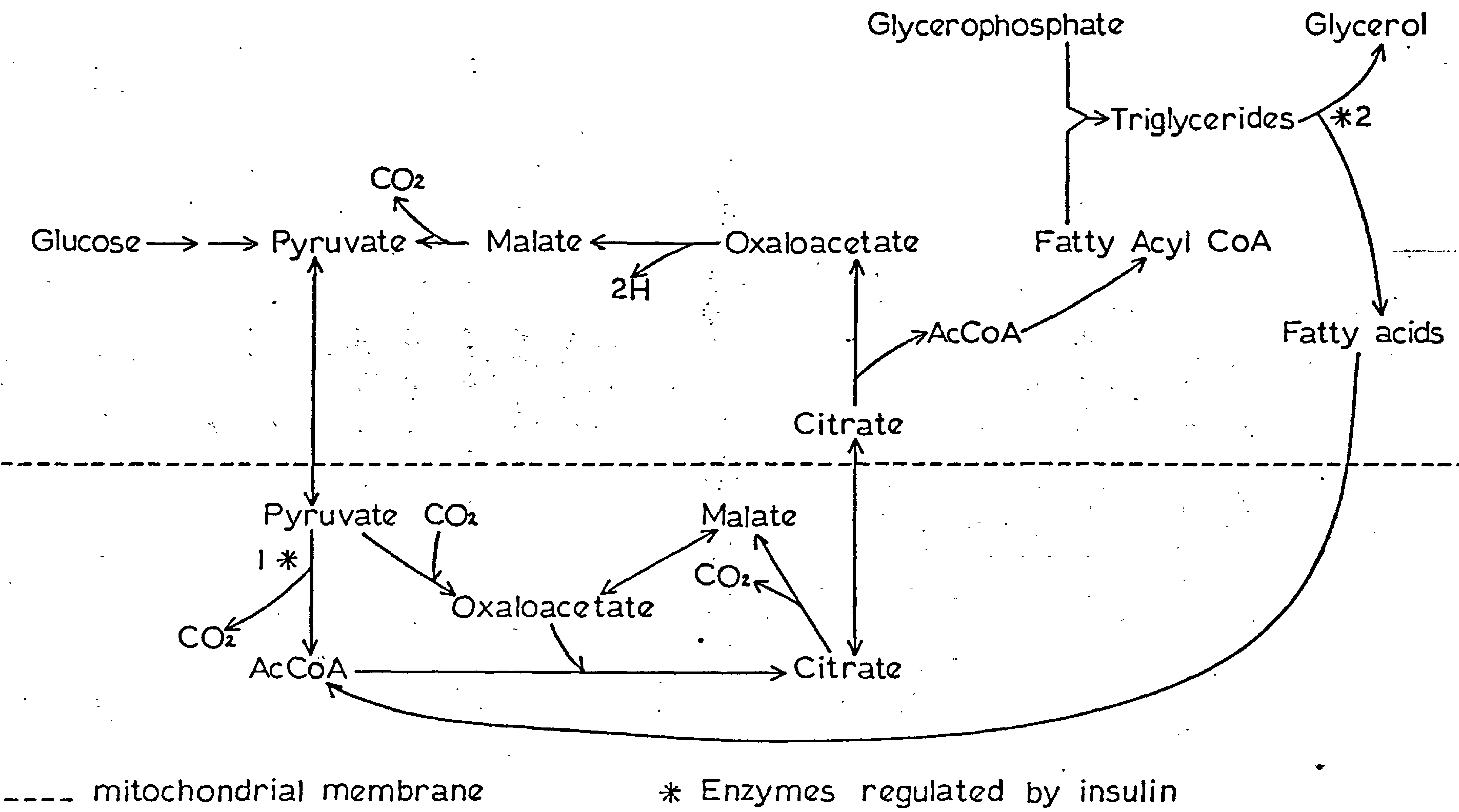
3. There is probably a sigmoid relationship between insulin concentration and the development of a metabolic directive effect on fatty acid synthesis.

4. Evidence is presented for the regulation of two enzymes involved in glucose storage in the form of triglycerides, pyruvate dehydrogenase and a lipase. This evidence is correlated with published *in vitro* enzyme studies.

5. Insulin promotes glucose storage in the adipocyte by increasing glucose uptake and by promoting its incorporation into fatty acids.

6. A simple model may be constructed for the insulin control of glucose storage in the adipocyte.

Sites at which insulin might act to promote glucose storage in the adipocyte.



---- mitochondrial membrane

* Enzymes regulated by insulin

1 pyruvate dehydrogenase (activated)

2 lipase (inactivated)

Chapter 5

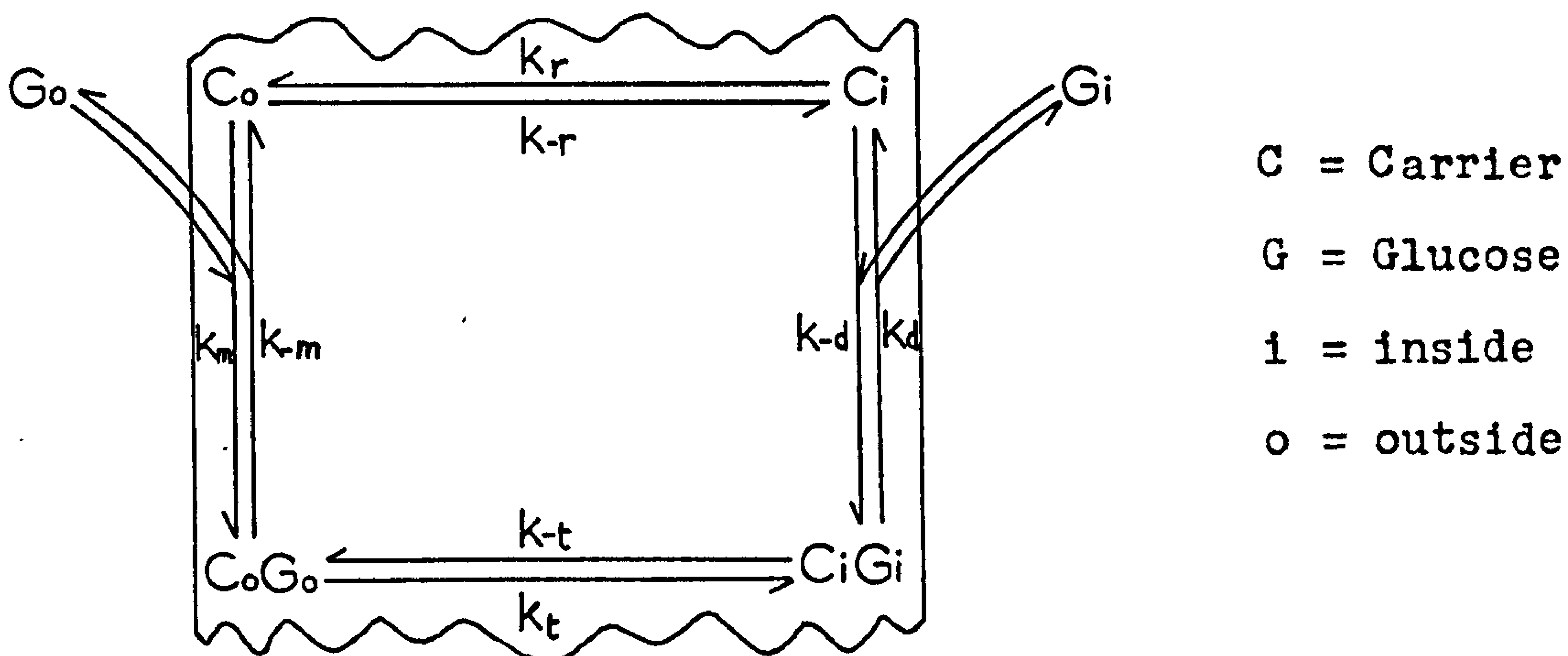
THE ACTION OF INSULIN ON GLUCOSE TRANSPORT IN THE ISOLATED ADIPOCYTE

5.1 Introduction

There is a sigmoid relationship between glucose utilisation in the isolated adipocyte and the concentration of insulin in the medium which is not directly related to the binding of the hormone to the cell since this process follows a hyperbolic saturation curve (Cuatrecasas, 1971; Kono and Barham, 1971 and Gammeltoft and Gliemann, 1973). In the previous Chapter of this thesis it was shown that a sigmoid relationship probably also exists between the concentration of insulin and the production of a metabolic directive effect on fatty acid synthesis.

Since the sigmoid relationships which have been observed do not seem to be related to the interaction between insulin and its receptor they are presumably related to subsequent processes by which the bound hormone produces its effects on the cell. In an attempt to elucidate these processes the effect of insulin on the glucose uptake process in the adipocyte was studied.

The data presented in this Chapter will be considered in the light of currently accepted models of carrier-mediated glucose transport across the plasma membrane. The model used is described overleaf:



C = Carrier

G = Glucose

i = inside

o = outside

The forward rate constants used are: km, the association of glucose with the carrier; kt, the transport of the carrier-glucose complex to the inner surface of the membrane; kd, the dissociation of the carrier-glucose complex with the release of glucose into the cell and kr, the return of the free carrier to the outer surface of the membrane. Since it is presumed that glucose cannot be transported against a concentration gradient it is a necessary constraint on the model that the products of the forward and backward rate constants should always be equal. Thus

$$\frac{km\ kd\ kr\ kt}{k\text{-}m\ k\text{-}d\ k\text{-}r\ k\text{-}t} = 1$$

If it is assumed that Gi is insignificant the expression which is derived from this model to describe the relationship between glucose concentration and transport into the adipocyte is of the form,

$$t = \frac{Tg}{K+g}$$

where t is the glucose transport at a glucose concentration g, T is the maximum rate of transport which can be observed and K is the substrate concentration giving half the maximal rate. The magnitude of T will be determined by the magnitude of various rate constants and the concentration of carriers in

the membrane, C.

The isolated adipocyte preparation described by Rodbell (1964) is suitable for studying the influence of insulin on the glucose transport system owing to its extreme sensitivity to insulin. The early work of Rodbell (1964) suggested that insulin increased T and decreased K for glucose utilisation. Gliemann (1970) confirmed these findings. However, these workers used the conversion of glucose to carbon dioxide and triglycerides as an index of glucose transport. Although it seems reasonable in view of the studies cited in Chapter 1 to assume that glucose utilisation is equivalent to glucose transport under the conditions used, the use of carbon dioxide and triglyceride production as an estimate of glucose utilisation has been criticised (see Chapter 2, Section 7).

Glucose utilisation was determined in these studies by the liberation of ^{3}H from 5-^{3}H-glucose (see Chapter 2, Section 7). The highest glucose concentration used was 1.0 mg/ml (5.55 mM). The relationship between glucose concentration and glucose utilisation in the adipocyte was studied in the absence of insulin and in the presence of submaximally and maximally stimulating concentrations of insulin (2×10^{-11}M and 10^{-9}M respectively). Glucose utilisation was not allowed to exceed 10% of the glucose initially present so that gross changes in the substrate concentration were avoided.

5.2 Theoretical models of the action of insulin on the glucose transport system

Insulin might facilitate glucose entry into the cell either by a direct stimulation of individual carriers or by the production of a more general change in the membrane structure which would lead to enhanced carrier turnover. According to the first model the stimulated carriers would turn over more rapidly than the unstimulated carriers. Thus at submaximally stimulating concentrations of insulin some of the carriers would transport glucose at a maximal rate while the remainder would exhibit a basal rate of transport. This model of the action of insulin on the glucose carrier system is described by the equation

$$t = \frac{(1-\alpha)\ T_b g}{K_b + g} + \frac{\alpha T_s g}{K_s + g} \qquad \text{Equation 1}$$

T_b, K_b and T_s, K_s are the constants which describe glucose transport in the unstimulated and fully stimulated states respectively. α is the proportion of C, the total carrier concentration, which is stimulated by insulin. In the absence of insulin α would be expected to be equal to 0 and in the presence of a maximally stimulating concentration of insulin α would approach a value of unity. At intermediate insulin concentrations α would assume a value between 0 and 1, according to the proportion of carriers which were stimulated.

Alternatively insulin might facilitate glucose transport by producing a progressive, concentration-dependent change in the whole membrane. Such a change would affect all carriers to the same extent and would produce a gradual change in K and T with increasing insulin concentration. This model is

described by the equation

$$t = \frac{Tg}{K + g} \quad \text{Equation 2}$$

where the values of K and T will depend upon the insulin concentration.

5.3 Studies of the influence of insulin on the glucose transport system of the adipocyte

The two models were tested by studying the effect of a submaximal insulin concentration on the proportion of the maximum possible stimulation of transport. This effect was calculated from the expression

$$e = \frac{ti-tb}{tm-tb} \quad \text{Equation 3}$$

tb = unstimulated glucose transport

ti = submaximally stimulated transport

tm = maximally stimulated transport

If the insulin stimulation of the glucose transport system is described by Equation 1, the substitution of this equation into Equation 3 gives an expression for e of the form

$$e = \frac{\left(\frac{(1-\alpha)T_b g}{K_b + g} + \frac{\alpha T_s g}{K_s + g}\right) - \frac{T_b g}{K_b + g}}{\frac{T_s g}{K_s + g} - \frac{T_b g}{K_b + g}} = \alpha$$

Thus, if insulin stimulates transport according to Equation 1 the effect will be independent of glucose concentration but will vary with the proportion of carriers stimulated.

However, substitution of Equation 2 into Equation 3

shows that

$$e = \frac{\left(\frac{T_i g}{K_i + g} - \frac{T_b g}{K_b + g}\right)}{\left(\frac{T_m g}{K_m + g} - \frac{T_b g}{K_b + g}\right)}$$

Providing that insulin affects both T and K significantly (Gliemann, 1970) then g cannot be eliminated from this equation until it is very large compared with the largest value of K. Thus the effect would be expected to increase with the glucose concentration until a limiting value was attained. This limiting value is defined as

$$e = \frac{Ti - Tb}{Tm - Tb}$$

The effect will, however, be independent of glucose concentration in any system in which insulin does not affect K.

Table 5.1 shows the results of three experiments performed to test the models described. In experiment 2, the effect of insulin was clearly not independent of glucose concentration, since it increased with glucose concentration. The effect of increasing glucose concentration was less clear in the other two experiments. However, the anomalous effect observed with a glucose concentration of 0.05 mg/ml in Experiment 3 may be overestimated. The value of ti at this glucose concentration was higher than the corresponding value observed at 0.1 mg/ml. If the value of ti at 0.05 mg/ml was overestimated then the value of effect at 0.05 mg/ml will in turn be overestimated. Examination of the three remaining values of effect in this experiment shows a progressive

Table 5.1 Influence of insulin on the adipocyte glucose uptake system: the relationship between glucose concentration and e*

Glucose concentration mg/ml		0.05	0.1	0.14	0.2	0.25	0.3	0.5	1.0
Experiment 1									
	tb	3.39	7.31	11.48	9.44				
	ti	8.59	20.32	23.65	26.15				
	tm	13.30	24.10	29.82	31.70				
	e	0.53	0.78	0.66	0.75				
Experiment 2									
	tb	1.75	2.85	3.48	5.67	5.97		5.12	10.25
	ti	4.59	8.52	11.51	14.24	18.51		22.51	29.87
	tm	19.40	36.41	48.14	52.95	71.89		74.93	72.37
	e	0.16	0.17	0.18	0.18	0.19		0.25	0.36
Experiment 3									
	tb	1.87	2.40		4.89		4.21		
	ti	12.06	11.13		19.02		20.59		
	tm	31.83	46.09		60.51		58.67		
	e	0.34	0.20		0.27		0.30		

*$e = \frac{ti-tb}{tm-tb}$

tb = glucose transport with insulin

ti = glucose transport with submaximal insulin stimulation

tm = glucose transport with maximal insulin stimulation

t is expressed in Nmol/hour

increase with increasing glucose concentration.

Since Equation 2 does not necessarily require that the insulin effect should be dependent upon the glucose concentration the results of all the experiments were consistent with this model. However, these results did not finally preclude Equation 1 as a viable model of the effect of insulin on the glucose transport system.

A more precise way of testing whether the experimental data are better fitted by a two term Michaelis Menten equation (Equation 1) or a one term Michaelis Menten equation is to examine the values of the constants T_b, T_s, K_b, K_s which can be used to fit the experimental data.

Equation 1 is an example of a two-component curve which can be analysed by the procedure described by Hoare (1972). This procedure was therefore used to determine the best fitting values of T and K. The advantage of Hoare's procedure is that it allows a comparison to be made of the goodness of fit obtained when data are fitted by different combinations of constants.

The essential details of the procedure as applied to this problem are as follows. Ranges of values of T_b and K_b were selected. For each combination of T_b and K_b a new set of values for the y variable was calculated from the formula

$$\frac{t}{g} - \frac{T_b}{K_b + g}$$

and values of K_s and T_s were then calculated by linear regression using the equation

$$\frac{1}{\frac{t}{g} - \frac{T_b}{K_b + g}} = \frac{K_s}{T_s} + \frac{g}{T_s}$$

These calculated values of K_s and T_s together with the selected values of K_b and T_b were then used to calculate the mean square error (S) between the observed values of t and those calculated from the equation

$$t = \frac{T_b g}{K_b + g} + \frac{T_s g}{K_s + g}$$

An Elliot 4130 computer was used to perform these calculations. A square matrix was printed out relating the values of S obtained with the calculated values of T_s and K_s to the selected values of T_b and K_b. In this way the values of T_b, T_s, K_b and K_s which minimised S were found. The F test can be used to determine whether this minimum value of S is significantly lower than values obtained with other sets of constants.

In some cases the lowest value of S was obtained by setting $T_b = 0$, which is equivalent to fitting the data with Equation 2 (see Table 5.2). In no case was the value of S for $T_b = 0$ significantly greater than the minimum S value and indeed the two values were usually extremely close.

This shows that in each case the data could be adequately fitted by a 1 term Michaelis-Menten equation (Equation 2) and that this model is therefore acceptable. However, no evidence could be obtained from this analysis to exclude Equation 1.

It appeared that Equation 1 might be excluded by an examination of the calculated values of T_b and T_s associated with selected values of K_b and K_s. The procedure described above was therefore repeated using a new set of y and x variables to calculate T_b and T_s by linear regression using the equation

Table 5.2 Comparison of the minimum mean square error (s) with the value obtained by setting T_b equal to 0.0

Insulin level		Basal	Submaximal	Maximal
Experiment 1	$T_b = 0$	0.1330	0.1226	0.1054
	Smin	0.1304	0.1222	0.0939
Experiment 2	$T_b = 0$	0.3287	0.0849*	0.1731
	Smin	0.2734		0.1676
Experiment 3	$T_b = 0$	0.2569*	0.1952	0.0968
	Smin		0.1489	0.0958

* The minimum value of S was associated with a value for T_b of 0.0.

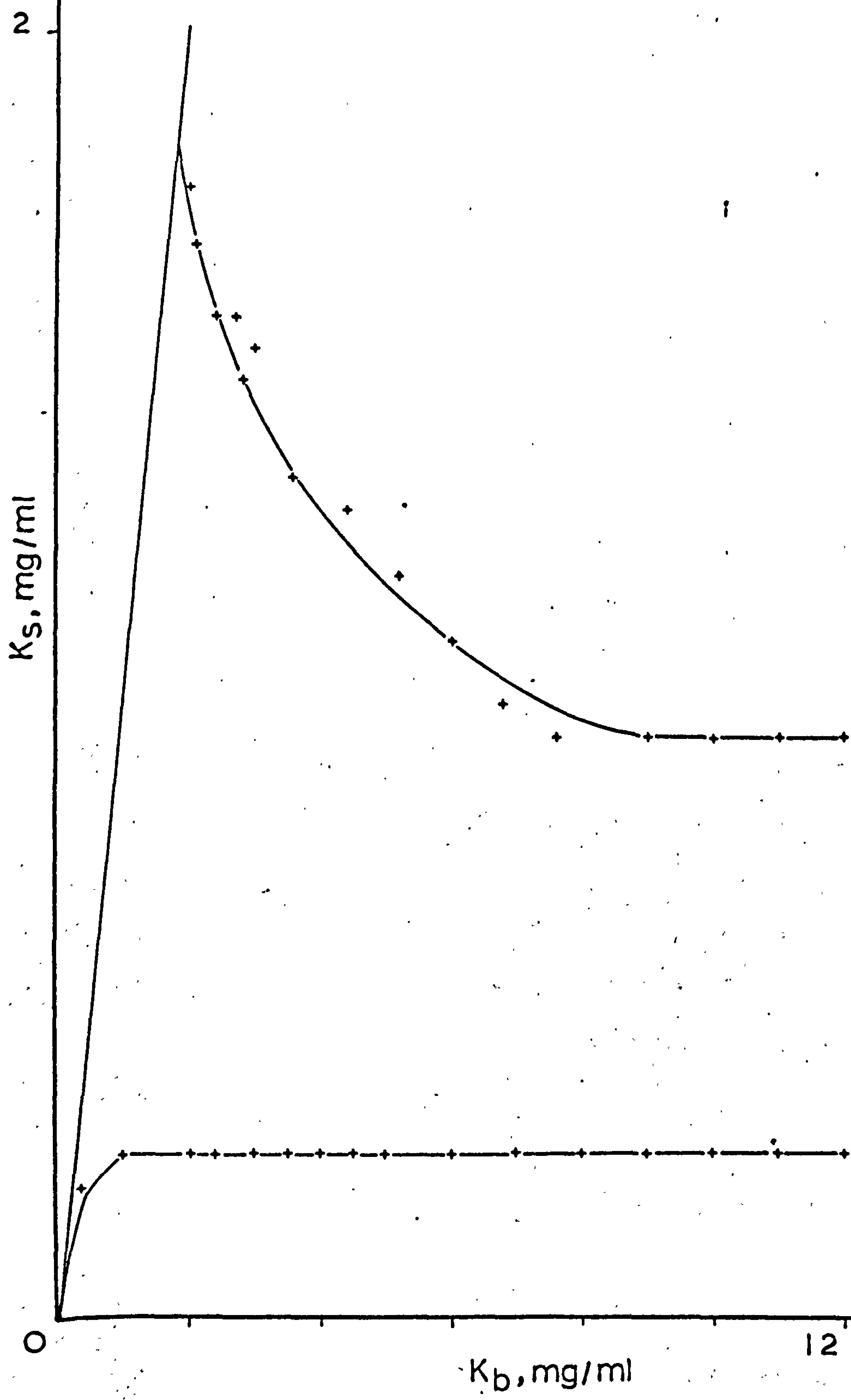
$$\frac{t}{g}(K_b + g) = T_b + T_s\left(\frac{K_b + g}{K_s + g}\right)$$

This treatment has the additional merit of allowing the possibility of heterogeneous carriers in the basal and maximally stimulated cells as might arise from Equation 1 if endogenous insulin was not completely washed off the adipocytes or if stimulation of all the carriers was not achieved.

The matrix relating S to K_b and K_s was plotted out as before. Hoare (1972) has described a procedure for plotting confidence contours on to this matrix to indicate the probability that the contours contain the correct combinations of values for the two selected constants. Figure 5.1 shows the matrix obtained for maximally stimulated transport data from Experiment 2. The 75% confidence contour enclosing acceptable combinations of K_b and K_s is indicated. 75% was chosen because it is a close approximation to one standard deviation. Since by definition $K_s < K_b$ only that part of the matrix to the right of a line of unit slope is shown.

It is clear from the Figure that the data can be fitted with a wide range of combinations of K_b and K_s providing that appropriate values of T_b and T_s are chosen. However, some of these combinations may be excluded according to the following criteria. According to the model (Equation 1) the basal and stimulated carriers in the submaximally stimulated adipocytes must display a K_b and K_s acceptable to the basal and maximally stimulated transport data. Thus, if the confidence regions describing all three hormonal conditions are combined, the only acceptable combinations of K_b and K_s will be those which occur in all three confidence regions.

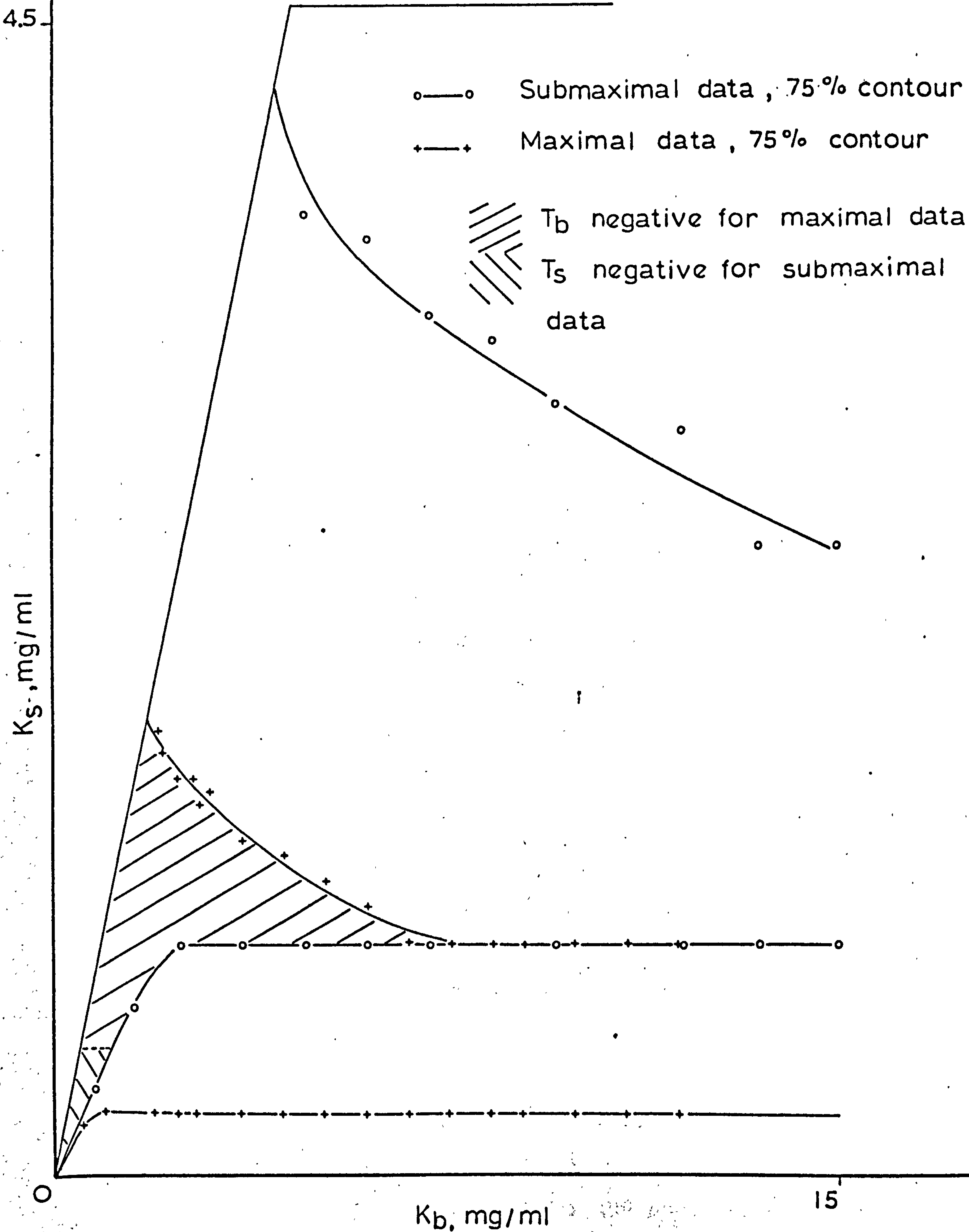
Figure 5.1 Matrix relating K_b and K_s to S, with the 75% confidence contour enclosing acceptable values for maximally stimulated transport data.



Two other criteria were used to assess the acceptability of various combinations of K_b and K_s. The first of these tests was based on the fact that, according to Equation 1, T_s must increase and T_b must decrease as the insulin concentration increases. The second test used was that all values of T_b and T_s must be positive. Since values of T were calculated which minimised S, negative T values were calculated in some instances, though such negative values of T can have no physical significance in the system under investigation. Where such negative values of T occurred the corresponding values of K were excluded as values of constants which adequately fit Equation 1 to the data.

The use of some of these criteria is illustrated in Figure 5.2. The confidence contours presented are those calculated from submaximally and maximally stimulated transport data of Experiment 2. The confidence contour for basal data lies outside the bounds of this Figure, so that all values within the submaximal and maximal confidence regions can be used to obtain an acceptable fit to basal data. Clearly a considerable region of the submaximal confidence contour contains values which are not acceptable since they lie outside the confidence contour for maximal data. By the same criterion much of the confidence region for maximally stimulated transport may also be excluded. However, exclusion of these regions leaves a considerable area of acceptable combinations of K_b and K_s where the two contours overlap. Thus, if the only criterion used is the selection of acceptable pairs of K values to fit all data it is possible to describe the data with a two-term transport equation. However, in order to fit the data with this model it is also necessary

Figure 5.2 Combinations of K_b and K_s which fit maximally and submaximally stimulated glucose transport data.



that the value of T_b should decrease with increasing insulin concentration and may fall to zero (but not below) in the maximally stimulated cells. Similarly, T_s must increase with increasing insulin concentration and may be as low as zero (but not below) in the basal cells.

An examination of the T values associated with combinations of K_b and K_s within this region of overlap showed that this was not the case. In order to fit the transport data with these combinations of affinity constants it was always necessary to assign a negative value to one of the T terms. This is shown in Figure 5.2.

Thus, the glucose transport data of Experiment 2 could never be fitted with a two-term transport equation using values of constants which satisfied the criteria used. These criteria were applied to the other two experiments and it was again found that there were no combinations of K_b and K_s which were acceptable to basal, submaximally and maximally stimulated transport data. It was therefore concluded that glucose transport into the adipocyte could not be described by a two-term transport equation.

5.4 Discussion

It was concluded that insulin does not stimulate individual carriers in a manner described by Equation 1 but that it produces a progressive change in the whole plasma membrane which facilitates the movement of all the glucose carriers. This progressive change is characterised by distinct pairs of values for K and T at submaximally stimulating insulin concentrations as described by Equation 2. Table 5.3 shows the values of K and T calculated for the three experiments

Table 5.3 The influence of insulin on the constants describing glucose transport

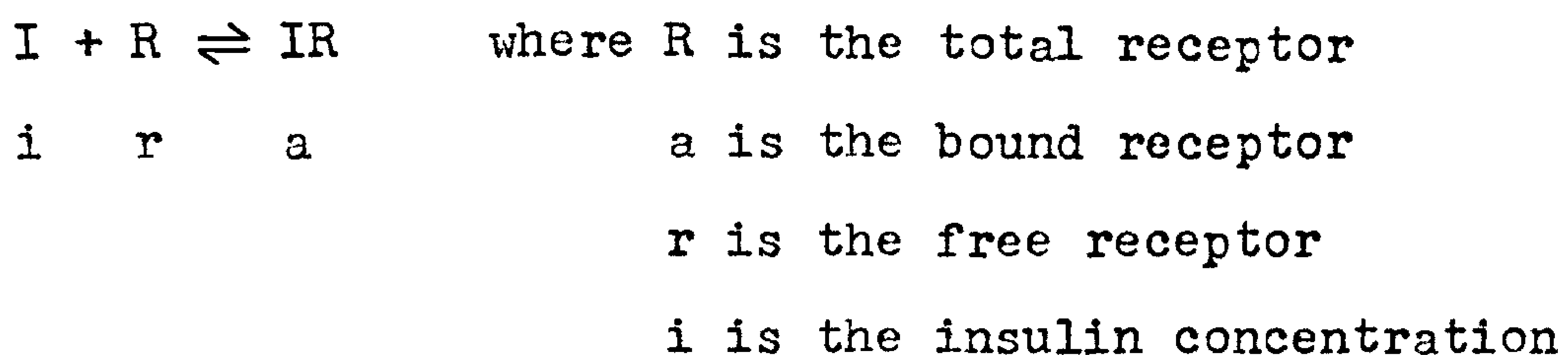
	K^1			T^2		
Insulin ($x10^{11}$M)	0	2	100	0	2	100
Experiment						
1	0.34	0.23	0.15	26.99	55.19	57.11
2	0.35	0.36	0.11	11.43	40.47	84.25
3	0.34	0.27	0.08	9.72	42.72	75.45

1 K in mg/ml

2 T in Nmol/hr/µmolTG

described in this Chapter. The calculation was performed with the Eadie equation. The Table shows that insulin progressively increased T in all the experiments performed. In two of the three experiments (1 and 3) insulin caused a progressive increase in the affinity of the transport system for glucose. In the remaining experiment, a maximally stimulating insulin concentration reduced K but a submaximally stimulating concentration did not.

Two models of the effect of insulin on the glucose transport system have been considered. The model postulating a direct interaction between insulin and the transport system has been excluded by transport studies. However, these studies were consistent with the model suggesting that the direct effect of insulin is on the membrane. This model has the additional merit that it can explain the origin of the sigmoid relationship between insulin concentration and its effect whereas the other model cannot. This can be demonstrated in the following way. The binding of insulin to its receptor appears to follow a hyperbolic saturation curve (see Section 1 of this Chapter) and binding can be described by the equation



$$\underset{i}{I} + \underset{r}{R} \rightleftharpoons \underset{a}{IR}$$

where R is the total receptor
a is the bound receptor
r is the free receptor
i is the insulin concentration

If binding is governed by the equilibrium constant K, then the fraction of the maximum receptor binding (a/R) is given by the equation

$$\frac{a}{R} = \frac{i}{i + K}$$

and can vary between 0 and 1. In the experimental system used in this work receptor binding has two limiting values. In the basal adipocyte no receptors are associated with insulin ($a/R = 0$) and in the maximally stimulated adipocyte the receptors are saturated with insulin ($a/R = 1$).

Consider first Equation 1

$$t = \frac{(1 - \alpha)T_b g}{K_b + g} + \frac{T_s g}{K_s + g}$$

In this model of the glucose transport system the fraction of stimulated carriers (α) is proportional to the receptor binding (a/R). Equation 1 can then be rewritten

$$t = \frac{\left(1 - \frac{i}{i + K}\right)T_b g}{K_b + g} + \frac{\left(\frac{i}{i + K}\right)T_s g}{K_s + g}$$

But at any given glucose concentration $T_b g\ (K_s + g)$, $T_s g\ (K_b + g)$ and $(K_b + g)(K_s + g)$ are all constants and can be given the symbols A, B and C. Under these circumstances Equation 1 simplifies to

$$t = \frac{Bi + AK}{Ci + CK}$$

which describes a hyperbolic relationship between i, the insulin concentration and t, the glucose transport. Since this relationship has been shown experimentally to be sigmoid (see Chapter 3) it is clear that this model does not adequately describe the response of the glucose transport system of the adipocyte to insulin.

However, the model in which a gradual change in the properties of the whole membrane in response to increasing concentrations of insulin was postulated leads to an entirely

different conclusion. In order to see how changing insulin concentrations will affect transport it is necessary to examine the transport equation more carefully. The equation for glucose transport derived from the carrier model in section 1.3 of this Chapter, assuming that the intracellular glucose concentration is negligible, can be expanded to include all the constituent rate constants:

$$t = \frac{\dfrac{k_d\, k_r\, k_t}{k_r\, k_t + k_d\, k_t + k_d\, k_r + k_{-t}\, k_r}\, C g}{\dfrac{k_r + k_{-r}}{k_m(k_r + k_t)} + g}$$

(where C is the concentration of carriers)

If it is assumed that the movement of the carrier through the plasma membrane is rate determining (see for example, Illiano and Cuatrecasas, 1971), because the rates of association and dissociation of the glucose-carrier complex are very large, then

$$t = \frac{\dfrac{k_r\, k_t}{k_t + k_r}\, C g}{\dfrac{k_r + k_{-r}}{k_m(k_r + k_t)} + g}$$

According to this model insulin must affect one or more of the rate constants in the equation describing glucose transport. The minimum number of rate constants which must be affected by insulin is two since the condition

$$\frac{k_m\, k_d\, k_r\, k_t}{k_{-m}\, k_{-d}\, k_{-r}\, k_{-t}} = 1$$

must always hold (see page 120).

Inspection of the rate constants making up T and K shows that the most probable way in which insulin could both increase T and decrease K (see Table 5.3) would be to cause an increase in kr (possibly associated with a proportional increase in k-t). This is consistent with a change in membrane structure in which the equilibrium position of the glucose carriers is changed so that the carriers (whether full or empty) preferentially move to the inner surface of the plasma membrane.

For the purpose of this Discussion it is proposed that insulin causes a gradual change in kr from kr to kr(1 + x). At submaximal concentrations of insulin x will have a value proportional to the degree of insulin stimulation. That is to say, it will have a value proportional to a/R or $\frac{i}{i + K}$.

The rate of glucose transport at any insulin concentration can then be written

$$t = \frac{\dfrac{kr\left[1 + \dfrac{i\,x}{i + K}\right] kt\;g\;C}{kr\left[1 + \dfrac{i\,x}{i + K}\right] + kt}}{\dfrac{kr\left[1 + \dfrac{i\,x}{i + K}\right] + k\text{-}r}{km\left[kr\left(1 + \dfrac{i\,x}{i + K}\right) + kt\right]} + g}$$

$$= \frac{\dfrac{kr\;ktg\left[i(1 + x) + K\right]}{i\;(kr + krx + kt) + K(kr + kt)}}{\dfrac{i\;(kr + krx + k\text{-}r + gkm + gkmx + gkm\;kt) + K(kr + k\text{-}r + gkm + gkm\;kt)}{Km\left[i(1 + x + kt) + K(1 + kt)\right]}}$$

At any given value of glucose concentration various terms in this expression are constant. By amalgamating the constant terms and replacing them with the symbols D to J the equation simplifies to

$$t = \frac{Di^2 + Ei + F}{Gi^2 + Hi + J}$$

The square term indicates that this expression describes a sigmoid relationship between glucose transport and the insulin concentration.

It is proposed that insulin interacts with the cell membrane in such a way as to produce a progressive change in the whole membrane. This change in the plasma membrane leads to a change in the rate constants describing the glucose transport system. The sigmoid relationship between glucose utilisation and insulin concentration is the inevitable result of this mechanism of action.

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