INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

University Microfilms International
300 North Zeeb Road
Ann Arbor, Michigan 48106 USA
St. John's Road, Tyler's Green
High Wycombe, Bucks, England HP10 8HR

77-8825

CHENG, Kang, 1946-
EFFECT OF INSULIN, INSULIN ANALOGS AND ANTIMITOTIC DRUGS ON GLUCOSE METABOLISM IN ISOLATED FAT CELLS.

City University of New York, Ph.D., 1977
Chemistry, biological

Xerox University Microfilms, Ann Arbor, Michigan 48106

EFFECT OF INSULIN, INSULIN ANALOGS AND ANTIMITOTIC DRUGS ON GLUCOSE METABOLISM IN ISOLATED FAT CELLS

by

KANG CHENG

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1976

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

NOV 30, 1976
date

P G Kadsayannis
Chairman of Examining Committee

November 30, 1976
date

Executive Officer

J. D. Chanley

The City University of New York

ABSTRACT

EFFECT OF INSULIN, INSULIN ANALOGS AND ANTIMITOTIC DRUGS ON GLUCOSE METABOLISM IN ISOLATED FAT CELLS

by

Kang Cheng

Adviser: Dr. Panayotis G. Katsoyannis

The effects of antimitotic drugs on glucose transport in isolated fat cells were studied. Colchicine, a potent antimitotic drug, inhibited glucose oxidation in isolated fat cells and fat cell ghosts but had no effect on glucose oxidation by fat cell homogenates. The concentration of colchicine which half-maximally inhibited the conversion of (1-^{14}C)glucose to $^{14}CO_2$ by isolated fat cells and fat cell ghosts was about 2 mM. Prolonged pre-incubation of fat cells with colchicine caused no further inhibition. The effect of colchicine on glucose oxidation was readily reversible upon the removal of colchicine from the medium. Furthermore, the response of pretreated cells to insulin was as good as untreated cells. Insulin and concanavalin A-stimulated glucose oxidation in isolated fat cells were also inhibited by colchicine. The inhibitory effect of colchicine on glucose oxidation was not reversed by increasing concentration of glucose, insulin or concanavalin A. These findings suggested

that colchicine did not compete for their binding sites on the plasma membrane of adipocytes.

Both insulin and concanavalin A significantly stimulated the initial rate of 2-deoxy-D-glucose influx in isolated fat cells. The enhancement of uptake varied from 50% to 100% increase over control levels. The uptakes of 2-deoxy-D-glucose by isolated fat cells in the presence and absence of colchicine were studied in an attempt to determine the effect of colchicine on sugar transport. Colchicine at concentration of 10 mM inhibited basal as well as insulin and concanavalin A-stimulated 2-deoxy-D-glucose uptakes by about 85% comparable to its effects on glucose oxidation. The effect of colchicine on sugar transport in isolated fat cells was specific for D-glucose, since the uptake of L-glucose was not affected by colchicine. In addition to its effect on the uptake of 2-deoxy-D-glucose, colchicine also inhibited the efflux of 3-O-methylglucose from isolated fat cells.

Lumicolchicine, which does not bind to microtubules, was more effective than colchicine in inhibiting glucose oxidation in isolated fat cells. Fifty per cent inhibition of glucose oxidation occurred with approximately 5×10^{-5} M. Significant inhibition by lumicolchicine was observed at concentrations as low as 1 to 5 μM. Lumicolchicine also inhibited the insulin and concanavalin A-stimulated glucose oxidations but to a greater extent than its effect on the basal rate. Griseofulvin, at concentration of 0.1 mM, acted in a manner similar to lumicolchicine, by inhibiting insulin

and concanavalin A-stimulated glucose oxidation more than the basal rate (75% vs. 50% inhibition). Both drugs inhibited the uptake of 2-deoxy-D-glucose by isolated fat cells but not the uptake of L-glucose. From this study, it is concluded that the effect of antimitotic drugs on glucose transport in isolated fat cells is not due to the disruption of microtubules. The site of action of these drugs is probably situated on the plasma membrane of adipocytes.

The biological activities of insulin analogs were determined on hormonally responsive isolated fat cells. The relative binding affinities of (A1-L-Ala)insulin, (A1-D-Ala) insulin, (A21-Arg)insulin, (B9-Leu)insulin and (B26-TyrNH_2) insulin are 12%, 100%, 33%, 27% and 28% of that of native insulin, respectively. The relative biological activities of these analogs in glucose oxidation are in agreement with their relative binding affinities. These results suggest that the lower biological activities of these analogs could be explained by the decrease in their binding affinities.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. Panayotis G. Katsoyannis for his advise, support and encouragement over the past four years. I am also in debt to Drs. A. Cosmatos and G. P. Schwartz for their kindly supply of insulin analogs.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE

The functions of adipose tissue are integrated with the activity of other tissues of the body under two different states; the fasting and fed states. These two states are as fundamental to adipose tissue as exercise and rest are to the cardiovascular system. During food deprivation the adipose cells release fatty acids and glycerol into the blood stream by a metabolic process called lipolysis to provide other tissues with a respiratory fuel (1,2). The fatty acids after exit from the cells are combined with serum albumin and circulated to other tissues. Almost all tissues excepting brain and red blood cells, can take up long-chain fatty acids from the blood stream and convert them to CO_2 and H_2O by the process of β-oxidation in mitochondria. This is accompanied by a release of chemical energy which is trapped by the metabolic machinery of the cell as "high energy" phosphate bonds in ATP.

The glycerol released from adipose tissue is taken up by the liver where it is converted into glucose by gluconeogenesis (3,4). The glucose is then released into the blood stream. Tissues which depend absolutely on glucose as a fuel, such as brain and red blood cells, are thus kept supplied during a fast.

Under the fed state, the primary functions of adipose tissue are to remove glucose from the blood stream, stimulated by the presence of insulin, and to assimulate

lipoprotein fatty acids produced by the liver and intestine and secreted into the blood stream as β- and pre-β-lipoproteins (5). Fatty acids and glucose, after entering the adipose cells are combined to form triglyceride by lipogenesis. At the same time as lipogenesis is initiated, lipolysis is inhibited so that there is a diminished release of free fatty acids and glycerol from the cells. Regulation of the adipose cells in the fed state is primarily mediated by insulin associated with reduced influence of lipolytic hormones. Due to the size of adipose tissue in the body and its sensitivity to insulin, it is obvious that adipose tissue plays a very important role in blood glucose hemeostasis.

Although the structure of plasma membrane is not completely understood as yet, it is generally agreed that the membrane is composed of proteins and lipid bilayer which is the barrier to water-soluble compounds, such as glucose. The presence of a glucose barrier in the peripheral tissues is very meaningful from the point of view of glucose hemeostasis of the organism. If a barrier to glucose entry did not exist in these tissues, there would be a continuous removal of blood glucose at the expense of the obligatory glucose-burning tissues, such as the brain, which would soon be perish from lack of fuel. On the other hand, it would not be surprising to find a "special mechanism" for glucose transport in the plasma membrane of peripheral tissues.

A. Glucose Transport in Adipose Tissue

Measurement of the penetration of glucose

independent of subsequent enzymatic steps has not been feasible. For this reason, a variety of poorly metabolizable sugars have been used as glucose models to study the transfer of sugars across the cell membrane. The most commonly employed analogs are 3-O-methylglucose and 2-deoxyglucose, both compete with glucose for the same membrane transport system (6-9). 3-O-Methylglucose has a high affinity for the glucose carrier in the membrane but has the disadvantage that a significant fraction of its membrane penetration occurs by simple diffusion. This is due to the fact that 3-O-methylglucose is more lipophilic than glucose (10). In rabbit erythrocytes, the diffusion constant for 3-O-methylglucose is eight times that for L-glucose (11). However, once inside the cell, 3-O-methylglucose is neither phosphorylated nor metabolized. Because of this property, it is a very useful glucose analog in studying the efflux of sugar from preloaded cells. On the other hand, 2-deoxyglucose is phosphorylated by hexokinase of adipose tissue with a K_m the same as glucose 3×10^{-5} M (12), to form the phosphate ester 2-deoxyglucose-6-phosphate, which accumulates within the cell, since it is not acted upon at an appreciable rate by other enzymes. By using these glucose analogs, a great deal of information about the kinetics of glucose transport in mammalian cells has been accumulated.

Glucose transport in adipose tissue, as in muscle and erythrocytes, occurs by a carrier-mediated facilitated diffusion mechanism (13) which has the following

characteristics:

1. Saturability:

At low substrate concentration, the rate of a carrier-mediated transport is usually proportional to substrate concentration and thus is first-order. However, as the substrate concentration is increased, the rate becomes zero-order with respect to substrate. Plots of the initial rate of the transport process vs. substrate concentration usually show a hyperbolic curve approaching a maximum, similar to the Michaelis-Menten curve of an enzyme. In contrast, in nonmediated transport the rate of transport is dependent primarily on the concentration gradient of the solute, with no tendency to show saturation. In adipose tissue (14), isolated fat cells (15) and fat cell ghosts (16), D-glucose transport has been shown to exhibit saturation kinetics of the Michaelis-Menten type. The net transport of glucose depends on relative rates of influx and efflux and is described by the following formula:

$$v = V_{max} \left(\frac{S_o}{S_o + K_m} - \frac{S_i}{S_i + K_m} \right)$$

In this formula, V_{max} expresses the maximal flux of glucose; K_m is the substrate concentration at which the flux is half that of maximal flux; S_o and S_i are extracellular and intracellular glucose concentrations, respectively. When intracellular glucose concentration is zero, net transport

is equal to the rate of influx. When intracellular and extracellular glucose concentrations are equal, net transport is zero.

2. Stereospecificity:

The glucose transport system in adipose tissue not only shows specificity among monosaccharides, it also shows stereospecificity by its impermeability to L-glucose as compared with transport of D-glucose (14,16,17). 2-Deoxy-D-glucose (17), 3-O-methyl-D-glucose (14,16,17) and D-mannose (14) are potent inhibitors of D-glucose uptake, while L-glucose (16,17) and D-fructose (17) have virtually no effect.

3. Counter-Transport:

A definitive criterion of a mobile-carrier hypothesis is that it should be possible, under certain conditions, to force one substrate to travel up a gradient of concentration by adding another substrate to the system. The counter-transports of 3-O-methyl-D-glucose in adipose tissue induced by the addition of glucose in the presence and absence of insulin have been demonstrated by Crofford and Renold (14). However, no counter-transport of 3-O-methyl-D-glucose has been induced by sucrose under any condition.

4. Susceptibility to Protein Reagents:

There is a considerable amount of evidence

indicating that at least some of the components of carrier-mediated transport system are protein in nature. Therefore, it would not be susprising that a carrier-mediated transport system shows high sensitivity to at least some of the protein reagents; and the appearance of such specific sensitivities in biological transport processes is often taken as evidence of involvement of carrier-mediated transport mechanism. Sulfhydryl reagents such as N-ethylmaleimide, arsenite, iodoacetate and parachloromercuribenzoate at low concentrations have been shown to stimulate the glucose ulitization in adipose tissue (18) and in isolated fat cells (19,20). At higher concentrations, N-ethylmaleimide (19) and arsenite (20) inhibited both basal and insulin-stimulated glucose metabolism. The site of action of these sulfhydryl reagents has been suggested at the level of cell membrane (19,20). More direct evidence about the effects of sulfhydryl reagents on fat cell glucose transport system has been demonstrated by Czech, et al (21,22). Treatment of fat cells with either 0.5 mM N-ethylmaleimide or 3 mM dithio(bis)-nitrobenzoic acid abolished the ability of insulin to activate 3-O-methyl-D-glucose transport. However, basal transport activity was not significantly influenced by these agents

In addition to the characteristics mentioned above, the glucose transport is also a temperature-dependent process which has been demonstrated in both fat cells (23) and fat cell ghosts (16,24). The rate of uptake at $4^{o}C$ was

about 20% as great as the rate observed at 25°C (16). Phlorizin, phloretin and cytochalasin B, agents known to block D-glucose transport in other cells (25-29), inhibited glucose transport in fat cells (23,24,30). In addition to its extremely sensitive to physiological concentrations of insulin, the glucose transport system in fat cells has also been observed in response to oxidants (31), lectins (32,33), and low concentrations of sulfhydryl reagents (18-20), proteases (34-38), phospholipases (39), and neuraminidase (40,41). The physiological significance of these effects is not known.

B. Evidence that Insulin Action Results from Its Interaction with the Plasma Membrane

The binding of insulin to hormone-sensitive tissue was first reported by Stadie et al (42) who observed that rat hemidiaphragms incubated with insulin for 10 sec and washed showed a significant stimulation of glycogen synthesis. The results were difficult to interpret, however, because of the uncertainty as to whether the hormone was actually bound or simply trapped in the extracellular spaces of the muscle. Crofford has shown that a rapid phase of insulin uptake by isolated fat cells occurred within the first minute (43). The rapidity of this uptake suggested that the site of hormone binding was on the plasma membrane.

Insulin effects have been mimicked by trypsin or certain other proteolytic enzymes at very low concentrations but not at high concentrations (34-38). However, Kono first

reported that incubation of isolated fat cells with 1 mg/ml of trypsin for 15 min completely abolishs the insulin stimulatory effect on glucose oxidation after inactivation of the enzyme with soybean trypsin inhibitor (44,45). Control studies revealed that the diminished effect of insulin was not caused by destruction of the insulin or nonspecific damage to the cell by trypsin. It was rather specific modification of insulin-effector system by trypsin since trypsin-treated cells still maintained normal levels of ATP, malate dehydrogenase, and lactate dehydrogenase. Furthermore, glucose oxidation in trypsin-treated cells was inhibited by 3-O-methyl-D-glucose, phloretin, or D-xylose, but not by L-xylose. The rate of glucose oxidation could still be augmented in normal fashion by raising the concentration of glucose in the medium. The lipolytic system of the cell remained highly responsive to epinephrine and ACTH, but the responsiveness of the system to glucagon was abolished entirely. After the inactivation of the enzyme, the insulin responsiveness of trypsin-treated cells was restored by incubating the cells at 37°C for more than 60 min (45). It was not necessary to add either glucose or insulin in the incubation medium during the recovery period. However, this recovery was inhibited by 0.1 mM puromycin or 0.01 mM cycloheximide. These findings indicated that protein synthesis was required in the restoration of the insulin-effector system.

It has been shown that trypsin can hydrolyze

specific proteins on the surface of the cells (46,47), but the enzyme does not penetrate into the living cells and attack intracellular proteins (48). Therefore, it has been concluded from the studies mentioned above that trypsin modifies receptor for insulin that are situated on the outer surface of the cell membrane.

The coupling of insulin to polymer, such as Sepharose, has been used as a tool for determining that insulin need not enter a cell to exert its biological effects. Cuatrecasas (49) has reported that insulin-Sepharose complex was almost as effective as native insulin in increasing glucose oxidation and inhibiting lipolysis in isolated fat cells. He claimed that the complex was stable during incubation with fat cells and that the Sepharose bead was not taken up by cells. Therefore, he concluded that all of the metabolic effects of insulin resulted exclusively from specific interactions with the cell membrane. The insulin-Sepharose complex also has been shown to stimulate RNA synthesis (50) & the uptake of α-aminoisobutyric acid (51) in isolated mammary cells and to activate glycogen synthetase in tadpole liver (52).

These data should be accepted with reservations. Davidson (53) and Kolb et al (54) have presented evidence that both biological as well as immunological activity of insulin has been recovered in the supernatant after the incubation of insulin-Sepharose with or without biological material. Katzen and Vlahakes (55) have recalculated the

data from original Cuatrecasas's report (49) on insulin-Sepharose and concluded that in several key experiments there was less than one insulin-Sepharose bead per incubation flask.

Recently, insulin has been covalently coupled to ferritin with the complex retaining equal biological and immunological activities (56,57). The former was determined by measuring the stimulation of glucose oxidation and the inhibition of epinephrine-induced lipolysis of adipocytes or the stimulation of the Mg^{2+}-ATPase. The ferritin-insulin complex is stable with negligible insulin dissociation during storage or incubation in the presence or absence of tissue. Electron microscopic studies indicated that the complex was not observed free inside the plasma membrane vesicles, suggesting that the complex was not capable of crossing the membrane. These data supported the theory that insulin does not enter a cell to exert its biological effects.

C. Transmission of the Insulin Signal

Specific high affinity receptors for insulin have been shown on the plasma membrane of adipocytes (58-60). The interaction of insulin with its receptor possibly initiates a conformational change in the plasma membrane. This perturbation may be responsible for altering the activities of membrane-bound enzymes, such as adenylate cyclase (61,62), adenosine 3':5'-cyclic monophosphate phosphodiesterase (63-66), Mg^{2+}-ATPase (67), and guanylate

cyclase (68), whose products or substrates may serve as a intracellular "second messenger" for insulin. There are two groups of compound (e.g. cyclic nucleotides and ions) which have been proposed as the possible "second messenger" for insulin in fat cells.

1. Cyclic Nucleotides:

Insulin can decrease the intracellular concentrations of cyclic AMP in fat cells if the nucleotide level has been elevated by epinephrine, ACTH, or glucagon in the presence of caffeine (69). Since the intracellular concentrations of cyclic AMP are determined by the rate of its formation and the rate of its degradation, the effect of insulin on cyclic AMP level can be achieved either by inhibiting adenylate cyclase or by stimulating adenosine 3':5'-cyclic monophosphate phosphodiesterase, or both. Hepp & Renner (61) and Illiano & Cuatrecasas (62) have reported that insulin in vitro inhibits lipolytic hormone-stimulated adenylate cyclase in adipocyte ghosts and in particulate preparations of isolated fat cells. However, Kipnis et al (70) and Vaughan & Murad (71) could not detect any effect of insulin on basal or epinephrine-stimulated adenylate cyclase. There have been reports that insulin stimulates cyclic AMP phosphodiesterase activity in homogenates of isolated fat cells pretreated briefly with insulin (63-66). The antilipolytic effect of insulin can be accounted for under certain conditions by a lowering of intracellular cyclic AMP (72,73). However, Sneyd et al (74) have shown that insulin stimulates sugar transport

apparently independently of the level of cyclic AMP, and Rodbell et al (75) has shown that various lipolytic hormones, which elevate cyclic AMP levels in fat cell ghosts, do not block insulin-stimulated glucose utilization. The possibility cannot be excluded that insulin affects transport through changes in cyclic AMP levels in a small compartment of the cell.

There have been reports that insulin increase cyclic GMP content in isolated fat cells (76-78). Recently, it has been demonstrated that membrane preparations from adipocytes contain a substantial quantity of guanylate cyclase activity (68,79) which can be directly stimulated by insulin (68). However, the physiological significance of the increased cyclic GMP levels by insulin in isolated fat cells has not been examined carefully.

2. Ions:

Ions have been proposed as possible second messengers for insulin (80-82), with a large body of indirect evidence to support this theory.

Krishna et al (83) have shown that norepinephrine causes a depolarization of the brown fat cell membrane potential. Insulin did not significantly change the membrane potential when added by itself, but counteracted the depolarization produced by norepinephrine. These authors suggested that norepinephrine and insulin modify membrane potential by modifying the entry of K^+ into the cell.

Touabi and Jeanrenaud (84) reported that epinephrine markedly reduces the net uptake of K^+ into fat cells and insulin counteracts this effect of epinephrine. Rodbell (85) reported a K^+ requirement for the insulin stimulation of glucose transport in isolated fat cells of the rat. However, Letarte & Renold (86) and Letarte et al (87) found no consistent effect of K^+ deficiency on the hormone effect in mouse adipocytes.

Recently, insulin was found to stimulate the Mg^{2+}-ATPase of the adipocyte plasma membrane (67). The stimulation of Mg^{2+}-ATPase by insulin could explain the observations made by Krahl that insulin caused an increased intracellular magnesium concentration in rat hemiuteri (88) and adipose tissue (89). The net increase of intracellular magnesium concentration then serves as "second messenger" to influence intracellular enzyme activities (80).

Insulin has been shown to affect cellular calcium distribution and that this may play an important role in the mechanism of insulin action (81,82,90). Agents which promote an increased concentration of intracellular calcium, such as ouabain, procaine, and calcium ionophores, have been reported to stimulate glucose transport (91) and to inhibit lipolysis in fat cells (82,92,93). Numerous intracellular enzymes isolated from adipocytes have been reported to be both insulin and calcium sensitive, such as pyruvate dehydrogenase (94), triglyceride lipase (95), adenylate cyclase (96), 3':5'-cyclic AMP phosphodiesterase (97), and glycogen synthetase (98).

D. Effect of Insulin on Glucose Transport

The intracellular glucose concentration is an index of the relative rates of glucose penetration and phosphorylation by hexokinase. An increase in the intracellular glucose concentration can result either from a relative increase in the rate of penetration or from a relative decrease in the rate of phosphorylation. In either circumstance, the presence of glucose within the cell would indicate that phosphorylation was rate limiting. On the other hand, the absence of free glucose from the intracellular compartment would mean that the transport of glucose across the cell membrane was the rate-limiting step in glucose utilization. By this analysis, glucose transport has been shown the rate-limiting step in glucose uptake by adipose tissue and the principal site of insulin action (99). In adipose tissue, insulin increases the affinity of the carrier without a change in V_{max} (14,100). However, an increase in V_{max} is observed in isolated fat cells (101). The significance of these apparent differences in the effect of insulin is uncertain.

The quantity of insulin required to accelerate glucose oxidation is extremely small. A detectable stimulation of glucose utilization has been observed with insulin concentrations as low as 1 μU/ml (7×10^{-12} M) (43, 102). Crofford et al (103) have calculated that binding of 3,000 molecules of insulin per cell would stimulate glucose utilization maximally, whereas as few as 100 insulin

molecules per cell can generate a detectable effect. Kono and Barham (59) have estimated that there are 160,000 insulin receptors in a single fat cell when the mean cell diameter is 50 μm and that glucose oxidation in intact fat cells is stimulated maximally when only approximately 2.4% of the total insulin receptors are occupied by insulin. The stimulatory effect of insulin on glucose transport in fat cells can be reversed either by the addition of anti-insulin serum or by washing the cell in an insulin-free buffer (43). Since no immunologically or biologically active insulin was released by washing, the insulin bound to the receptor was probably inactivated by the cell (43). It is still not clear that whether this inactivation of insulin by the cells represents a non-specific proteolysis or is a part of the physiological mechanism for the termination of the cell response to insulin.

Avruch et al (104) have reported that pre-incubation of isolated fat cells with insulin led to an accelerated rate of both D-glucose uptake and release by the isolated plasma membrane vesicles. Addition of insulin directly to plasma membrane was without effect. At equilibrium, D-glucose space was identical in both insulin-treated and control plasma membrane preparations. Spectroscopic studies on both membrane preparations did not reveal any differences.

E. Enzymatic Modification of Insulin Effects

Exposure of fat cells to low concentrations of

trypsin causes a selective fall in the affinity of the insulin-receptor interaction, whereas the total amount of receptor and the maximal insulin response are unaffected (105). Treatment of fat cells with trypsin or other proteases in very high concentrations results in a loss of insulin binding and of biological effects (44,45,105). Such digestion decreases the total amount of receptor available for binding (59).

Like trypsin, low concentrations of neuraminidase has insulin-like effect on glucose transport in isolated fat cells (40,41). However, drastic digestion of the cell with higher concentrations of neuraminidase causes a fall in the enhanced basal rate of glucose transport and abolishes the effects of insulin on glucose transport and lipolysis. This enzymatic digestion has no effect either on the total number of insulin receptor on the membrane or on the affinity of the insulin-receptor interaction (41). These results suggest that membrane sialic acid is not involved in the recognition of insulin by the receptor, but may be involved in transmitting the insulin effects.

At low concentrations, phospholipase C has been found to stimulate the conversion of glucose to carbon dioxide (39,40). Increasing concentrations of phospholipase C blocked the response of fat cells to insulin (39,40) while even higher concentrations of the enzyme caused lysis of the cells as indicated by the release cellular fat (39). Cuatrecasas (106) reported that digestion of isolated fat

cells and fat cell membranes with high concentrations of phospholipase C or A caused a dramatic increase in insulin binding. The increase in the specific binding of insulin to fat cells or membranes after enzymatic digestion reflected an increase in the total binding capacity of the membranes. The new exposed insulin receptors were probably identical with those normally present in the surface of the membranes (106).

F. Stimulation of Glucose Transport by Concanavalin A

Concanavalin A has been shown to mimic the action of insulin on glucose transport system in white (33,107) and brown fat cells (32), whereas the lectin did not modify the equilibrium distribution of glucose (32). Evidence indicated that the effects of insulin and concanavalin A on glucose transport in fat cells were mediated by different mechanism. For example, addition of α-methylmannoside to the incubation medium completely prevented the ability of concanavalin A to activate fat cell glucose metabolism while insulin action was only slightly affected (33,107). Furthermore, trypsinzation of fat cells, which is known to abolish the binding of insulin to receptors (45) and thus completely inhibit insulin action, had little effect on the action of concanavalin A on isolated fat cells (32,33,107). The binding of ^{125}I-concanavalin A to brown fat cell surface receptors and the lectin-mediated agglutination of these cells were also not affected by trypsinzation (32).

G. Effect of Antimitotic Drugs on Cellular Functions

Colchicine and other antimitotic drugs have been shown to inhibit the secretion of insulin from the pancreas (108), catecholamines from adrenal medulla (109), histamine from human leukocytes (110) and from rat peritoneal mast cells (111), thyroid hormone from thyroid gland (112), norepinephrine and dopamine-β-hydroxylase from sympathetic nerves (113), the release of free fatty acids from adipocytes (114), and the secretion of albumin and other plasma proteins from rat hepatocytes (115). These effects in connection with the well-known property of colchicine and other antimitotic drugs to disrupt the microtubules prompted the speculation that the microtubular system might be involved in the secretory processes (116). More recent work has shown that colchicine is involved in a variety of other metabolic processes. For example, it affects the transport of nucleosides in several mammalian cell lines (117), inhibits the metabolism of glucose by isolated fat cells (30) and the insulin-stimulated metabolism of glucose to fatty acids (118), and inhibits the Mg^{2+}-ATPase of synatic membranes (119).

PART I. EFFECT OF ANTIMITOTIC DRUGS ON GLUCOSE TRANSPORT AND OXIDATION IN ISOLATED FAT CELLS AND FAT CELL GHOSTS.

INTRODUCTION

Concanavalin A, a plant lectin, mimics the effects of insulin on glucose metabolism, lipolysis, and protein synthesis in isolated adipocytes (32,33,107). It has been established that concanavalin A binding sites are in the glycocalyx (120,121), but appeared to be chemically distinct from the insulin receptor. Trypsin treatment of fat cells prevents insulin binding (45) but not the concanavalin A binding (122) or its ability to stimulate glucose transport (32). Concanavalin A binding and stimulation of glucose transport is prevented by α-methylglucoside which only slightly inhibits insulin-stimulated glucose oxidation (33, 107). The ability of concanavalin A to block insulin binding (122) may only be due to the close association of the two receptors and the large size of concanavalin A or induced conformational changes in the glycocalyx.

Recently, it has been demonstrated that concanavalin A inhibits the mobility of cell surface receptors (123). This effect can be reversed by addition of α-methyl-D-mannoside, a competitive inhibitor of concanavalin A binding, or colchicine and other antimitotic agents. Furthermore, antimitotic drugs have been shown to inhibit the mitogenic stimulation of lymphocytes by concanavalin A as measured by the incorporation of thymidine (124). Since antimitotic drugs are known to bind to microtubular protein, it is proposed that the microtubules might be involved in regulating the mobility and distribution

of membrane surface receptors and their interactions with cytoplasma (123,124). These results prompted us to study the effects of antimitotic drugs on insulin-stimulated glucose transport in isolated fat cells.

MATERIALS AND METHODS

A. Animal

Animals used in all experiments were male Sprague-Dawley (150-200 grams) which had free access to food and water.

B. Isolation of Fat Cells

Plastic or siliconized glass tubes and flasks were used in the isolation and incubation of fat cells.

Rats were killed by decapitation, the epididymal fat pads were removed and rinsed in 0.85% NaCl solution. Isolated fat cells were prepared essentially according to the method of Rodbell (15) using, however, a lower concentration of collagenase. Specifically, epididymal fat pads were digested with collagenase at a concentration of 1.5 mg per ml of Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, which contained 120 mM NaCl; 4.8 mM KCl; 1.3 mM $CaCl_2$; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 24.6 mM $NaHCO_3$; 3% bovine albumin (fraction V) and 0.5 mM glucose, unless otherwise indicated. Incubations were carried out for 30 to 45 min at 37°C in a metabolic shaker with a speed of 80 cycles per min. At the end of the incubation, the contents of incubation flasks were filtered though two layers of cheesecloth and then through one layer of Japanese silk without pressure. Cells were washed four times with warm KRB buffer and then suspended in the same buffer solution. Fat cells used in the transport studies were washed and suspended in the glucose-free KRB buffer.

C. Preparation of Fat Cell Ghosts

The ghosts were prepared by treating the isolated fat cells with hypotonic medium as described by Rodbell (85, 125), except that the medium contained no ATP, NAD^+ and $NADP^+$. The final ghosts were resuspended in KRB buffer.

D. Preparation and Incubation of Fat Cell Homogenates

Fat cell homogenates were prepared in 0.25 M sucrose, as described by Zinman and Hollenberg (65). Glucose oxidation by fat cell homogenates was carried out as described by Potter (126); briefly, the incubation medium contained 1 mM ATP; 0.2 mM NAD^+; 40 mM nicotinamide; 2 mM fructose-1,6-diphosphate; 25 mM $KHCO_3$; 2 mM KH_2PO_4; 4 mM $MgCl_2$; 1 μCi (1-^{14}C)glucose and 0.1 ml fat cell homogenate (10% in 0.25 M sucrose); final volume 1 ml, pH 7.4; gas phase 95% O_2-5%CO_2. The reaction mixtures were incubated at 37°C for one hour. The reaction was stopped as described in E.

E. Glucose Oxidation by Isolated Fat Cells or Fat Cell Ghosts

Glucose oxidation by isolated fat cells (or fat cell ghosts) was measured by conversion of (1-^{14}C)glucose to $^{14}CO_2$. Unless indicated otherwise in legends to figures and tables, fat cells (or fat cell ghosts) were incubated in 1 ml KRB buffer with 0.5 mM (1-^{14}C)glucose (200 μCi/mmole) at 37°C for two hours. All incubations were carried out in plastic vials capped by rubber stoppers with center wells containing a roll of filter paper. The gas phase was 95% O_2-5% CO_2. At the end of the incubation, 0.2 ml of Hyamine was

injected onto the filter paper and 0.2 ml of 5 N H_2SO_4 into the incubation medium. After the vials were shaken for 15 min at room temperature, the paper strips were transferred to 10 ml of scintillation fluid containing 42 ml of Liquifluor (New England Nuclear) per liter of toluene.

F. Assay of Hexose Transport in Isolated Fat Cells

The uptake of 2-deoxy-D-(^{3}H)glucose by isolated fat cells was measured by the oil filtration separation technique described by Gliemann, et al (127). The reaction was started by adding 2-deoxy-D-(^{3}H)glucose (20 μCi/mmole), final concentration 1 mM, to intact cells suspended in KRB buffer without glucose. All assays were carried out in the plastic tubes and incubated at 25°C for time periods indicated. The reaction was stopped by transferring 0.2 ml aliquot of cell suspension to a cellulose acetate butyrate tube containing 0.2 ml of dinonyl phthalate followed by centrifugation for 15 sec. The cells were rapidly washed by adding 0.2 ml of the same ice-cold buffer followed by immediate centrifugation. After centrifugation, the cells were transferred to a counting vial containing 1 ml of 10% sodium dodecyl sulfate. After the vials were shaken for 20 min at room temperature, radioactivity was determined in the presence of 10 ml ScintiVerse (Fisher Scientific Co.).

G. Assay of Hexose Transport in Fat Cell Ghosts

The reaction mixture usually consisted of about 150-200 μg of membrane protein in 0.1 ml glucose-free KRB

buffer. The reaction was initiated by the addition of the radioactive 2-deoxy-D-glucose, final concentration 1 mM. All assays were incubated at either 25°C or 4°C for times periods indicated. The reaction was stopped by adding 3 ml ice-cold KRB buffer, followed rapidly by filtration on E-A Millipore filters. The filters were immediately washed with three 5 ml ice-cold KRB buffer. The filters were then transferred to scintillation counting vials which contained 1 ml 10% sodium dodecyl sulfate. The vials were shaken in room temperature for 20 min. Radioactivity was determined in the presence of 10 ml ScintiVerse.

H. Efflux of 3-O-Methylglucose

Two milliliters of 20% fat cell suspension was incubated with 1 ml 3-O-(^{3}H)methyl-D-glucose (2 μCi/μmole) for 30 min at 37°C. The cell suspension was then cooled to 15°C for 10 min and centrifuged at 400 x g for 30 sec. After the centrifugation, the infranatant below the cell was carefully removed. The efflux of 3-O-methylglucose was started by adding 2 ml of KRB buffer without 3-O-methylglucose, temperature 15°C, to the cell. At various periods of time, 0.2 ml aliquots of cell suspension were removed and the cells were rapidly separated from incubation buffer by the oil centrifugation method. The radioactivity remaining in the cell pellet was determined as previously described.

I. Estimation of Fat Cells

Dry weights of isolated fat cells were determined

by filtering an aliquot of fat cell suspension through a pre-weighed Millipore filter paper (EAWP 02500, Millipore Filter Corp.). The filters were washed with 10 ml of KRB buffer without bovine albumin and dried over silica gel under reduced pressure for overnight, and weighed. The averages of three determinations were used in calculating the weights of fat cells used in each experiment.

J. Protein Determination

The protein content of fat cell ghosts was determined by the method of Lowry, et al (128), using bovine serum albumin as standard.

K. Chemicals

(1-^{14}C)Glucose, 2-deoxy-D-(^{3}H)glucose, 3-O-(^{3}H)methyl-D-glucose and Hyamine hydroxide were purchased from New England Nuclear. Bovine insulin, bovine serum albumin, bovine serum albumin (fraction V), L-glucose, 2-deoxy-D-glucose, 3-O-methyl-D-glucose, fructose-1,6-diphosphate, nicotinamide, ATP, NAD^{+} and $NADP^{+}$ were obtained from Sigma Chemical Co. Crude bacterial collagenase was from Worthington Biochemical Corp. Colchicine was purchased from Calbiochem. Dinonyl phthalate was obtained from Eastman Kodak Co. Concanavalin A was from Miles. Griseofulvin was purchased from Aldrich Chemical Co., Inc. Lumicolchicine was prepared by a method described by Wilson and Friedkin (129).

RESULTS

(I). In Isolated Fat Cells

A. Selection of Experimental Conditions

It has been noted in this laboratory that different lots of collagenase and bovine serum albumin (fraction V) have different effects on the responsiveness of isolated fat cells to insulin. Therefore, all the preparations of collagenase and albumin were screened beforehand and only those which gave more than 5-fold of maximal stimulation by insulin in the glucose oxidation assay were used.

The conversion of (1-^{14}C)glucose to $^{14}CO_2$ by isolated fat cells prepared in this laboratory proceeded at a constant rate for at least 3 hours as shown in Figure 1. In the presence of 50 μU/ml of insulin, there was a lag phase before 1/2 hour of incubation. However, maximal stimulation was reached around 2 hours. Consequently, all the later experiments on glucose oxidation were incubated for 2 hours, unless otherwise indicated.

Experiments were performed in order to determine what glucose concentration allowed the maximum insulin effect. As shown in Table I, a glucose concentration of 0.5 mM maximized the effect of insulin on glucose oxidation. Lower or higher concentrations of glucose decreased the insulin effect. These observations were in agreement with the reports of Gliemann (130) and Crofford (43).

B. Effects of Colchicine on Basal and Insulin-Stimulated

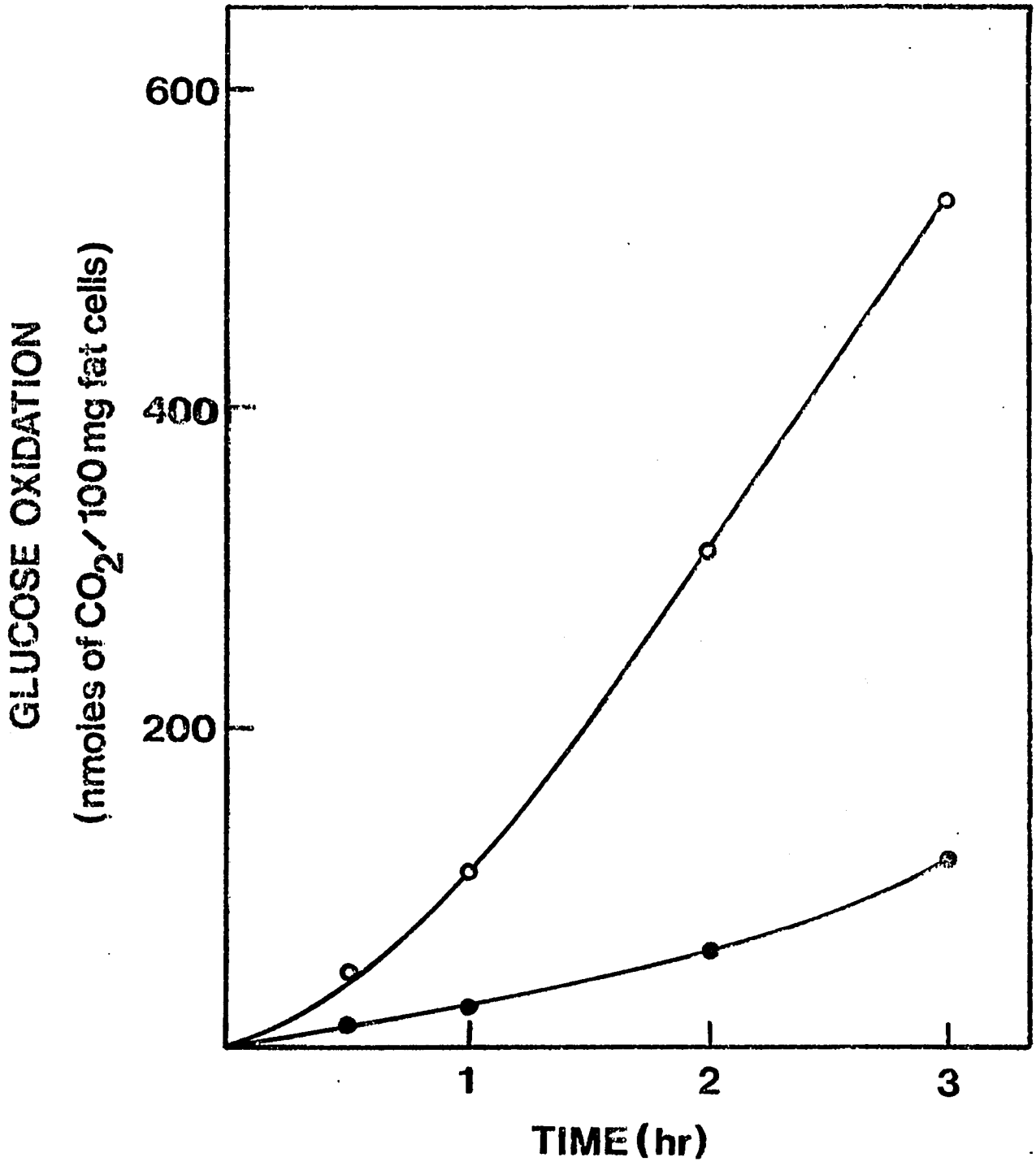


Figure 1. Time course of glucose oxidation by isolated fat cells. Fat cells (5.05 mg) were incubated with 0.5 mM (1-^{14}C)glucose (200 µCi/mmole) in the presence (○) and absence (●) of 50 µU/ml insulin at 37°C for time periods indicated.

Table I

Effect of Glucose Concentration on Insulin-Stimulated Glucose Oxidation by Isolated Fat Cells

Glucose Concentration (mM)	Insulin	Glucose Oxidation	Percent Stimulation
0.1	-	8	
	+	16	200
0.25	-	16	
	+	49	305
0.5	-	32	
	+	212	665
1.0	-	69	
	+	335	485
5.0	-	212	
	+	774	365
10.0	-	309	
	+	837	270

Fat cells (4.8 mg) were incubated with various concentrations of glucose at 37°C for 2 hours. Insulin, when present, was at 50 μU/ml. Glucose oxidation is expressed in nmoles of CO_2/100 mg fat cells/2 hr.

Rates of Glucose Oxidation

The concentration dependence for inhibition of glucose oxidation by colchicine in isolated fat cells is shown in Figure 2. In this experiment, (1-^{14}C)glucose and colchicine were added simultaneously to the cell and the reaction mixtures were incubated at 37°C for 2 hours. The concentration of colchicine which half-maximally inhibited glucose oxidation by fat cells was about 2 mM.

It has been reported that in some system colchicine requires to be in contact with the tissue for a long time to display its effect (131). Therefore, in order to see the effect of prolonged preincubation of fat cells with colchicine, experiments were performed in which fat cells were incubated with the drug for various times prior to addition of labeled glucose. As shown in Figure 3, isolated fat cells which were preincubated with 1 mM colchicine for one hour had the same activity as cells without preincubation.

The time course of the conversion of (1-^{14}C)glucose to $^{14}CO_2$ by isolated fat cells is shown in Figure 4. The production of CO_2 increased proportionately with incubation time, both in the presence and absence of insulin. Colchicine, at concentration of 2 mM, inhibited the basal rate about 55% at various incubation periods. However, the same concentration of colchicine inhibited insulin-stimulated glucose oxidation about 70%.

C. Effect of Colchicine on Glucose Oxidation by Fat Cell Homogenates

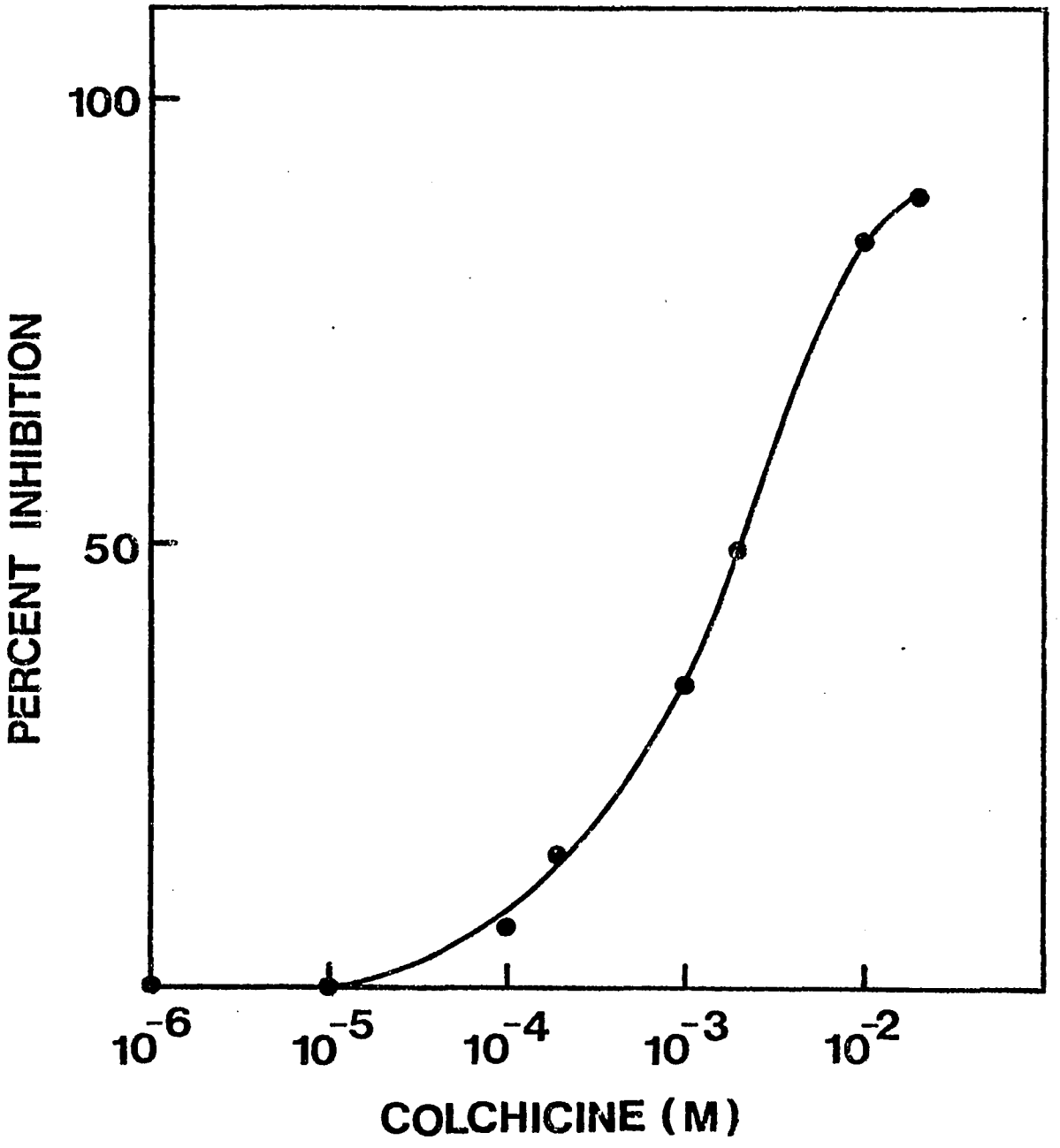


Figure 2. Effect of colchicine on glucose oxidation by isolated fat cells. Aliquots of a single pool of isolated fat cells were incubated for 2 hours at 37°C. Each incubation flask contained 4.8 mg of fat cells, 0.5 mM (1-^{14}C)glucose (200 µCi/mmole), various concentrations of colchicine, and a total volume of 1 ml. Isolated fat cells did not pre-incubate with colchicine. Control value: 35.7 nmoles/100 mg fat cells/2 hr.

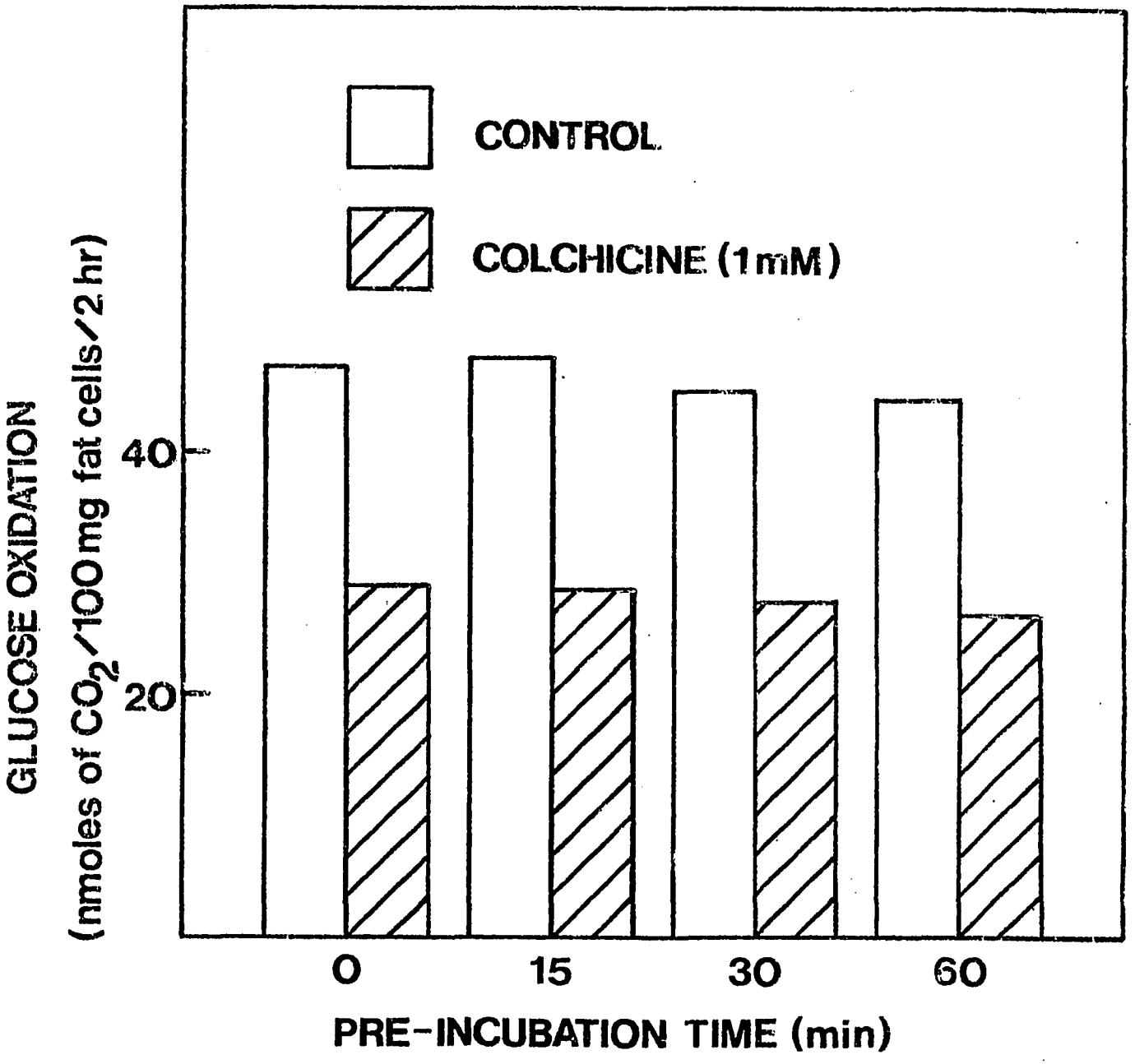


Figure 3. Effect of pre-incubation with colchicine on the glucose oxidation by isolated fat cells. Fat cells (3.5 mg) were pre-incubated with (▨) or without (□) 1 mM colchicine at 37°C in 1 ml KRB buffer for time periods indicated. Labelled glucose (0.1 µCi), final concentration 0.5 mM, was added to each flask and incubation was continued for additional 2 hr.

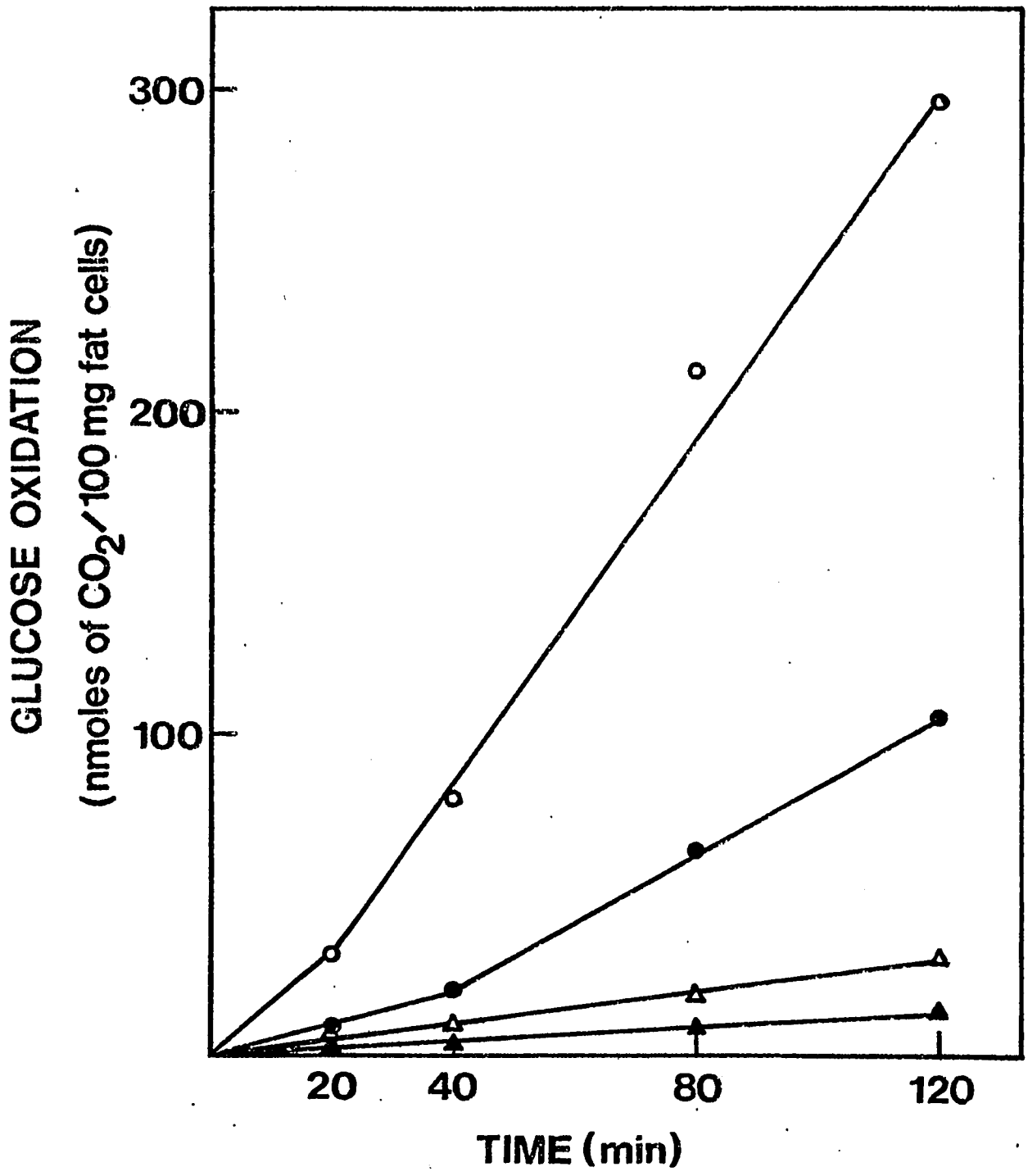


Figure 4. Time course of basal and insulin-stimulated glucose oxidation by isolated fat cells in the presence and absence of colchicine. Fat cells (4.5 mg) were incubated at 37°C in 1 ml KRB buffer containing 0.5 mM (1-^{14}C) glucose (200 µCi/mmole) for time periods indicated. Control (Δ); 2 mM colchicine (▲); 100 µU/ml insulin (O); 100 µU/ml insulin and 2 mM colchicine (●).

Fat cell homogenates were prepared as described by Zinman and Hollenberg (65). Glucose oxidation by fat cell homogenates was studied in the presence of a wide range concentrations of colchicine. As shown in Table II, colchicine at concentration of 1 mM had no effect on glucose oxidation by fat cell homogenates but significantly inhibited glucose oxidation by isolated fat cells (Figure 2). Even 10 mM colchicine caused only 15% inhibition of glucose oxidation by fat cell homogenates. This clearly indicated that the effect of colchicine on glucose oxidation by isolated fat cells was not at enzymatic levels but rather at the transport process.

D. Reversibility of Colchicine Effect

The effect of colchicine on glucose oxidation by isolated fat cells is reversible. Isolated fat cells were incubated for 30 min in the presence of 10 mM colchicine and washed thoroughly to remove the drug in the medium before measuring glucose oxidation for 15 min. The total washing procedure took no longer than 10 min. As shown in Figure 5, colchicine-treated fat cells showed 100% recovery after removing the drug and the response to insulin was as good as that of the untreated cells.

E. Effects of Glucose, Insulin and Concanavalin A on Glucose Oxidation Inhibited by Colchicine

The glucose oxidation by rat adipocytes in the presence and absence of 10 mM colchicine was monitored at

Table II

Glucose Oxidation by Fat Cell Homogenates

Colchicine (M)	Conversion of (1-^{14}C)Glucose into CO_2 (% of control)
-	100
10^{-5}	102
5×10^{-5}	102
10^{-4}	100
5×10^{-4}	100
10^{-3}	97
5×10^{-3}	94
10^{-2}	86

The incubation medium contained 1 mM ATP; 0.2 mM NAD^+; 40 mM nicotinamide; 2 mM fructose-1,6-diphosphate; 25 mM $KHCO_3$; 2 mM KH_2PO_4; 4 mM $MgCl_2$; 1 µCi(1-^{14}C)-glucose and 0.1 ml fat cell homogenate (10% in 0.25 M sucrose); final volume 1 ml, pH 7.4; gas phase 95% O_2; 5% CO_2. The samples were incubated at 37° for one hour.

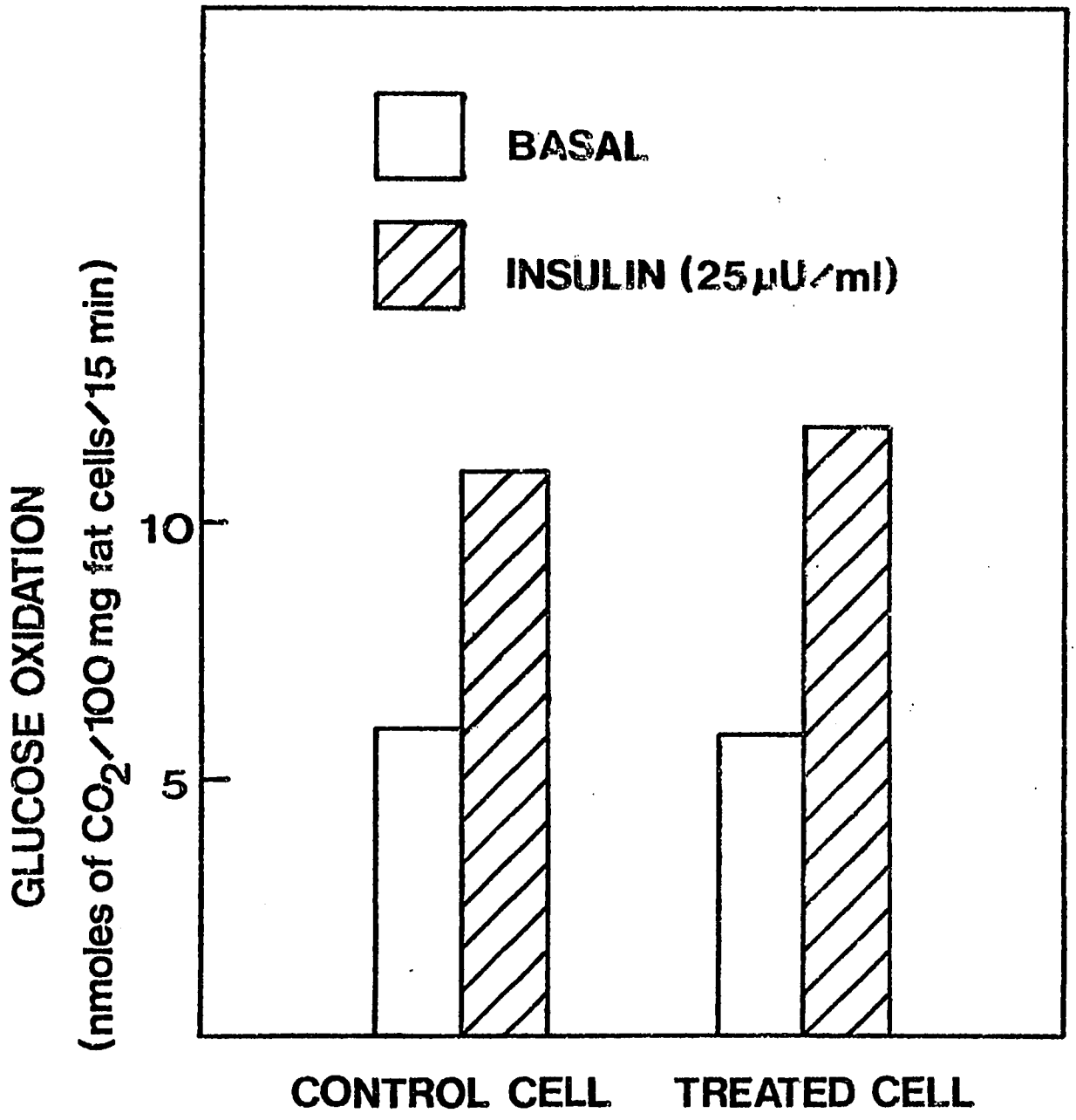


Figure 5. Reversibility of the effect of colchicine on glucose oxidation by isolated fat cells. Fat cells (6.8 mg) were incubated with or without 10 mM colchicine for 30 min at 37°C. Then the cells were washed three times with KRB buffer and resuspended in 1 ml of the fresh buffer. Labelled glucose (1 μCi), final concentration 0.5 mM, was added to each flask quickly. All the samples were incubated at 37°C for 15 min in the presence (▨) or absence (□) of 25 μU/ml insulin.

glucose concentrations between 0.1 mM and 100 mM. The rate of CO_2 formation increased with increasing glucose concentrations and reached the maximum at 50 mM glucose (Figure 6). In the presence of colchicine, the rate of CO_2 formation also increased proportionately with glucose concentrations up to 100 mM. However, the ratio of the slopes of these two curves decreased gradually from 9.14 (between 0.1 mM and 0.5 mM glucose) to 0.72 (between 50 mM and 100 mM glucose). This finding indicated that the entry of glucose into isolated fat cells by simple diffusion was not affected by colchicine. The data presented in Figure 6 also suggested that the inhibition of glucose oxidation by colchicine is of the non-competitive type.

In Figure 4, the same concentration of colchicine inhibited insulin-stimulated glucose oxidation more than the basal rate. It will be interesting to see whether colchicine not only inhibits glucose transport but also competes with insulin for insulin receptor. The effects of colchicine on glucose oxidation by isolated fat cells were studied in the presence of various concentrations of insulin. As shown in Figure 7, the rate of glucose oxidation was linear in the presence of insulin up to 100 μU/ml at a glucose concentration of 0.5 mM. The maximum response was observed with an insulin concentration of 125 μU/ml (approximately 8.3×10^{-10} M). In the presence of colchicine, even concentrations of insulin as high as 1 mU/ml (not shown in the Figure) were ineffective in overcoming the inhibition. The concentration of insulin

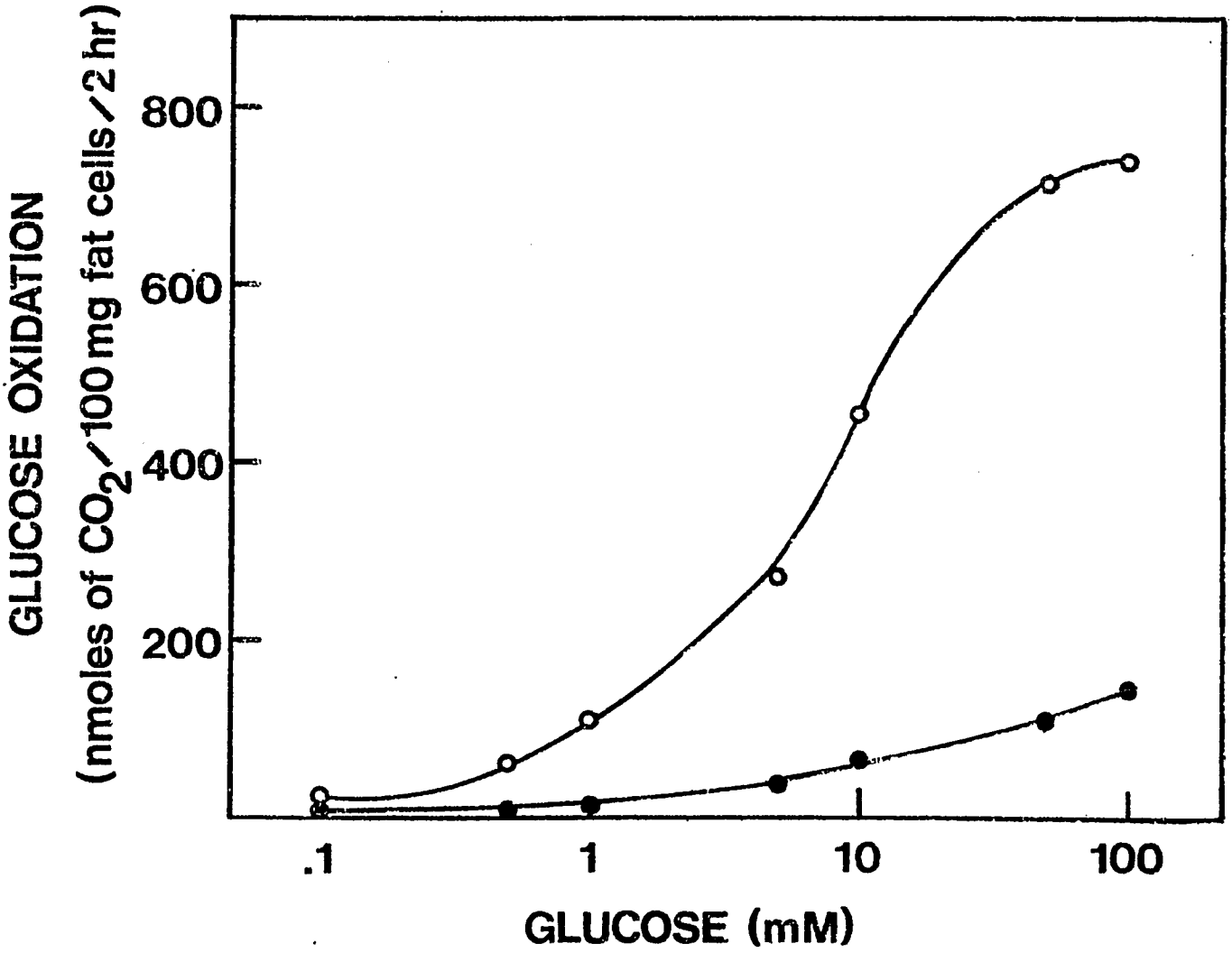


Figure 6. Effect of glucose concentration on glucose oxidation by isolated fat cells in the presence (●) and absence (○) of colchicine. Aliquots of a single pool of fat cells were incubated for 2 hours at 37°C. Each incubation flask contained 4.3 mg of fat cells and various concentration of glucose as indicated, with a total volume of 1 ml. Colchicine, when present, was 10 mM.

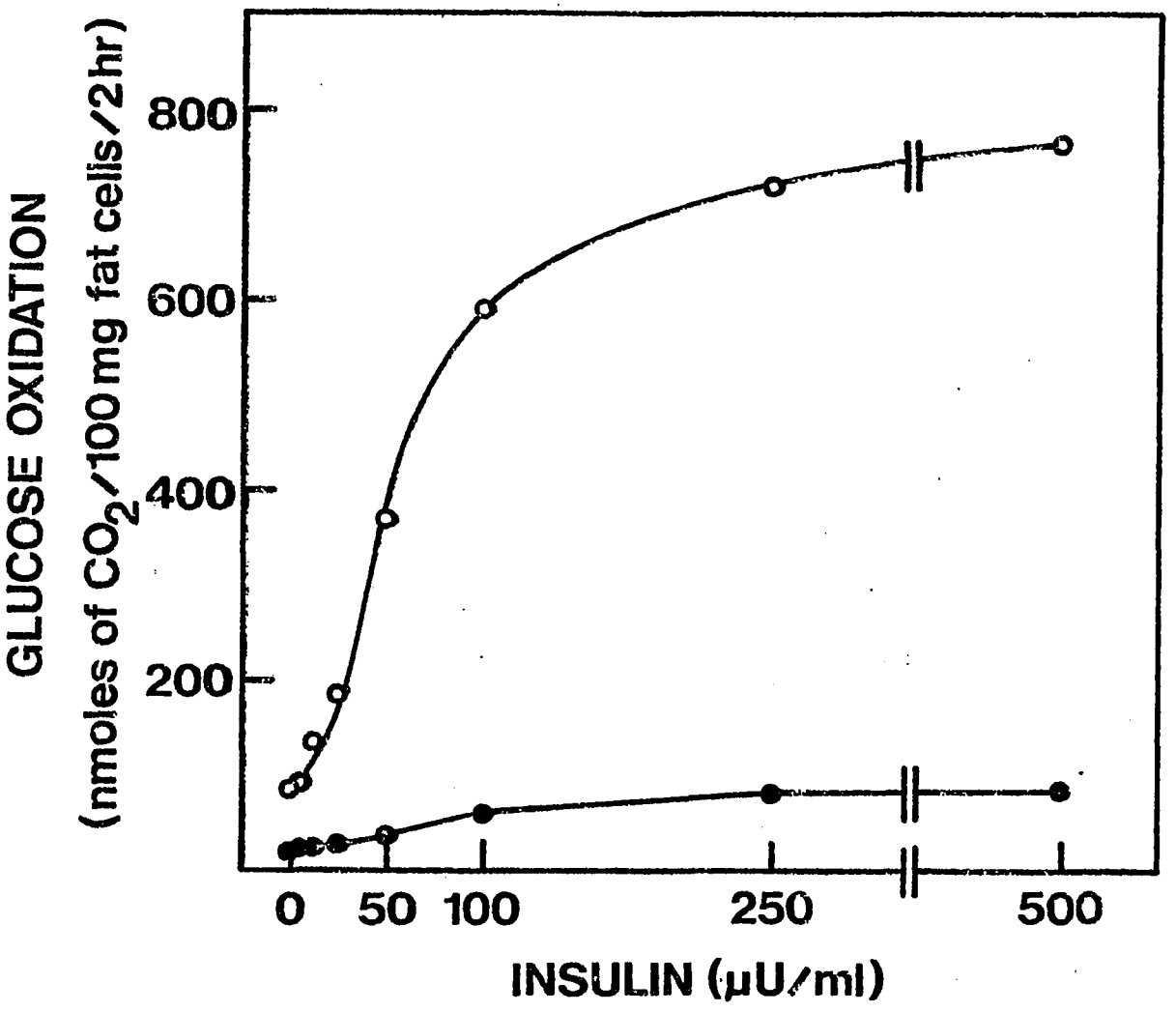


Figure 7. Effect of insulin concentration on glucose oxidation by isolated fat cells in the presence (●) and absence (○) of colchicine. Fat cells (3.9 mg) were incubated for 2 hours at 37°C in 1 ml of KRB buffer containing 0.5 mM (1-^{14}C)glucose (200 μCi/mmole) and various concentrations of insulin as indicated. Colchicine, when present, was 10 mM.

required to achieve a half-maximal response was not altered by colchicine, suggesting that colchicine does not affect the affinity of the insulin receptor for the hormone.

Although it has been shown that concanavalin A as well as other plant lectins mimic the effects of insulin on glucose oxidation and antilipolysis (33,107) and high concentrations of these plant lectins competitively displace the binding of insulin to receptor in fat cell membranes (33, 122), it is still uncertain that the insulin-like effects of these plant lectins result from direct perturbations of insulin receptors. Figure 8 shows that concanavalin A-stimulated glucose oxidation in fat cells was also inhibited by colchicine. Higher concentrations of concanavalin A failed to overcome the inhibition.

F. Effects of Colchicine on 2-Deoxy-D-glucose Transport

The results that colchicine has no effect on glucose oxidation by fat cell homogenates but significantly inhibits the glucose oxidation by intact fat cells suggest that colchicine is altering glucose oxidation by affecting glucose transport. However, more conclusive evidence was obtained by direct measurements of the uptake of the non-metabolized glucose analog, 2-deoxy-D-glucose, by isolated fat cells in the presence and absence of colchicine.

Insulin consistently stimulated the initial 2-deoxy-D-glucose influx rate in isolated fat cells, as shown in Figure 9. This enhancement of uptake by the hormone varied from 70% to 100% increase over basal levels. No

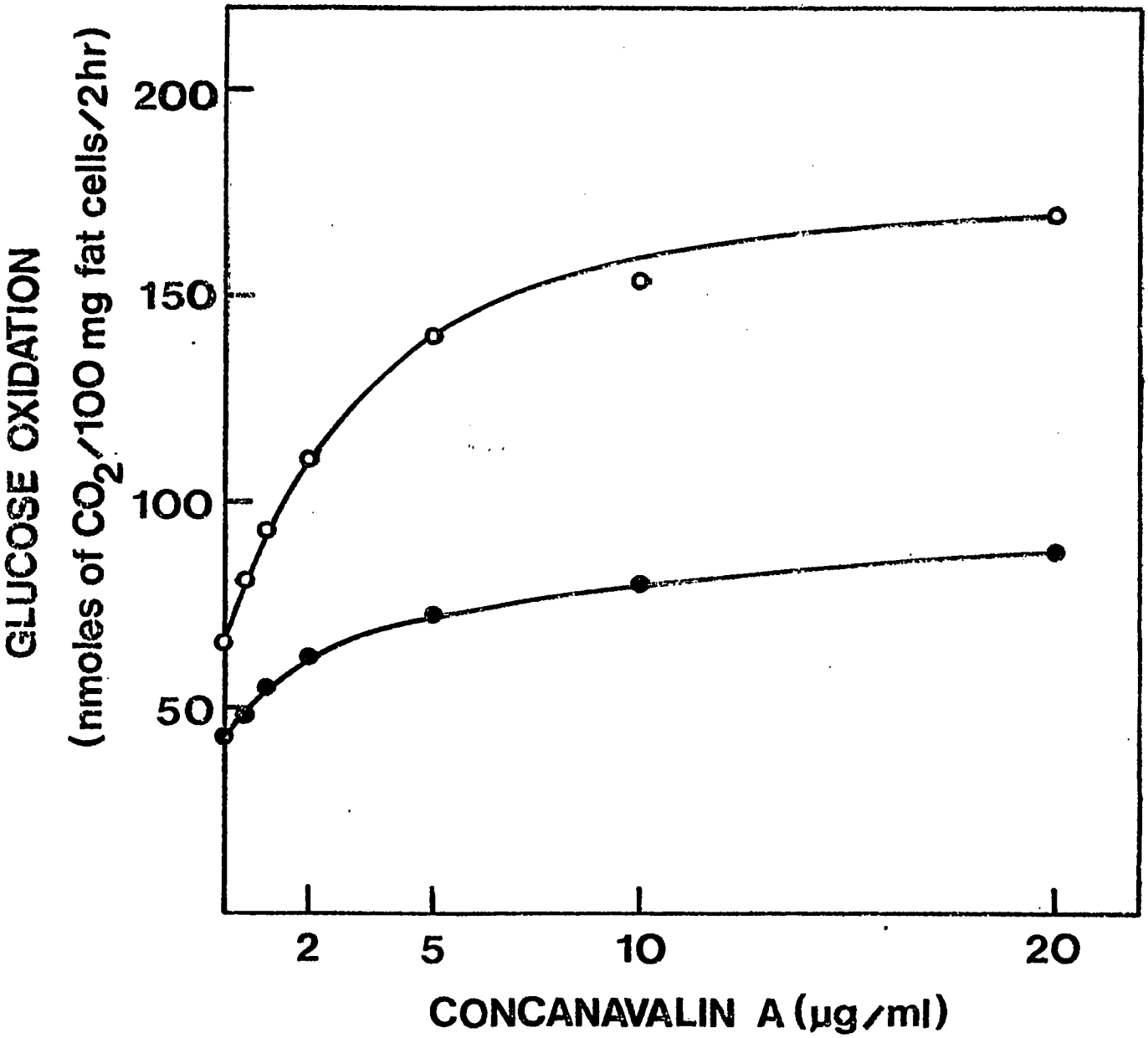


Figure 8. Effect of concanavalin A concentration on glucose oxidation by isolated fat cells in the presence (●) and absence (○) of colchicine. The assay conditions were as described in Figure 7 except that insulin was replaced by concanavalin A. Colchicine, when present, was 2 mM.

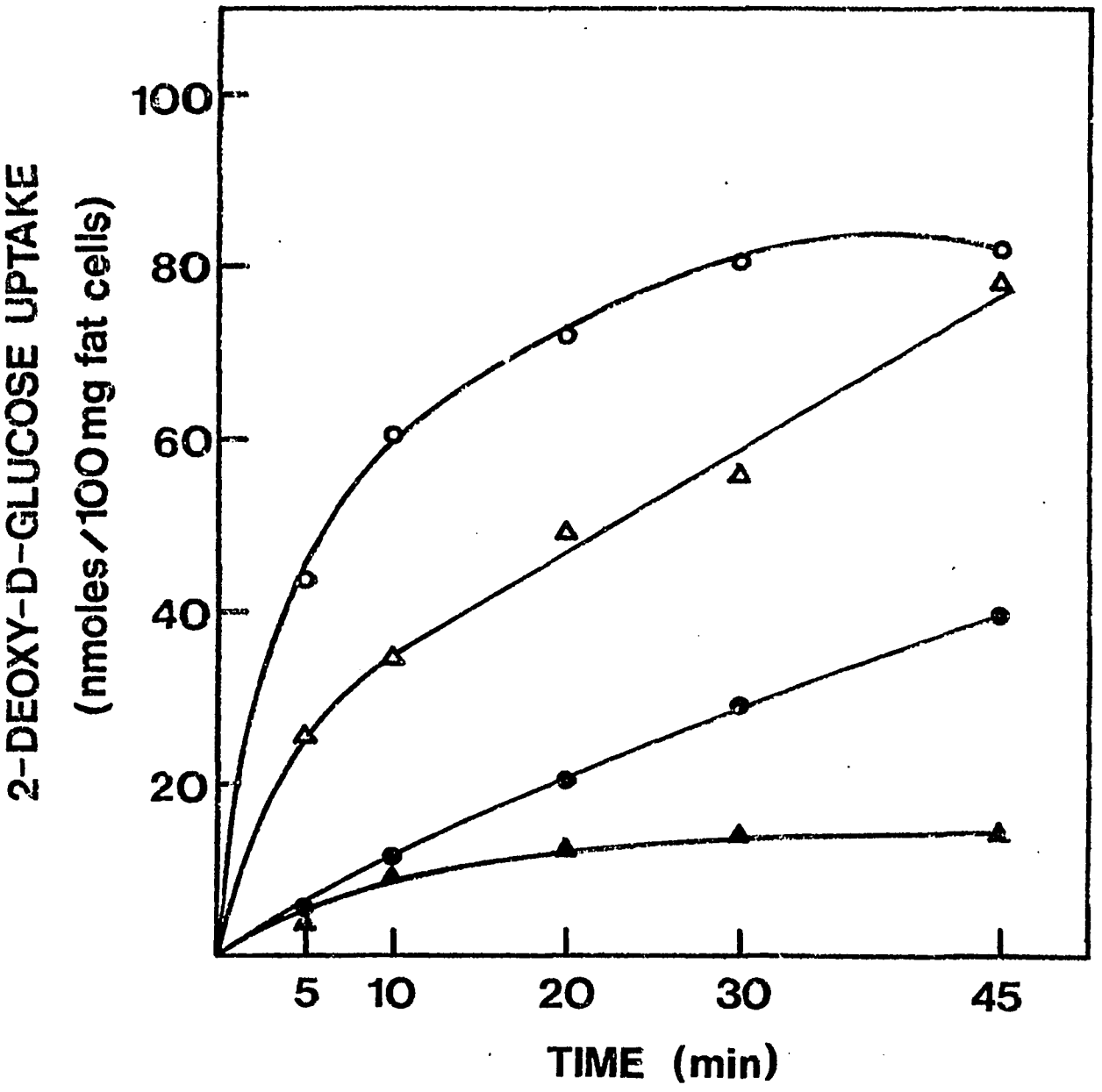


Figure 9. Effect of colchicine on basal and insulin-stimulated 2-deoxy-D-glucose transport in isolated fat cells. Isolated fat cells were pre-incubated at 25°C for 25 min in the presence (o, ●) and absence (△, ▲) of 1 mU/ml of insulin. Colchicine, 10 mM (●, ▲), was added and the uptake was initiated by the addition of 1 mM 2-deoxy-D-(^{3}H)glucose (20 μCi/umole). The reaction was stopped as described in Materials and Methods. Each point represents the average of triplicates.

significant effect of the hormone was apparent after the rate of 2-deoxy-D-glucose transport had reached equilibrium. Colchicine, at concentration of 10 mM, inhibits both basal and insulin-stimulated 2-deoxy-D-glucose uptake by about 85%. Insulin effects can be detected even in the presence of 10 mM colchicine and become apparent after long incubation. The effects of colchicine on the basal and insulin-stimulated 2-deoxy-D-glucose uptake confirm the data obtained from the glucose oxidation studies. The inhibition of glucose transport by colchicine is specific for D-glucose, since the uptake of L-glucose by isolated fat cells was unaffected by colchicine as shown in Table III. These findings suggest that the effect of colchicine on glucose transport is not due to its nonspecific interactions with plasma membrane of fat cells.

It has been demonstrated that the effects of concanavalin A on glucose oxidation by fat cells is the result of a direct stimulatory action of the lectin on the glucose transport system (32). Earlier experiments performed in this laboratory failed to show any significant stimulation of 2-deoxy-D-glucose uptake by fat cells in the presence of 5 μg/ml of concanavalin A. Similar observation has been reported by Czech, et al (32). However, even lower concentrations of concanavalin A significantly stimulate glucose oxidation by isolated fat cells (Figure 8). The reason for this discrepancy is not known. Therefore, high concentration of concanavalin A (100 μg/ml) was used in all the experiments on transport study.

Table III

Effects of Colchicine, Lumicolchicine and Griseofulvin on the Uptake of L-Glucose by Isolated Fat Cells

Additions	L-Glucose Uptake (% of control)
Colchicine (10 mM)	96
Lumicolchicine (0.1 mM)	97
Griseofulvin (0.1 mM)	92

Isolated fat cells (3.4 mg) were incubated with 1 mM (^{3}H)-L-glucose (25 µCi/µmole) at 25°C for one hour. The reaction was stopped by oil centrifugation as described in Materials and Methods. Control value: 30.4 nmoles of L-glucose/100 mg fat cells/hr.

Concanavalin A at concentration of 100 μg/ml significantly stimulated the uptake of 2-deoxy-D-glucose by fat cells as shown in Figure 10. Like insulin, concanavalin A did not modify the equilibrium distribution of the sugar. In the presence of 10 mM colchicine, the initial rate of sugar uptake was inhibited by about 80% and the equilibrium was reached at 10 min. However, the insulin-stimulated 2-deoxy-D-glucose uptake in the presence of 10 mM colchicine was relatively linear for at least 45 min (Figure 9).

G. Effect of Colchicine on the Efflux of 3-O-Methylglucose from Isolated Fat Cells

The glucose transport system in isolated fat cells is bidirectional; that means glucose can be transferred not only from outside to inside of the cell but also in the reverse direction. In order to study the efflux of sugar from isolated fat cells, the sugar must be remained unmodify inside the cell. Since 3-O-methylglucose is not a substrate for hexokinase, it can be used in this study. Table IV shows the results obtained when the efflux of 3-O-methylglucose from fat cells is measured in the presence of 10 mM colchicine. Colchicine decreased the rate of efflux as indicated by the larger amount of 3-O-methylglucose present in the treated cells during the first 5 min of the assay. These results, in together with 2-deoxy-D-glucose uptake studies, suggest that colchicine rapidly and directly alter the sugar transport system in isolated fat cells.

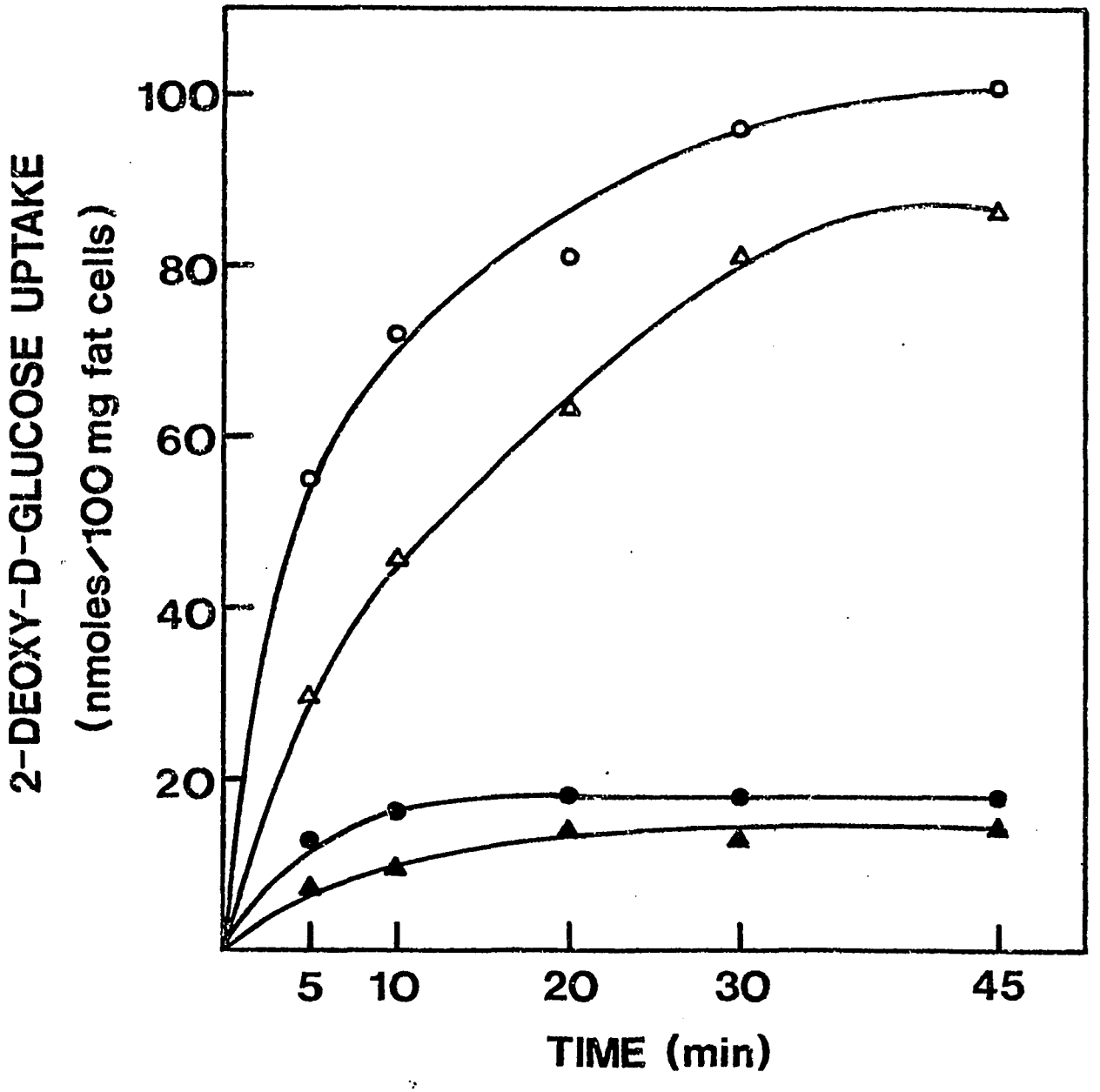


Figure 10. Effect of colchicine on concanavalin A-stimulated 2-deoxy-D-glucose transport in isolated fat cells. The assay conditions were as described in Figure 9. Control (△); 10 mM colchicine (▲); 100 µg/ml of concanavalin A (○); 10 mM colchicine and 100 µg/ml of concanavalin A (●).

Table IV

Relative Effect of Colchicine on 3-O-Methylglucose Efflux from Adipocytes

Treatment	Period of Efflux (sec)	Cell Content of 3-O-Methylglucose (% of equilibrium value)
Control	45	155
	120	140
	300	133
Colchicine (10 mM)	45	204
	120	165
	300	154

Experimental conditions were as described in Materials and Methods.

H. Effects of Lumicolchicine and Griseofulvin on Glucose Oxidation and 2-Deoxy-D-glucose Transport

Lumicolchicine, a photoisomer of colchicine which does not have antimitotic activity or bind to the microtubule subunits (132), was more effective than colchicine in inhibiting glucose oxidation as shown in Figure 11. Fifty per cent inhibition of glucose oxidation occurred with approximately 5×10^{-5} M lumicolchicine. Significant inhibition by lumicolchicine was observed at concentration as low as 1 to 5 μM. About 85% glucose oxidation activity was inhibited by 1 mM of lumicolchicine. This finding suggests that the effects of colchicine and lumicolchicine on glucose oxidation appear unrelated to the microtubule disruption.

The effects of lumicolchicine on insulin and concanavalin A-stimulated glucose oxidation by fat cells are shown in Table V. Lumicolchicine, at the concentration of 50 μM, inhibited the basal rate by 50%. Identical concentrations of lumicolchicine, but in the presence of insulin ranging from 25 μU/ml to 1 mU/ml, inhibited the insulin-stimulated glucose oxidation by about 65%. Under the same conditions, 0.1 mM of lumicolchicine inhibited both basal and insulin-stimulated glucose oxidation by 65% and 85%, respectively. In another set of experiments, 50 μM of lumicolchicine inhibited basal rate by 40%. However, its effects on concanavalin A-stimulated glucose oxidation progressively increased with increasing concanavalin A concentrations from 1 μg/ml to 100 μg/ml.

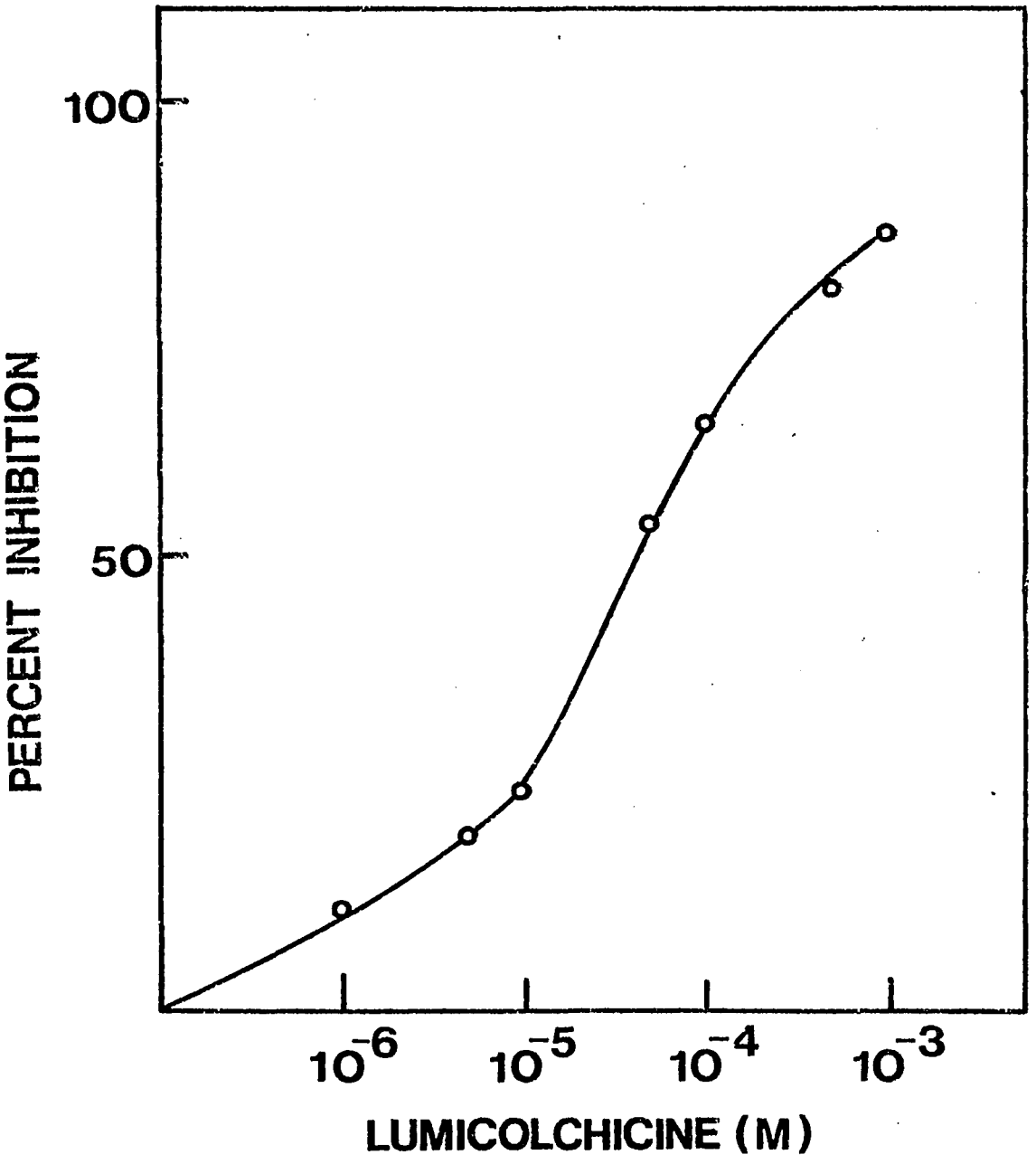


Figure 11. Effect of lumicolchicine on glucose oxidation by isolated fat cells. The assay conditions were as described in Figure 2 except that colchicine was replaced by lumicolchicine. Each incubation flask contained 3.8 mg of fat cells.

Table V

Effects of Lumicolchicine on Insulin and Concanavalin A-Stimulated Glucose Oxidation by Isolated Fat Cells

	Glucose Oxidation		
		Lumicolchicine (M)	
Additions	None	$5x10^{-5}$	10^{-4}
Insulin (μU/ml)			
None	42.8	21.4	14.7
25	236.4	70.9	39.1
100	286.6	100.9	45.5
1000	369.7	132.9	50.4
Concanavalin A (μg/ml)			
None	34.3	20.8	17.8
1	40.8	21.3	18.4
10	160.0	66.1	51.1
100	422.0	130.2	90.6

Experimental conditions were as described in Figure 2. Glucose oxidation is expressed in nmoles of CO_2/100 mg fat cells/2 hr.

Table VI shows the effects of lumicolchicine on basal as well as insulin and concanavalin A-stimulated uptake of 2-deoxy-D-glucose by fat cells. Lumicolchicine at concentration of 0.1 mM inhibited the uptake of 2-deoxy-D-glucose by 68% which is comparable to its effect on glucose oxidation. The same concentrations of lumicolchicine inhibited the insulin and concanavalin A-stimulated 2-deoxy-D-glucose uptake by about 55% and 60%, respectively.

The effects of griseofulvin, an antimitotic drug which acts differently from colchicine (133), on glucose oxidation are shown in Table VII. Since griseofulvin was dissolved in dimethylsulfoxide (DMSO), therefore, we studied the effects of this solvent on glucose oxidation by fat cells in the presence and absence of insulin. The final concentration of DMSO in the samples treated with griseofulvin was 1%. At this concentration, DMSO lowered the basal rate by about 15% but had no effect on insulin-stimulated glucose oxidation (1220% in the presence of 1% DMSO vs. 1135% in the absence of DMSO when insulin concentration was 100 μU/ml). Griseofulvin acted in a manner similar to lumicolchicine, by inhibiting insulin and concanavalin A-stimulated glucose oxidation more than the basal rate (75% vs. 50% inhibition). However, its effects on basal as well as insulin and concanavalin A-stimulated uptake of 2-deoxy-D-glucose were about the same (Table VIII).

Table VI

Effects of Lumicolchicine on the Uptake of 2-Deoxy-D-Glucose by Isolated Fat Cells

	2-Deoxy-D-Glucose Uptake (nmoles/100 mg fat cells/5 min)	
Additions	None	Lumicolchicine (0.1 mM)
None	24.6	7.9
Insulin (1 mU/ml)	50.6	23.0
Concanavalin A (100 µg/ml)	39.1	16.3

The assay conditions were as described in Figure 9. The uptake was measured for 5 min.

Table VII

Effects of Griseofulvin on Basal as well as Insulin and Concanavalin A-Stimulated Glucose Oxidation by Isolated Fat Cells

	Glucose Oxidation		
Additions	None	DMSO (1%)	Griseofulvin (0.1 mM)
Insulin (μU/ml)			
None	47.6	40.3	24.4
25		229.9	78.0
100	545.0	489.3	114.7
1000		565.4	127.9
Concanavalin A (μg/ml)			
None		118.1	50.3
10		294.4	79.5
100		593.0	126.3

Experimental conditions were as described in Figure 2. Glucose oxidation is expressed in nmoles of CO_2/100 mg fat cells/2 hr. The final concentration of dimethylsulfoxide (DMSO) in the samples treated with griseofulvin was 1%.

Table VIII

Effects of Griseofulvin on the Uptake of 2-Deoxy-D-Glucose by Isolated Fat Cells

Additions	2-Deoxy-D-Glucose Uptake (nmoles/100 mg fat cells/5 min)	
	DMSO (1%)	Griseofulvin (0.1 mM)
None	36.4	14.8
Insulin (1 mU/ml)	71.1	30.8
Concanavalin A (100 µg/ml)	51.6	23.8

Experimental conditions were as described in Figure 9 and Table VI. The final concentration of dimethylsulfoxide (DMSO) in the samples treated with griseofulvin was 1%.

(II) In Fat Cell Ghosts

A. Effects of Colchicine on Glucose Oxidation by Fat Cell Ghosts

Colchicine, at concentration of 1 mM, had no effect on glucose oxidation by fat cell homogenates (Table II), but significantly inhibited the glucose oxidation by fat cell ghosts as shown in Table IX. Fifty per cent inhibition of glucose oxidation was observed approximately at 2 mM colchicine which is similar to the value obtained from fat cell studies. The inhibition of glucose oxidation in fat cell ghosts by colchicine was also reversible. As shown in Table X, the treated ghosts were fully active after thorough washing to remove colchicine from the medium. The poor responsiveness of the ghosts to insulin, which is apparant in Table X, has also been observed by other investigators (16,85). Insulin-stimulated glucose oxidation by fat cell ghosts was inhibited by colchicine to a similar extent (72%) to the basal inhibition (75%) as shown in Figure 12.

B. Effects of Colchicine on 2-Deoxy-D-glucose Uptake by Fat Cell Ghosts

The uptake of 2-deoxy-D-glucose was studied in an attempt to determine the effect of colchicine on sugar transport in fat cell ghosts. This glucose is transported by the same carrier system as the parent molecule and is phosphorylated but is not further metabolized (29,134,135). As shown in Figure 13, both the basal and insulin-stimulated 2-deoxy-D-glucose initial uptake were inhibited by colchicine

Table IX

Effect of Colchicine on Glucose Oxidation by Fat Cell Ghosts

Colchicine (M)	Conversion of (1-^{14}C)Glucose into CO_2 (% of control)
-	100
10^{-5}	96
10^{-4}	91
10^{-3}	66
10^{-2}	28

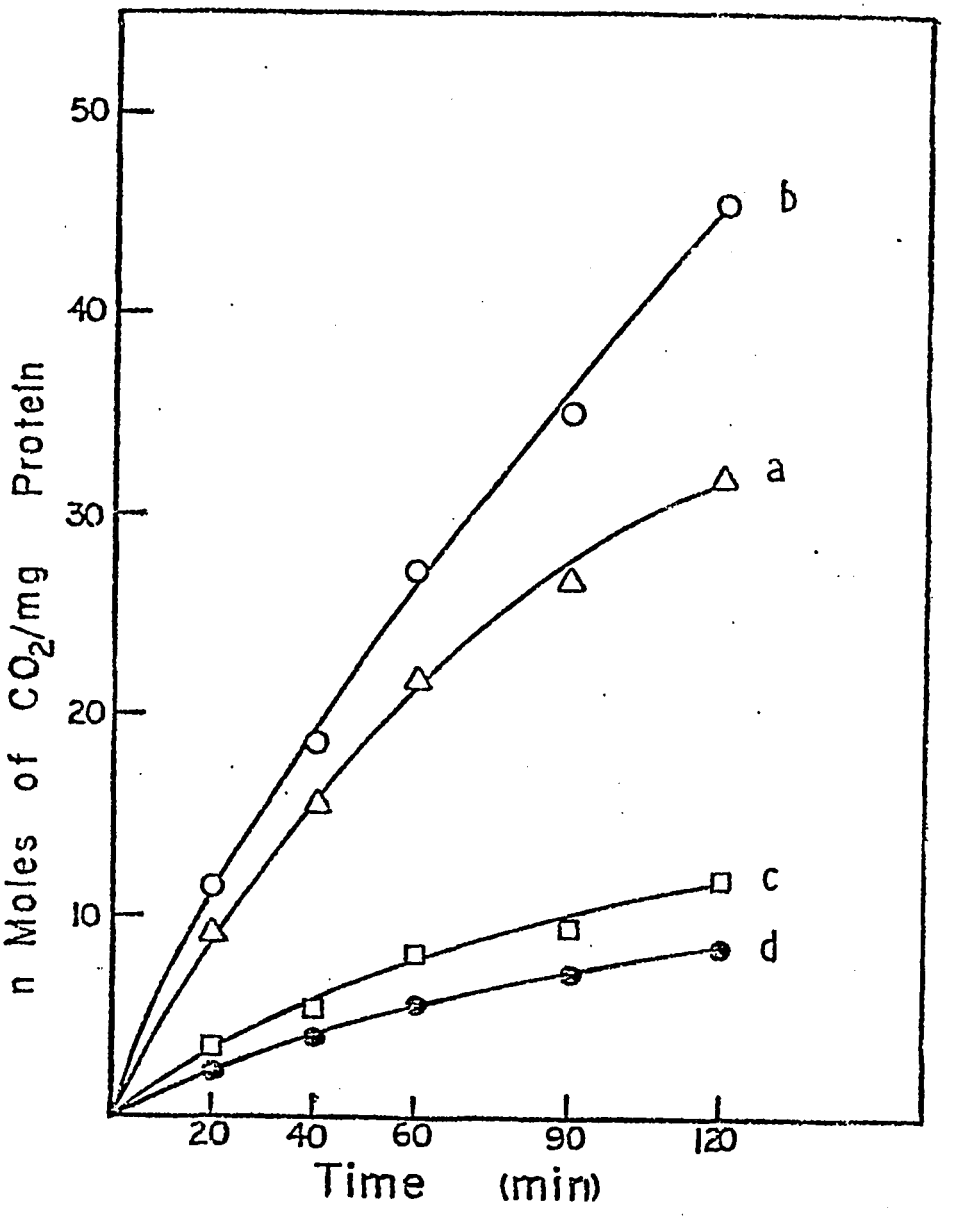
Fat cell ghosts (101 µg of protein) were incubated for 2 hours at 37°C in 1 ml KRB buffer containing 0.5 mM (1-^{14}C)glucose (0.1 µCi) and various concentrations of colchicine as indicated.

Table X

Reversibility of the Effect of Colchicine on Glucose Oxidation by Fat Cell Ghosts

Conditions	Glucose Oxidation (nmoles of CO_2/mg protein/15 min
Control ghosts	10.1
Control ghosts + Insulin (1 mU/ml)	12.0
Treated ghosts	10.5
Treated ghosts + Insulin (1 mU/ml)	12.4

Fat cell ghosts (212 µg of protein) were pre-treated with 10 mM colchicine (final volume 0.1 ml) at 37°C for 0.5 hr. Cells were isolated by centrifugation (900 x g for 15 min) and washed three times with KRB buffer . All samples were incubated at 37°C for 15 min.



Figuer 12. Time course of basal and insulin-stimulated glucose oxidation by fat cell ghosts in the presence and absence of colchicine. The incubation mixture contained 0.5 mM (1-^{14}C)glucose (0.1 µCi) and 105 µg of protein of fat cell ghosts, with a total volume of 1 ml. Basal (△); 1 mU/ml insulin (○); 10 mM colchicine (●); 1 mU/ml insulin and 10 mM colchicine. (□).

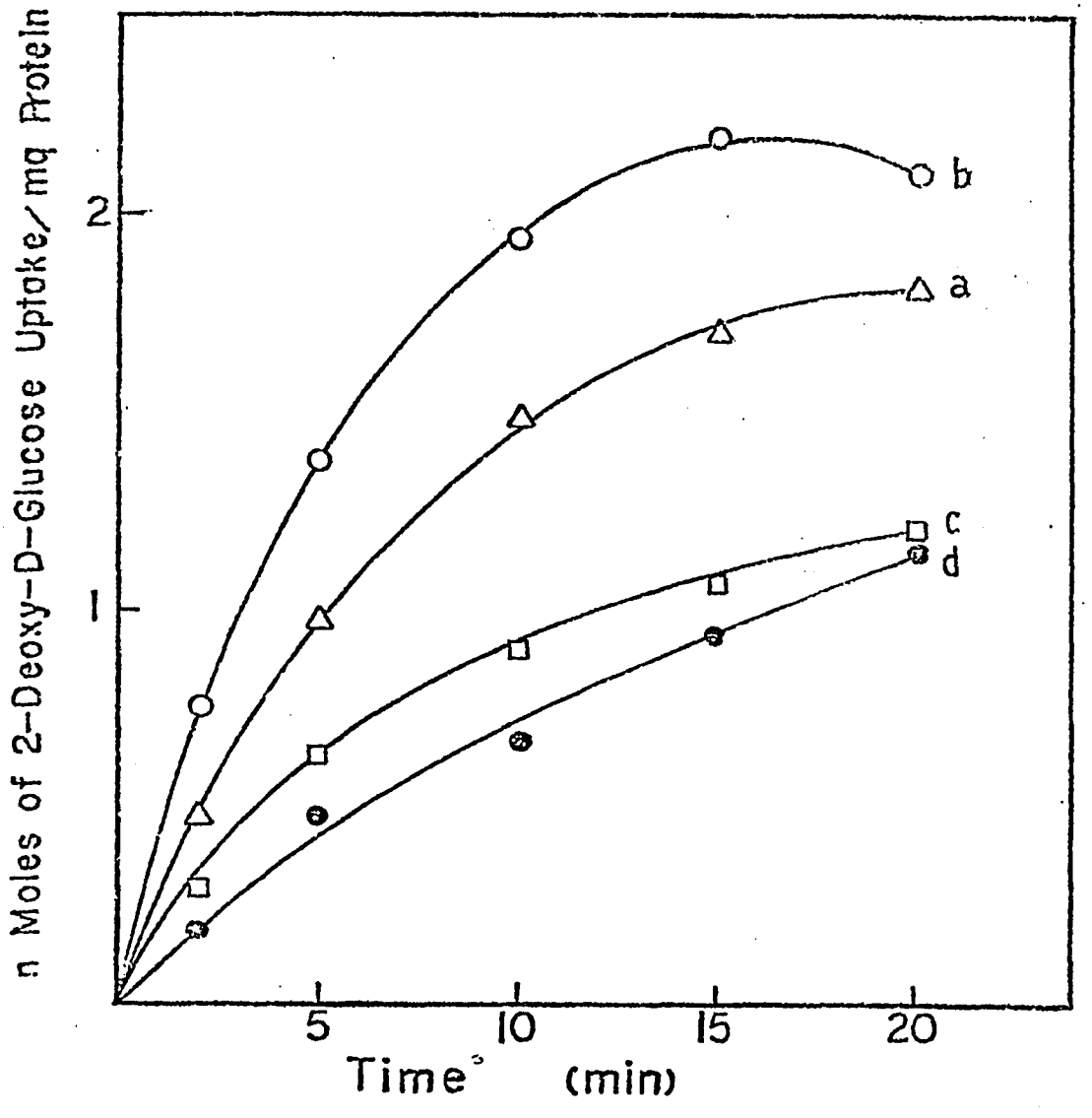


Figure 13. Effect of colchicine on basal and insulin-stimulated 2-deoxy-D-glucose transport in fat cell ghosts. Fat cell ghosts, about 150-200 µg of protein, were incubated for various times at 25°C in 0.1 ml KRB buffer containing 1 mM 2-deoxy-D-(^{3}H)glucose (2 µCi/ml), and other substances as indicated. Basal (△); 1 mU/ml insulin (○); 10 mM colchicine (●); 1 mU/ml insulin and 10 mM colchicine (□).

to the same extent. The inhibitory effect on transport occurred at the earliest time point measured (2 min). It has been shown that microtubule are disrupted by cold (136,137). Therefore, the uptake of 2-deoxy-D-glucose by fat cell ghosts was studied at 4°C in the presence and absence of colchicine in order to see whether microtubules are involved in sugar transport. Since the rate of the uptake of 2-deoxy-D-glucose by fat cell ghosts at 4°C is much slower than that at 25°C, the uptake was measured at 30 min and 60 min. As shown in Table XI, the inhibition (44% and 47%) by colchicine of 2-deoxy-D-glucose transport at 4°C is comparable to that observed at 25°C. These results suggest that the effect of colchicine on sugar transport is not due to the disruption of microtubules.

Table XI

Effect of Colchicine on the Uptake of 2-Deoxy-D-Glucose by Fat Cell Ghosts at 4°C

Incubation Time (min)	Uptake of 2-Deoxy-D-(^{3}H)Glucose by Fat Cell Ghosts (cpm)		
	Control	Colchicine (10 mM)	Percent Inhibition
30	760	420	44
60	1200	640	47

The assay conditions were as described in Figure 13 except that the incubations were performed at 4°C. The amount of fat cell ghosts used was 192 µg of protein per assay.

DISCUSSION

Low concentrations of colchicine, which are sufficient to disrupt microtubules (138), had no effect on glucose oxidation by both isolated fat cells and fat cell ghosts. Small but significant inhibition of glucose oxidation by colchicine was observed at concentration of 0.1 mM. These results are similar to those reported by Soifer, et al (118), who reported that colchicine at concentration of 5×10^{-5} M did not inhibit glucose oxidation in isolated fat cells, and by Loten & Jeanrenaud (30), who observed significant inhibition of the conversion of glucose into CO_2, total lipids and glyceride fatty acids by 0.1 mM colchicine. Fifty per cent inhibition of glucose oxidation occurred with approximately 2 mM colchicine. Experiments in which isolated fat cells were preincubated with 10 mM colchicine up to one hour showed that colchicine did not result in any greater inhibitory effect on glucose oxidation. The penetration of colchicine into isolated fat cells was not a problem, sine colchicine has been found to be taken up by fat cells and to bind to tubulin (139), a protein dimer of which the microtubule is composed.

Since colchicine inhibits glucose oxidation by isolated fat cells and fat cell ghosts but has no effect on glucose oxidation by fat cell homogenates, it seems logical to assume that it is the glucose transport process being inhibited by colchicine. Direct measurements of the uptake of 2-deoxy-D-glucose by isolated fat cells and fat cell ghosts in the presence of colchicine confirmed this assumption.

The onset of the inhibitory effect of colchicine on transport is immediate (within 2 min) not requiring pre-incubation of isolated fat cells or fat cell ghosts with the drug. The percent inhibition in sugar transport by colchicine in isolated fat cells and fat cell ghosts are comparable to its effect on glucose oxidation in respective cells. These results indicate that the action of the drug is probably confined to the plasma membrane. In agreement with such a postulate was the finding that the effect of colchicine on glucose oxidation was rapidly reversed after washing off the drug. Furthermore, the insulin-stimulated glucose oxidation in colchicine pretreated cells was as good as that in untreated cells. These results suggest that colchicine is not covalently bound to the membrane and the pretreatment does not affect either the glucose transport system or insulin receptor. The effect of colchicine on glucose transport system is specific, since the uptake of L-glucose, which gets into the cell by simple diffusion, is not affected by colchicine. In addition to its effect on the uptake of 2-deoxy-D-glucose by isolated fat cells, colchicine also inhibits the efflux of 3-O-methylglucose from fat cells. This is not unusual, since it has been shown that glucose transport in isolated fat cells is bidirectional (14) and phlorizin & phloretin, known inhibitors of glucose transport (23,24), also inhibit the rate of 3-O-methylglucose efflux from adipocytes (140) and fat cell membranes (16).

The finding that colchicine inhibits not only basal but also insulin and concanavalin A-stimulated glucose

oxidation suggests that the effect of this drug is on glucose transport rather than on the effector systems for insulin and concanavalin A. The possibility of colchicine directly competing with glucose for its carrier is ruled out by the finding that higher concentrations of glucose in the medium fail to overcome the inhibition. This result, together with the observation that glucose oxidation by fat cell homogenates is not affected by colchicine, indicates that the site of colchicine action is a step after the binding of glucose to its carrier but before the phosphorylation of glucose by hexokinase.

Lumicolchicine, which does not disrupt microtubules (132), is about 40 times more potent than colchicine in inhibiting glucose oxidation by isolated fat cells. The inhibition constant for lumicolchicine is approximately 5×10^{-5} M. Identical concentration of lumicolchicine inhibit insulin-stimulated glucose oxidation more than the basal rate. However, high concentration of insulin were also ineffective in overcoming the inhibition. The effect of lumicolchicine on concanavalin A-stimulated glucose oxidation was different from the one stimulated by insulin. Although greater inhibition was also observed in the presence of the lectin, but the percent inhibition was gradual increase with increasing concentration of the lectin. In the case of insulin, the percent inhibition remained the same regardless of the insulin concentration. Griseofulvin, an antimitotic drug which acts differently from colchicine in antimitosis and does not block

the *in vitro* polymerization of tubulin (133), was also more effective than colchicine in inhibiting glucose oxidation by isolated fat cells. Fifty per cent inhibition occurred with approximately 0.1 mM griseofulvin. Similar to lumicolchicine, griseofulvin also had greater effect on insulin and concanavalin A-stimulated glucose oxidation than the basal rate. Both lumicolchicine and griseofulvin inhibited the uptake of 2-deoxy-D-glucose by isolated fat cells in the presence or absence of insulin or concanavalin A. However, the uptake of L-glucose by isolated fat cells was not affected by either one. These results suggest that the effects of these two drugs on glucose transport system are not result of a non-specific action either.

From this study, it is concluded that the effects of antimitotic drugs on glucose transport in isolated fat cells is not due to the disruption of microtubules. The conclusion is based on the following evidences:

1. It has been indicated that the binding of colchicine to tubulin, a subunit of microtubule, at 37°C is almost irreversible (133). In this study, however, isolated fat cells which had been preincubated with 10 mM colchicine at 37°C for 30 min were still active in glucose oxidation upon removal of colchicine from the medium.

2. Cytoplasmic microtubules, which have been identified in electromicrographs of isolated fat cells (118,141), are known to be disrupted by low concentrations of colchicine (below

10^{-5} M) (138). In the present investigation, low concentrations of colchicine have no effect on glucose oxidation by isolated fat cells or fat cell ghosts. There has been reported that the inhibition of nucleotide transport in mammalian cells by colchicine appears unrelated to an action on microtubules (117). Furthermore, the concentration range of colchicine for nucleotide transport inhibition is at least one to two orders of magnitude higher than that necessary for disruption of microtubules (117,138).

3. It has been demonstrated that microtubules are also disrupted by exposure to low temperatures (136,137). Therefore, colchicine should not inhibit glucose transport at 4°C, provided that the inhibition observed at 25°C was due to the disruption of microtubules. On the contrary, the uptake of 2-deoxy-D-glucose by fat cell ghosts at 4°C is inhibited by colchicine and the percent inhibition at 4°C is comparable to that observed at 25°C. This finding suggests that the inhibition of glucose transport by colchicine at 25°C is not due to the disruption of microtubules.

4. Both lumicolchicine, which does not bind to the tubulin (132), and griseofulvin, are more effective than colchicine in inhibiting glucose transport in isolated fat cells.

It has been shown that the uptake and release of putative transmitters by synaptosomes are inhibited by colchicine at concentrations similar to that used in this study (119). Furthermore, the Mg^{2+}-ATPase activity of

synaptosomal actomyosin-like protein is also inhibited by colchicine at mM concentrations (119). Since magnesium ion has been proposed as a possible second messenger for insulin action (80) and the Mg^{2+}-ATPase activity of adipocyte plasma membrane has been shown to stimulate by both insulin and concanavalin A (67), it is possible that the effects of colchicine on glucose transport is the result of interactions between colchicine and the Mg^{2+}-ATPase of adipocyte plasma membrane.

PART II. THE BIOLOGICAL ACTIVITIES OF INSULIN ANALOGS DETERMINED ON ISOLATED FAT CELLS

INTRODUCTION

The studies of structure-activity relationships of insulin have been extensively carried out in mentor's laboratory and others'. The activity of insulin or insulin analog was determined by the conventional method, mouse convulsion assay (142). This method requires large quantity of insulin or insulin analog and a great number of mice in order to obtain accurate results. Recently, with the development of methods for preparation of biological active labeled insulin (143,144) and hormonally responsive isolated fat cells (15), direct study of insulin activities at cell-level has become possible. This *in vitro* assay has the following adventages:

1. The preparation of isolated fat cells is relative simple and the results are reliable.

2. The isolated fat cells are highly responsive to insulin, therefore, only minute quantity of the hormone or its analog is needed to perform this assay.

3. In addition to stimulating the uptake of glucose by isolated fat cells, other insulin effects can also be examined at the cell-level, such as receptor binding and antilipolysis, which are difficult to study *in vivo*.

It is not necessary that insulin or insulin analog behaves the same both *in vitro* and *in vivo*. Therefore, it is by no means that the *in vitro* assay can completely replace

the *in vivo* assay. However, the former will provide very important information about the mechanism of insulin action. In this study, the biological activities of five insulin analogs, which were synthesized in mentor's laboratory, were determined on isolated fat cells.

MATERIALS AND METHODS

A. Biological Activity of Insulin Analogs Determined by the Stimulation of Glucose Oxidation in Isolated Fat Cells

The fat cells preparation and incubation were the same as described previously. For every analog, a complete dose-response curve, with at least 6 concentrations, was carried out in addition to the dose-response curve of native insulin. The 0% and 100% stimulation represent the $^{14}CO_2$ production in the absence and in the presence of 40 ng/ml of native insulin. With 40 ng/ml of native insulin, the $^{14}CO_2$ production was 3- to 12-fold greater than that observed in the absence of insulin. The relative potency of analog was calculated as following:

$$\text{Relative potency of analog} = \frac{\text{conc. of analog (ng/ml)}}{\text{conc. of insulin (ng/ml)}} \text{ to}$$

$$\text{achieve 50\% stimulation} \times 100\%$$

B. Relative Binding Affinity of Insulin Analogs Determined by the Inhibition of the Binding of (^{125}I)Insulin to Isolated Fat Cells

The binding of (^{125}I)insulin to isolated fat cells was assayed as described by Gliemann and Gammeltoft (145). Briefly, 0.2 ml of isolated fat cells (30 µl/ml), which were suspended in KRB buffer, were incubated 45 min at 37°C with (^{125}I)insulin (5×10^{-10} M) in the presence and absence of native insulin and insulin analogs in various concentrations.

The cells were separated from the buffer solution by oil centrifugation technique as described previously. The cells were solubilized with 1 ml of 10% sodium dodecyl sulfate and the radioactivities were counted in the presence of 10 ml ScintiVerse. The results were expressed in % of inhibition. The 0% and 100% inhibition represent the radioactivities of (^{125}I)insulin associated with isolated fat cells in the absence and in the presence of 4000 ng/ml of unlabeled native insulin. The relative binding affinity of analog was calculated as following:

$$\text{Relative binding affinity} = \frac{\text{conc. of analog (ng/ml)}}{\text{conc. of insulin (ng/ml)}} \text{ to}$$

$$\text{achieve 50\% inhibition} \times 100\%$$

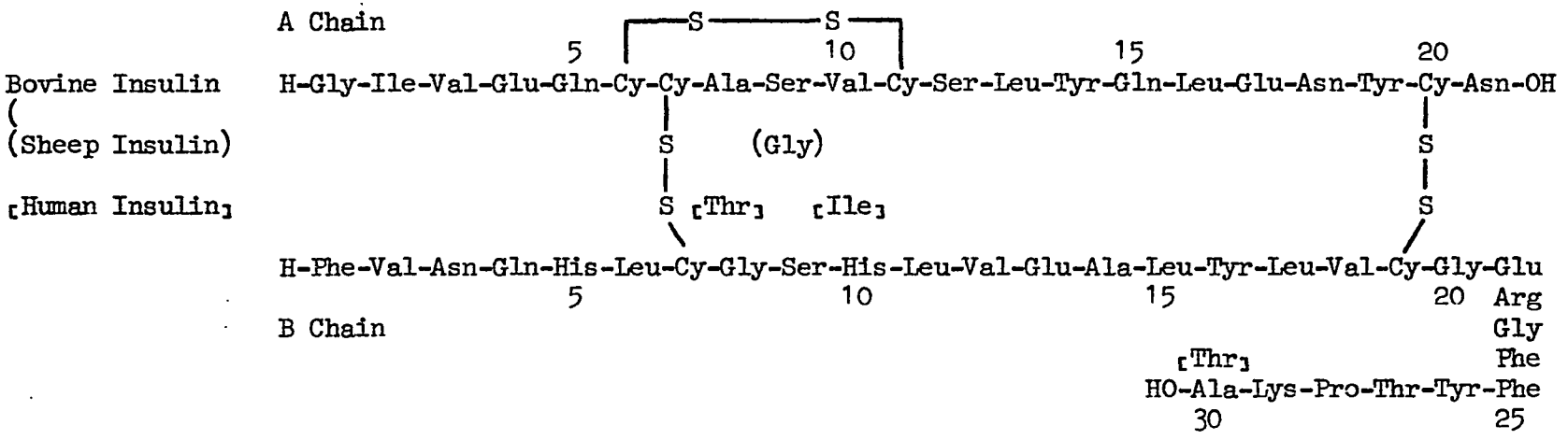
C. Materials

(^{125}I)Insulin was purchased from New England Nuclear. Insulin analogs used in this study were kindly provided by Drs. A. Cosmatos and G. P. Schwartz. Table XII gives the partial structure of these insulin analogs.

Table XII

Partial Structure of Insulin Analogs

(Primary Structure of Bovine, Sheep and Human Insulins are Shown on Top.)



Analog	Partial Structure
(A1-L-Ala) Sheep Insulin	H-Ala-Ile----------Cy-Asn-OH 1 2 20 21
(A1-D-Ala) Sheep Insulin	H-(D-Ala)-Ile----------Cy-Asn-OH 1 2 20 21
(A21-Arg) Sheep Insulin	H-Gly-Ile----------Cy-Arg-OH 1 2 20 21
(B9-Leu) Human Insulin	H-Phe-Val----------Leu----------Lys-Ala-OH 1 2 9 29 30
(B26-TyrNH$_2$) Bovine Insulin	H-Phe-Val----------Phe-TyrNH$_2$ 1 2 25 26

RESULTS AND DISCUSSION

In more than 10 experiments, the concentrations of native insulin required to achieve 50% of maximal stimulation of glucose oxidation in isolated fat cells are in the range of 0.23 ng/ml to 0.8 ng/ml. The average concentration for half-maximal stimulation is 0.42 ng/ml (or 70 pM) which is in agreement with the value reported by various investigators (43,102,146). This indicates that the fat cells prepared for this study are highly sensitive to the hormone and therefore, serve the purpose to be used in the estimation of biological activities of insulin analogs.

All the insulin analogs have the same maximal activity as native insulin in glucose oxidation and their dose-response curves appear parallel to that of insulin as shown in Figures 14A, 15A, 16A, 17A and 18A. The relative potencies of (A1-L-Ala)insulin, (A1-D-Ala)insulin, (A21-Arg) insulin, (B9-Leu)insulin and (B26-TyrNH_2)insulin in glucose oxidation are 9%, 95%, 34%, 20% and 31% of that of native insulin (Table XIII). The values shown in Table XIII are the average of at least two determinations which are in the reasonable range.

The ability of these insulin analogs to inhibit the binding of (^{125}I)insulin to isolated fat cells was studied in order to estimate their relative binding affinities. The binding curves for (^{125}I)insulin in the presence of (A1-L-Ala) insulin, (A1-D-Ala)insulin, (A21-Arg)insulin, (B9-Leu)insulin and (B26-TyrNH_2)insulin are shown in Figures 14B, 15B, 16B,

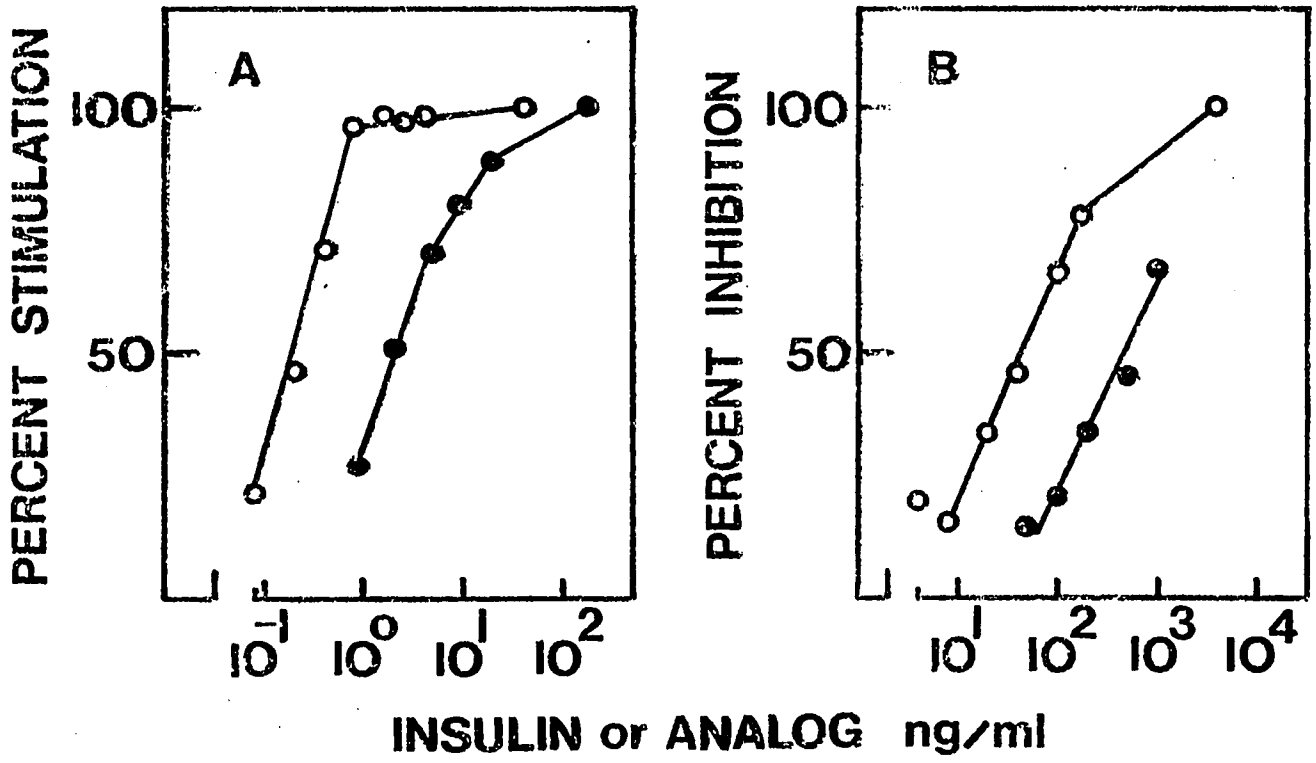


Figure 14. A. Stimulation of glucose oxidation in isolated fat cells by native insulin (○) and (A1-L-Ala)insulin (●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of duplicates.

B. Inhibition of (^{125}I)insulin binding to isolated fat cells by native insulin (○) and (A1-L-Ala) insulin (●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of triplicates.

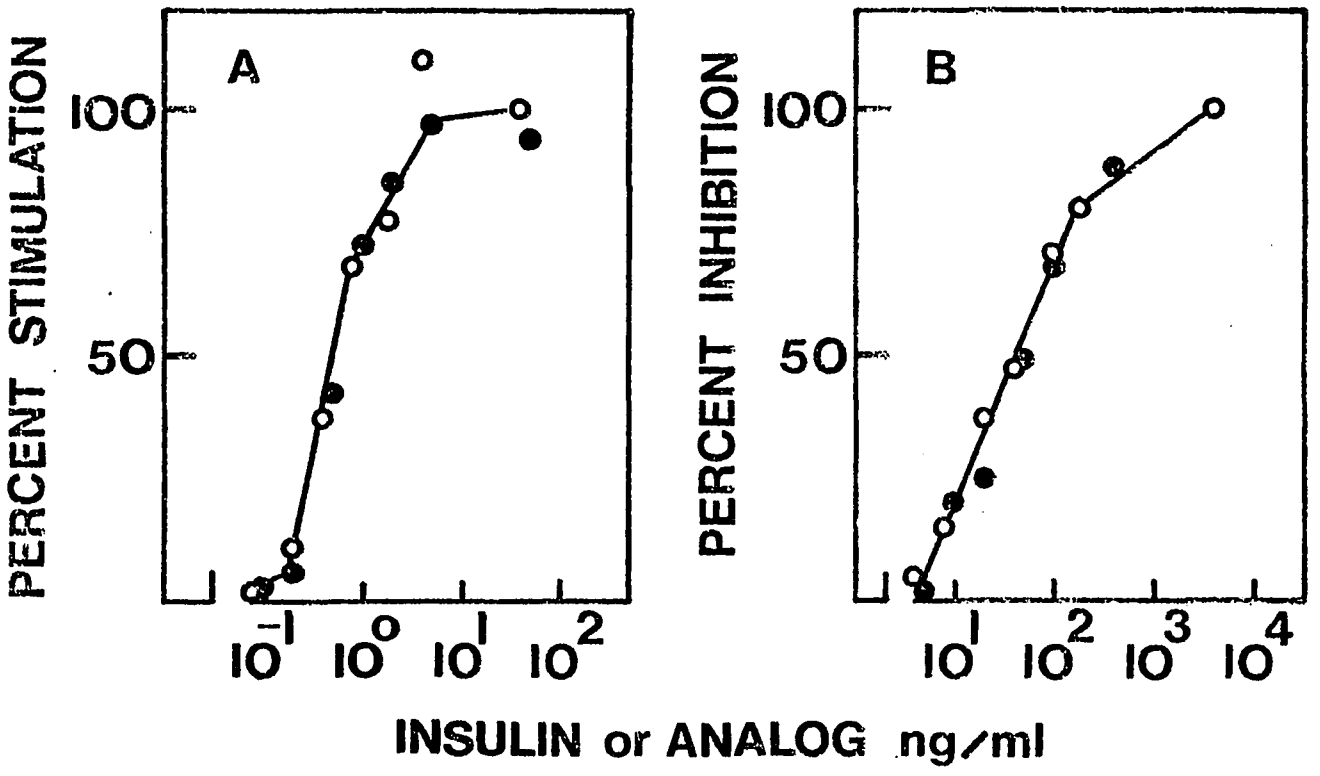


Figure 15. A. Stimulation of glucose oxidation in isolated fat cells by native insulin (o) and (A1-D-Ala)insulin (●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of duplicates.

B. Inhibition of (^{125}I)insulin binding to isolated fat cells by native insulin (o) and (A1-D-Ala)insulin(●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of triplicates.

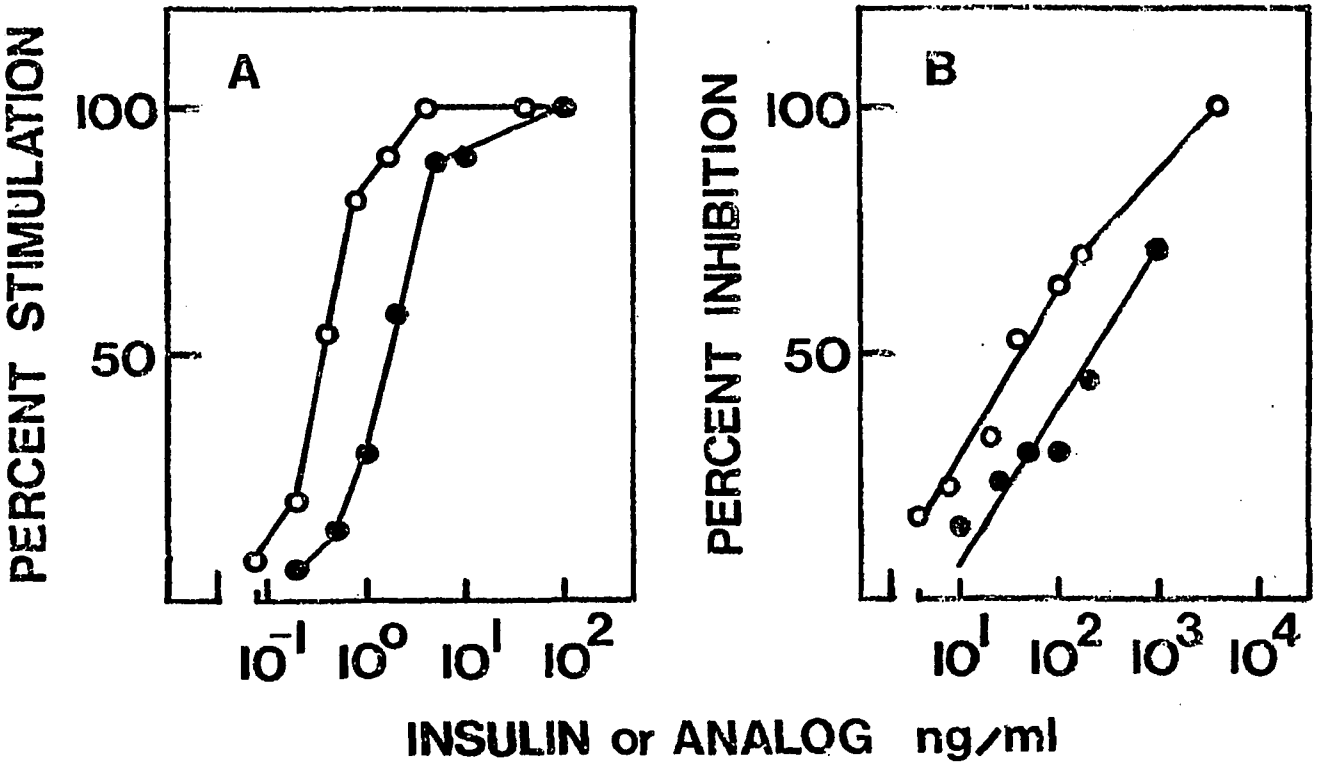


Figure 16. A. Stimulation of glucose oxidation in isolated fat cells by native insulin (o) and (A21-Arg)insulin (●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of duplicates.

B. Inhibition of (^{125}I)insulin binding to isolated fat cells by native insulin (o) and (A21-Arg)insulin(●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of triplicates.

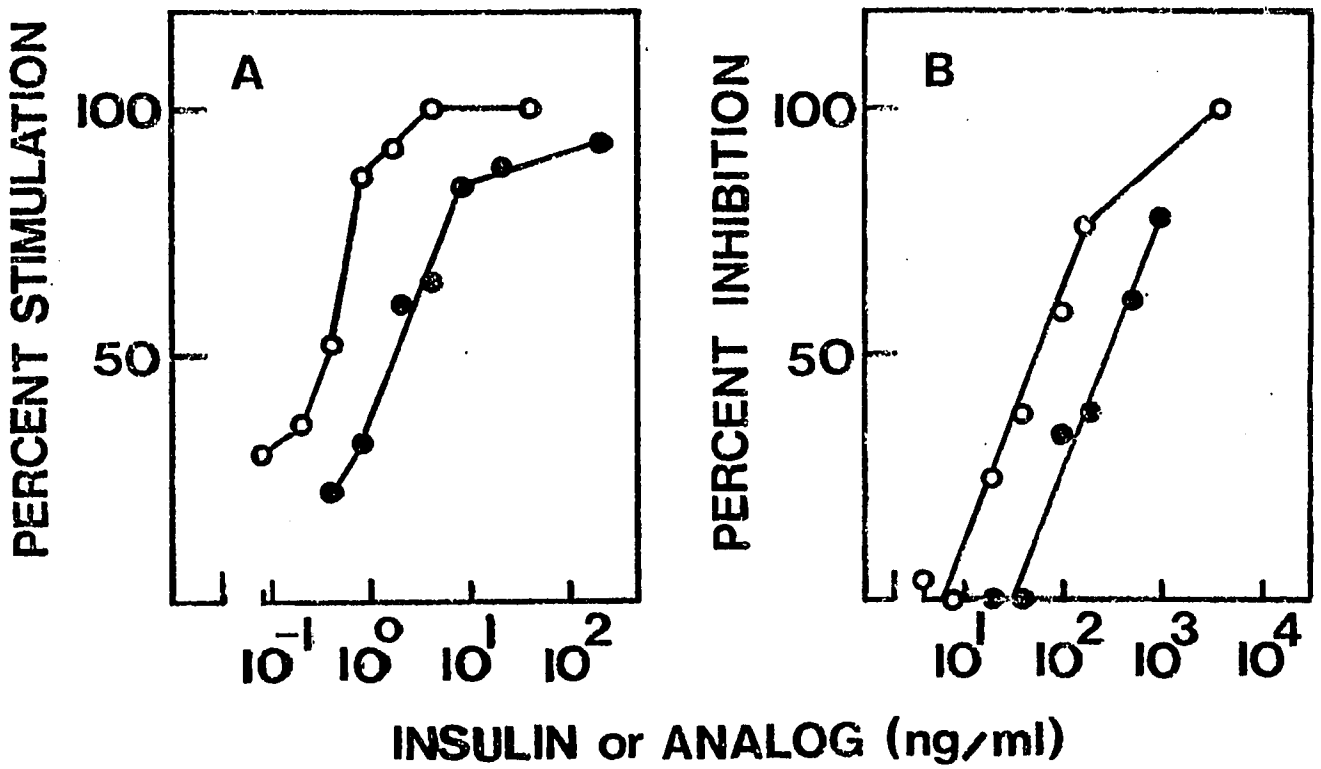


Figure 17. A. Stimulation of glucose oxidation in isolated fat cells by native insulin (○) and (B9-Leu)insulin (●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of duplicates.

B. Inhibition of (^{125}I)insulin binding to isolated fat cells by native insulin (○) and (B9-Leu)insulin (●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of triplicates.

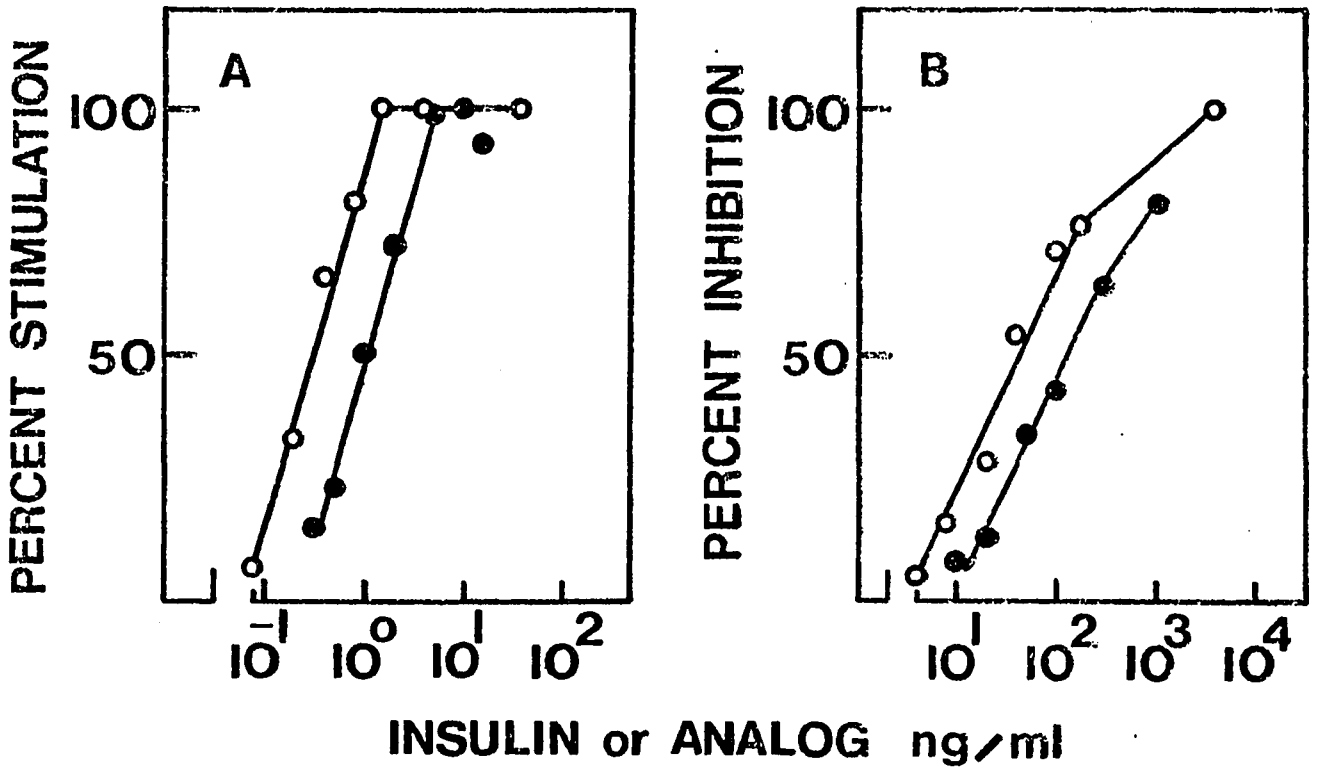


Figure 18. A. Stimulation of glucose oxidation in isolated fat cells by native insulin (○) and (B26-TyrNH_2) insulin (●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of duplicates.

B. Inhibition of (^{125}I)insulin binding to isolated fat cells by native insulin (○) and (B26-TyrNH_2) insulin (●). Experimental conditions were as described in Materials and Methods. Each point represents the mean of triplicates.

Table XIII

Relative Biological Activities of Insulin Analogs in Glucose Oxidation and Insulin-Receptor Binding in Isolated Fat Cells

	Relative Biological Activity (% of native insulin)	
Analog	Glucose Oxidation	Receptor Binding
(A1-L-Ala)insulin	9	12
(A1-D-Ala)insulin	95	100
(A21-Arg)insulin	34	33
(B9-Leu)insulin	20	27
(B26-TyrNH_2)insulin	31	28

Values were obtained from Figure 14 to Figure 18 and calculated as described in Materials and Methods. Each analog was assayed at least twice.

17B and 18B, respectively. The finding of parallel inhibition curves suggest a simple competitive inhibition. The relative binding affinities of (A1-L-Ala)insulin, (A1-D-Ala)insulin, (A21-Arg)insulin, (B9-Leu)insulin and (B26-TyrNH_2)insulin are 12%, 100%, 33%, 27% and 28% of that of native insulin, respectively (Table XIII). It is apparent that the relative binding affinities of these five analogs are in agreement with their relative biological potencies in glucose oxidation. Similar data on insulins from different species and other insulin analogs have been reported from other laboratories (147-149). These results suggest that the lower biological activities in glucose oxidation of these analogs, compared to that of native insulin, could be explained by the decrease in their binding affinities.

Recently, two analogs with A1-glycine being substituted by D-alanine and by L-alanine were made by Geiger et al from porcine insulin (150). (A1-D-Alanine)insulin has the same activity as native insulin in lowering rabbit blood glucose level and is more active than its parent molecule in stimulating the glucose uptake by rat diaphragm. However, the relative binding affinity of this analog to insulin receptor of rat liver membranes is only 38% of native insulin which is different from the value reported in this study. It is possible that there may be significant structural differences in insulin receptors from different organs of the same species. The relative binding affinity of (A1-L-Ala) insulin to insulin receptor of rat liver membranes is 5-7% of native insulin as reported by Geiger et al.

ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
Ala	Alanine
Arg	Arginine
Asn	Asparagine
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine triphosphatase
Cy	Cysteine
Cyclic AMP	Adenosine-3':5'-phosphate
Cyclic GMP	Guanosine-3':5'-phosphate
DMSO	Dimethylsulfoxide
g	Gravity
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
hr	Hour
Ile	Isoleucine
K_m	Michaelis-Menten constant
KRB buffer	Krebs-Ringer bicarbonate buffer
Leu	Leucine
Lys	Lysine
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar

mU	Milliunit
N	Normal
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
nmole	Nanomole
Phe	Phenylalanine
pM	Picomolar
Pro	Proline
RNA	Ribonucleic acid
sec	Second
Ser	Serine
S_i	Intracellular substrate concentration
S_o	Extracellular substrate concentration
Thr	Threonine
Tyr	Tyronine
µCi	Microcurie
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
µmole	Micromole
µU	Microunit
Val	Valine
V_{max}	Maximum velocity

REFERENCES

1. Jeanrenaud, B., and Renold, A. E. (1960) J. Biol. Chem. 235, 2217.
2. Goodman, H. M., and Knobil, E. (1961) Am. J. Physiol. 201, 1.
3. Robinson, J., and Newsholme, E. A. (1969) Biochem. J. 112, 449.
4. Robinson, J., and Newsholme, E. A. (1969) Biochem. J. 112, 455.
5. Windmueller, H. G., and Levy, R. I. (1967) J. Biol. Chem. 242, 2246.
6. Battaglia, F. C., and Randle, P. J. (1960) Biochem. J. 75, 408.
7. Fisher, R. B., and Lindsay, D. B. (1956) J. Physiol. (London). 131, 526.
8. Fisher, R. B., and Zachariah, P. (1961) J. Physiol. (London). 158, 73.
9. Park, C. R., Reinwein, D., Henderson, M. J., Cadenas, E., and Morgan, H. E. (1959) Am. J. Med. 26, 674.
10. Hillman, R. S., Landan, B. R., and Ashmore, J. (1959) Am. J. Physiol. 196, 1277.
11. Regen, D. M., and Morgan, H. E. (1964) Biochim. Biophys. Acta 79, 151.
12. Hernandez, A., and Sols, A. (1963) Biochem. J. 86, 166.
13. Lefevre, P. G. (1975) Curr. Top. Membranes Transp. 7, 109.
14. Crofford, O. B., and Renold, A. E. (1965) J. Biol. Chem. 240, 3237.

15. Rodbell, M. (1964) J. Biol. Chem. 239, 375.

16. Illiano, G., and Cuatrecasas, P. (1971) J. Biol. Chem. 246, 2472.

17. Carter, J. R., Jr., Avruch, J., and Martin, D. B. (1972) J. Biol. Chem. 247, 2682.

18. Lynn, W. S., Jr., Earnhardt, E., and Brown, R. (1961) J. Clin. Invest. 40, 1059.

19. Carter, J. R., Jr., and Martin, D. B. (1969) Biochim. Biophys. Acta 177, 521.

20. Kuo, J. F., Dill, I. K., and Holmlund, C. E. (1967) Biochim. Biophys. Acta 148, 683.

21. Czech, M. P., Lawrence, J. C., Jr., and Lynn, W. S. (1974) Proc. Nat. Acad. Sci. USA 71, 4173.

22. Czech, M. P. (1976) J. Biol. Chem. 251, 1164.

23. Czech, M. P., Lawrence, J. C., Jr., and Lynn, W. S. (1974) J. Biol. Chem. 249, 5421.

24. Czech, M. P., Lynn, D. G., and Lynn, W. S. (1973) J. Biol. Chem. 248, 3636.

25. Lefevre, P. G. (1961) Pharmacol. Revs. 13, 39.

26. Rickenberg, H. V., and Maio, J. J. (1961) in Membrane Transport and Metabolism, ed. A. Kleinzeller and A. Kotyk, p. 409, Czechoslovak Academy of Sciences, Prague, Academic Press, Inc., New York.

27. Jervis, E. L., Johnson, F. R., Sheff, M. F., and Smyth, D. H. (1956) J. Physiol. (London). 134, 675.

28. Mizel, S. B., and Wilson, L. (1972) J. Biol. Chem. 247, 4102.

29. Kletzien, R. F., and Perdue, J. F. (1973) J. Biol. Chem. 248, 711.

30. Loten, E. G., and Jeanrenaud, B. (1974) Biochem. J. 140, 185.

31. Czech, M. P., Lawrence, J. C., Jr., and Lynn, W. S. (1974) J. Biol. Chem. 249, 1001.

32. Czech, M. P., Lawrence, J. C., Jr., and Lynn, W. S. (1974) J. Biol. Chem. 249, 7499.

33. Cuatrecasas, P., and Tell, G. P. E. (1973) Proc. Nat. Acad. Sci. USA 70, 485.

34. Rieser, P., and Rieser, C. H. (1964) Proc. Soc. Exp. Biol. and Med. 116, 669.

35. Kuo, J. F., Holmlund, C. E., and Dill, I. K. (1966) Life Sciences 5, 2257.

36. Kuo, J. F., Holmlund, C. E., Dill, I. K., and Bohonos, N. (1966) Arch. Biochem. Biophys. 117, 269.

37. Kuo, J. F., Dill, I. K., and Holmlund, C. E. (1967) J. Biol. Chem. 242, 3659.

38. Kuo, J. F. (1968) J. Biol. Chem. 243, 211.

39. Rodbell, M. (1966) J. Biol. Chem. 241, 130.

40. Rosenthal, J. W., and Fain, J. N. (1971) J. Biol. Chem. 246, 5888.

41. Cuatrecasas, P., and Illiano, G. (1971) J. Biol. Chem. 246, 4938.

42. Stadie, W. C., Haugaard, N., Marsh, J. B., and Hills, A. G. (1949) Am. J. Med. Sci. 218, 265.

43. Crofford, O. B. (1968) J. Biol. Chem. 243, 362.

44. Kono, T. (1969) J. Biol. Chem. 244, 1772.

45. Kono, T. (1969) J. Biol. Chem. 244, 5777.

46. Cook, G. M. W. (1968) Biol. Rev. 43, 363.

47. Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, C. A., and Weber, P. (1967) Biochemistry 6, 2195.

48. Northrop, J. H. (1926) J. Gen. Physiol. 9, 497.

49. Cuatrecasas, P. (1969) Proc. Nat. Acad. Sci. USA 63, 450.

50. Turkington, R. W. (1970) Biochim. Biophys. Res. Commun. 41, 1362.

51. Oka, T., and Topper, Y. J. (1971) Proc. Nat. Acad. Sci. USA 68, 2066.

52. Blatt, L. M., and Kim, K. H. (1971) J. Biol. Chem. 246, 4895.

53. Davidson, M. B., Gerschenson, L. E., and Van Herle, A. J. (1972) Diabetes 21: Suppl. 1, 335.

54. Kolb, H. J., Renner, R., Hepp, K. D., Weiss, L., and Wieland, O. H. (1975) Proc. Nat. Acad. Sci. USA 72, 248.

55. Katzen, H. M., and Ulahakes, G. J. (1973) Science 179, 1142.

56. Jarett, L., and Smith, R. M. (1974) J. Biol. Chem. 249, 7024.

57. Jarett, L., and Smith, R. M. (1975) Proc. Nat. Acad. Sci. USA 72, 3526.

58. Cuatrecasas, P. (1971) Proc. Nat. Acad. Sci. USA 68, 1264.

59. Kono, T., and Barham, F. W. (1971) J. Biol. Chem. 246, 6210.

60. Gammeltoft, S., and Gliemann, J. (1973) Biochim. Biophys. Acta 320, 16.

61. Hepp, K. D., and Renner, R. (1972) FEBS Letters 20, 191.

62. Illiano, G., and Cuatrecasas, P. (1972) Science 175, 906.

63. Loten, E. G., and Sneyd, J. G. T. (1970) Biochem. J. 120, 187.

64. Manganiello, V., and Vaughan, M. (1973) J. Biol. Chem. 248, 7164.

65. Zinman, B., and Hollenberg, C. H. (1974) J. Biol. Chem. 249, 2182.

66. Sakai, T., Thompson, W. J., Lavis, V. R., and Williams, R. H. (1974) Arch. Biochem. Biophys. 162, 331.

67. Jarett, L., and Smith, R. M. (1974) J. Biol. Chem. 249, 5195.

68. Cuatrecasas, P. (1974) Ann. Rev. Biochem. 43, 169.

69. Butcher, R. W., Baird, C. E., and Sutherland, E. W. (1968) J. Biol. Chem. 243, 1705.

70. Cryer, P. E., Jarett, L., and Kipnis, D. M. (1969) Biochim. Biophys. Acta 177, 586.

71. Vaughan, M., and Murad, F. (1969) Biochemistry 8, 3092.

72. Vaughan, M. (1972) in Insulin Action, ed. I. B. Fritz, p. 297, Academic Press, New York.

73. Pilkis, S. J., and Park, C. R. (1974) Ann. Rev. Pharmacol. 14, 365.

74. Sneyd, J. G. T., Gorbin, and Park, C. R. (1968) in Int. Symp. Pharmacol. Horm. Polypeptides Proteins, p.367, Milan.

75. Rodbell, M., Jones, A. B., Chiappe de Cingolani, G. E., and Birnbaumer, L. (1968) Recent Progr. Horm. Res. 24, 215.

76. Illiano, G., Tell, G. P. E., Siegel, M. I., and Cuatrecasas, P. (1973) Proc. Nat. Acad. Sci. USA 70, 2443.

77. Vydelingum, N., Kissebah, A. H., and Wynn, V. (1975) Diabetologia 11, 382.

78. Fain, J. N., and Butcher, F. R. (1976) J. Cyclic Nucl. Res. 2, 71.

79. Levilliers, J., Pairault, J., Lecot, F., and Laudat, M.-H. (1976) FEBS Letters 63, 323.

80. Krahl, M. E. (1972) Diabetes 21, 695.

81. Clausen, T., Elbrink, J., and Martin, B. R. (1974) Acta Endocr. 191, 137.

82. Kissebah, A. H., Hope-Gill, H., Vydelingum, N., Tulloch, B. R., Clarke, P. V., Fraser, T. R. (1975) Lancet 1, 144.

83. Krishna, G., Moskowitz, J., Dempsey, P., and Brodie, B. B. (1970) Life Sci. 9, 1353.

84. Touabi, M., and Jeanrenaud, B. (1969) Biochim. Biophys. Acta 173, 128.

85. Rodbell, M. (1967) J. Biol. Chem. 242, 5751.

86. Letarte, J., and Renold, A. E. (1969) Biochim. Biophys. Acta 183, 350.

87. Letarte, J., Jeanrenaud, B., and Renold, A. E. (1969) Biochim. Biophys. Acta 183, 357.

88. Lostroh, A. J., and Krahl, M. E. (1973) Biochim. Biophys. Acta 291, 260.

89. Jacobs, B. O., and Krahl, M. E. (1973) Biochim. Biophys. Acta 319, 410.

90. McDonald, J. M., Bruns, D. E., and Jarett, L. (1976) Proc. Nat. Acad. Sci. USA 73, 1542.

91. Ho. R.-J., Jeanrenaud, B., and Renold, A. E. (1966) Experientia 22, 86.

92. Ho, R.-J., and Jeanrenaud, B. (1967) Biochim. Biophys. Acta 144, 61.

93. Kissebah, A. H., Tulloch, B. R., Vydelingum, N., Hope-Gill, H., Clarke, P., and Fraser, T. R. (1974) Horm. Metab. Res. 6, 357.

94. Randle, P. J., and Denton, R. M. (1973) Symp. Soc. Exp. Biol. 27, 401.

95. Wade, D. R., Chalmers, T. M., and Hales, C. N. (1970) Biochim. Biophys. Acta 218, 496.

96. Birnbaumer, L., Pohl, S. L., and Rodbell, M. (1969) J. Biol. Chem. 244, 3468.

97. Kono, T., Robinson, F. W., and Sarver, J. A. (1975) J. Biol. Chem. 250, 7826.

98. Hope-Gill, H., Kissebah, A., Clarke, P., Vydelingum, N., and Fraser, T. R. (1975) in Advances in Cyclic Nucleotide Research, ed. G. I. Drummond, P. Greengard and G. A. Robinson, vol. 5, p.805, Raven Press, New York.

99. Crofford, O. B., and Renold, A. E. (1965) J. Biol. Chem. 240, 14.

100. Baker, W. K., and Rutter, W. J. (1964) Arch. Biochem. Biophys. 105, 68.

101. Letarte, J., and Renold, A. E. (1969) Biochim. Biophys. Acta 183, 366.

102. Gliemann, J. (1967) Diabetologia 3, 382.

103. Crofford, O. B., Minemura, T., and Kono, T. (1970) in Advances in Enzyme Regulation, ed. G. Weber, vol. 8, p.219, Pergamon Press, New York.

104. Avruch, J., Carter, J. R., and Martin, D. B. (1972) Biochim. Biophys. Acta 288, 27.

105. Cuatrecasas, P. (1971) J. Biol. Chem. 246, 6522.

106. Cuatrecasas, P. (1971) J. Biol. Chem. 246, 6532.

107. Czech, M. P., and Lynn, W. S. (1973) Biochim. Biophys. Acta 297, 368.

108. Malaisse, W. J., Malaisse-Lagae, F., Walker, M. O., Lacy, P. E. (1971) Diabetes 20, 257.

109. Poisner, A. M., and Bernstein, J. (1971) J. Pharmacol. Exp. Ther. 177, 102.

110. Gillespie, E., and Lichtenstein, L. M. (1972) J. Clin. Invest. 51, 2941.

111. Gillespie, E., Levine, R. J., and Malawista, S. E. (1968) J. Pharmacol. Exp. Ther. 164, 158.

112. Williams, J. A., and Wolff, J. (1972) J. Cell Biol. 54, 157.

113. Thoa, N. B., Wooten, G. F., Axelrod, J., and Kopin, I. J. (1972) Proc. Nat. Acad. Sci. USA 69, 520.

114. Schimmel, R. J. (1974) J. Lipid Res. 15, 206.

115. Redman, C. M., Banerjee, D., Howell, K., and Palade, G. E. (1975) J. Cell Biol. 66, 42.

116. Olmsted, J. B., and Borisy, G. G. (1973) Ann. Rev. Biochem. 42, 507.

117. Mizel, S. B., and Wilson, L. (1972) Biochemistry 11, 2573.

118. Soifer, D., Braun, T., and Hechter, O. (1971) Science 172, 269.

119. Nicklas, W. J., Puszkin, S., and Berl, S. (1973) J. Neurochem. 20, 109.

120. Bernhard, W., and Avrameas, S. (1971) Exptl. Cell Res. 64, 232.

121. Oseroff, A. R., Robbins, P. W., and Burger, M. M. (1973) Ann. Rev. Biochem. 42, 647.

122. Cuatrecasas, P. (1973) J. Biol. Chem. 248, 3528.

123. Edelman, G. M., Yahara, I., and Wang, J. L. (1973) Proc. Nat. Acad. Sci. USA 70, 1442.

124. Wang, J. L., Gunther, G. R., and Edelman, G. M. (1975) J. Cell Biol. 66, 128.

125. Rodbell, M. (1967) J. Biol. Chem. 242, 5744.

126. Potter, V. R. (1972) in Manometric and Biochemical Techniques, Fifth Ed., ed. W. W. Vmbreit, R. H. Burris and J. F. Stauffer, p.177, Burgess Publishing Co., Minnesota.

127. Gliemann, J., Østerlind, K., Vinten, J., and Gammeltoft, S. (1972) Biochim. Biophys. Acta 286, 1.

128. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265.

129. Wilson, L., and Friedkin, M. (1966) Biochemistry 5, 2463.

130. Gliemann, J. (1965) Diabetes 14, 643.

131. Butcher, F. R., and Goldman, R. H. (1972) Biochem. Biophys. Res Commun. 48, 23.

132. Wilson, L., and Friedkin, M. (1967) Biochemistry 6, 3126.

133. Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M., and Creswell, K. M. (1974) Federation Proc. 33, 158.

134. Kipnis, D. M., and Cori, C. F. (1959) J. Biol. Chem. 234, 171.

135. Smith, D. E., and Gorski, J. (1968) J. Biol. Chem. 243, 4169.

136. Inoue, S. (1964) in Primitive Motile System in Cell Biology, ed. R. D. Allen and N. Kamiya, p.549, Academic Press, New York.

137. Tilney, L. G., and Porter, K. R. (1967) J. Cell Biol. 34, 327.

138. Schindler, R. (1965) J. Pharmacol. Exp. Ther. 149, 409.

139. Schimmel, R. J. (1975) Biochim. Biophys. Acta 399, 181.

140. Livingston, J. N., and Lockwood, D. H. (1975) J. Biol. Chem. 250, 8353.

141. Cushman, S. W. (1970) J. Cell Biol. 46, 326.

142. Katsoyannis, P. G., Trakatellis, A. C., Zalut, C., Johnson, S., Tometsko, A., Schwartz, G., and Gino, J. (1967) Biochemistry 6, 2656.

143. Garratt, C. J. (1964) Nature 201, 1324.

144. Izzo, J. L., Roncone, A., Izzo, M. J., and Bale, W. F. (1964) J. Biol. Chem. 239, 3749.

145. Gammeltoft, S., and Gliemann, J. (1973) Biochim. Biophys. Acta 320, 16.

146. Hepp, D., Poffenbarger, P. L., Ensinck, J. W., and Williams, R. H. (1967) Metabolism 16, 393.

147. Freychet, P., Roth, J., and Neville, D. M., Jr., (1971) Proc. Nat. Acad. Sci. USA 68, 1833.

148. Freychet, P., Brandenburg, D., and Wollmer, A. (1974) Diabetologia 10, 1.

149. Gliemann, J., and Gammeltoft, S. (1974) Diabetologia 10, 105.

150. Geiger, R., Geisen, K., Summ, H.-D., and Langer, D. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 1635.