Insulin Stimulation of Glucose Transport in Adipose Cells. An Energy-Dependent Process†

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ABSTRACT: Glucose transport can be measured in membrane vesicles prepared from isolated adipose cells of the rat. As previously reported (Martin, D. B., and Carter, J. R. (1970), *Science 167*, 873) stimulation of transport is observed if whole cells are exposed to insulin prior to disruption and isolation of membrane vesicles, but not if the hormone is added to the isolated membranes. To explain this phenomenon, we have examined the relationship between intracellular energy metabolism and the cellular response to insulin, utilizing both isolated membrane vesicles and whole cells to assess glucose transport. Inhibition of energy metabolism in the intact cell by sodium azide prior to addition of insulin blocks the response to the hormone as measured in isolated membrane vesicles. 2-Deoxyglucose, an analogue of glucose which is a substrate for hexokinase, also blocks insulin action. The glucose analogue 3-*O*-methylglucose, which is not phosphorylated by hexokinase, has no effect on the cellular response to insulin. Incubation of isolated fat cells in the presence of glucose under an atmosphere of N_2 does not inhibit insulin responsiveness, suggesting that oxidative electron flow per se is not required. To verify the significance of these findings, similar studies were done utilizing a recently described method for measuring sugar transport in intact isolated adipocytes. Insulin stimulation, which is readily demonstrated by measuring the 5-s uptake of 3-*O*-methylglucose into control and hormone-exposed cells, is blocked by both cyanide and 2,4-dinitrophenol; basal transport is unaffected. Intact adipocytes, like other tissues, show enhanced oxygen uptake in the presence of 2,4-dinitrophenol, indicating that respiratory electron flow is not sufficient for the energy transfer required for insulin effect. Binding of ^{125}I-labeled insulin to isolated adipocytes is not significantly altered by pretreatment of the cells with cyanide, 2,4-dinitrophenol, or 2-deoxyglucose. It is suggested that high-energy phosphate bonds are necessary for insulin stimulation of glucose transport, probably via phosphorylation–dephosphorylation of membrane proteins. While ATP is likely involved, there is no convincing evidence as yet that it is the proximate phosphate donor. The energy requirement appears to be for the step(s) linking hormone binding to accelerated glucose transport.

Despite decades of study, the fundamental mechanism by which insulin enhances glucose transport is unknown. While certain actions of the hormone, such as its antilipolytic effect on adipose tissue, appear related to lowered intracellular levels of cyclic AMP,[1] there is no convincing evidence relating the adenylate cyclase (or guanylate cyclase) system to glucose transport. The study of this basic action of insulin has been hampered by two major problems. First, most of the assays used to measure glucose "transport" in fact measure some aspect of intracellular glucose metabolism, such as CO_2 or lipid production from labeled glucose, and thus are many steps removed from the site of insulin action at the plasma membrane. Second, a wide variety of presumably nonspecific perturbations of plasma membrane structure, such as those induced by proteolytic (Kuo et al., 1967; Kono and Barham, 1971) or lipolytic (Rodbell, 1966) enzymes, sulfhydryl reagents (Carter and Martin, 1969a), etc., have "insulin-like" effects on glucose transport; the relation of these chemically induced changes to the hormonal action is obscure.

The importance of studying insulin action directly at the level of sugar transport has recently been emphasized (Livingston and Lockwood, 1974; Czech, 1975, 1976); large rat adipocytes "resistant" to the action of insulin by the usual assay methods (conversion of [^{14}C]glucose to $^{14}CO_2$) in fact showed a normal response to the hormone when transport was measured directly (3-*O*-methylglucose uptake).

We have previously described (Carter and Martin, 1969b) a method for assessing glucose transport in membrane vesicles prepared from isolated fat cells of the rat and shown that the bulk of the transport activity resides in vesicles of plasma membrane (Carter et al., 1972). Such vesicles, when prepared from insulin-stimulated cells, show enhanced glucose uptake comparable to that seen in whole cells (Martin and Carter, 1970), indicating that the insulin-induced change in the glucose transport system is stable to cell disruption and membrane isolation procedures. However, disruption of the cells prior to exposure to the hormone abolished the response.

To assess the reasons for the unresponsiveness of broken cell preparations, we have studied the relation of intracellular metabolism in the isolated adipocyte to the cell's response to insulin. The recent development (Gliemann et al., 1972) of a method permitting the direct measurement of sugar transport in whole fat cells has enabled us to study this both in isolated membrane vesicles and in whole cells. The experiments reported below indicate that intact energy metabolism, presumably involving high-energy phosphate bonds, is required for insulin stimulation of glucose transport. In a recent preliminary report, Kono et al. (1976) have arrived at a similar conclusion.

Materials and Methods

^{14}C- and ^{3}H-labeled sugars were obtained from New England Nuclear Corp. Crude bacterial collagenase was obtained

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[1] Abbreviations used are: cyclic AMP, adenosine 3′,-5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate.

from Worthington Biochemicals and albumin (fraction V) from Sigma. All collagenase and albumin preparations had to be tested for their ability to produce isolated adipocytes responsive to insulin, since many such preparations available commercially produce insulin-unresponsive cells. Firefly lanterns for ATP assay were obtained from Sigma. Insulin was kindly provided by the Eli Lilly Co. All other chemicals were reagent grade.

Glucose Uptake by Membrane Vesicles. Isolated fat cells were prepared from rat epididymal pads (Rodbell, 1964) in Krebs–Ringer bicarbonate buffer (Cohen, 1951) using 0.5–1.0 mg of collagenase per mL and 2.5–4.0% bovine albumin. For studies of glucose transport in membrane vesicles, the methods reported previously (Carter and Martin, 1969b) were used. Briefly, these can be summarized as follows: after incubation with the appropriate inhibitor (where indicated) at 37 °C for 30 min in a Dubnoff shaker, one-half of the control and inhibitor-treated cells were exposed to insulin at 50 or 100 μU per mL for 15 min. The cells were then homogenized without further washing in a Potter-Elvejhem homogenizer and the "microsomal" fraction isolated by differential centrifugation. The isolated membranes were resuspended in a small volume of Krebs–Ringer phosphate buffer and incubated briefly (30–60 s) with L-[^{14}C]glucose and D-[^{3}H]glucose at a final concentration of 5 mM each. The reaction was stopped by rapid filtration and washing of the membranes on Millipore filters. The filters were then counted in a liquid scintillation counter. By using L-glucose to correct for nonspecific trapping of sugar, this method allows for a measure of the rate of transport of D-glucose into the intravesicular space (Carter and Martin, 1969b). Membranes prepared from insulin-treated cells consistently show enhanced rates of glucose uptake but no change in the equilibrium glucose "space" (Martin and Carter, 1970). However, even using very short time points (5 or 10 s), initial rates cannot be determined and hence exact kinetic parameters of transport are not determined.

Sugar Uptake by Whole Cells. We have used a modification of the method of Gliemann et al. (1972) similar to that of Livingston and Lockwood (1974) but adapted for very short time points. Isolated fat cells were prepared and preincubated with or without inhibitors as above, followed by exposure of one-half the cells to insulin at 100 μU/mL. For uptakes of 15 s or longer, cells suspended in Krebs–Ringer phosphate buffer containing 2% albumin were incubated at 24 °C with shaking with equimolar concentrations of [^{14}C]sucrose and 3-*O*-[^{3}H]methylglucose. Aliquots (0.2 ml) of the incubation mixture were rapidly layered over two drops of castor oil in a 0.45-ml conical plastic tube and centrifuged at 10 000 rpm for 30 s in a Beckman microcentrifuge. The transfer of cells to the microtube requires about 5 s, and the end of the incubation was taken as the time the centrifuge was started, since cells are separated from the medium within 2 s (Gliemann et al., 1972). The tubes were then sliced through the castor oil layer separating the incubation medium (bottom) from the fat cells (top); the upper half of the tube containing the fat cells was placed in a liquid scintillation vial and mixed briefly on a vortex mixer with 0.2 ml of Hyamine hydroxide, and radioactivity determined in a toluene-based scintillation mixture. With this technique, sucrose serves as a marker for extracellular medium trapped with the fat cells. Intracellular uptake of 3-*O*-methylglucose was determined by subtracting the amount of sucrose from the 3-*O*-methylglucose present; normally, trapped extracellular 3-*O*-methylglucose represented about one-half of the total 3-*O*-methylglucose present in the adipocytes, comparable to figures reported by Gliemann et al. (1972).

For 5- and 10-s time points a different technique was used. The fat cell suspension (0.2 ml) was layered over two drops of castor oil in the 0.45-ml microfuge tube; the labeled sugars in a total volume of 10 μL were added in a single drop to the inside of the tube, being careful that there was no contact with the fat cell suspension. The tube was capped and the incubation begun by vigorously mixing the contents of the tube by hand. Termination of the incubation and subsequent handling were done as above. All assays were done in triplicate or quadruplicate.

Measurement of ATP. To determine the effect of various inhibitors on cellular levels of ATP, aliquots of control and inhibitor-treated cells were extracted with glycine buffer pH 11.0 and ATP levels determined by a luciferase assay (Bihler and Jeanrenaud, 1970). Appropriate blanks containing the inhibitor were also assayed, as these often gave higher values than plain buffer. All assays were performed in triplicate. We noted wide variation in the preincubation level of ATP between different preparations of fat cells. Fat cells were counted in a hemocytometer and ATP levels expressed per 10^5 cells. In a given experiment the fat cells from several rats were pooled and divided into equal aliquots. Protein was measured by the method of Lowry et al. (1951) using crystalline bovine albumin as standard.

Binding of ^{125}I-Labeled Insulin to Adipocytes. ^{125}I-Labeled insulin was prepared essentially according to the method of Cuatrecasas (1971) except that labeled hormone was separated from unreacted $^{125}I^-$ by initial passage over a Sephadex G-25 column rather than by adsorption to talc. The labeled insulin was further purified by passage over a Sephadex G-50 column and the fractions containing the major peak of radioactivity pooled. The ^{125}I-labeled insulin so prepared was >97% precipitable by 7% Cl_3CCOOH and >97% adsorbed to talc. It cochromatographed as a single peak with unlabeled insulin on Sephadex G-50. Biological activity, assessed by the ability of the labeled hormone to stimulate $^{14}CO_2$ production from D-[U-^{14}C]glucose in isolated fat cells, was identical with unlabeled hormone over the range of physiological concentrations (10–100 μU/mL).

To measure binding, isolated fat cells ($\sim 5 \times 10^5$ cells/mL) suspended in Krebs–Ringer bicarbonate buffer with 2% bovine albumin were incubated with shaking at 24 °C for 30 min in the presence of increasing concentrations of ^{125}I-labeled insulin. Aliquots (0.2 ml) of cell suspension were transferred to microfuge tubes containing 2 drops of castor oil and cells separated from medium as described above. Radioactivity bound to the cells was determined by slicing the tubes and counting the upper portion in a γ counter. "Nonspecific" binding was corrected for by carrying out identical incubations in the presence of excess (50 μg/mL) unlabeled hormone.

Results

Studies with Membrane Vesicles. The first indication that metabolic energy might be directly related to insulin stimulation of glucose transport came from studies with azide poisoned cells. When intact adipocytes were incubated with 10 mM NaN_3 for 30 min prior to exposure to insulin at physiological concentrations, the membrane vesicles prepared from these cells failed to show the characteristic stimulation of glucose transport (Table I, experiment 1). Basal, i.e. non-insulin-stimulated, rates of transport in the inhibitor-treated vesicles were the same as or slightly greater than in control vesicles but did not approach the levels observed in vesicles prepared from control cells exposed to insulin. In nine of ten similar experiments done with different batches of fat cells,

TABLE I: Effect of Sodium Azide and Anaerobiasis on Insulin Stimulation of Glucose Uptake Measured in Microsomal Vesicles.[a]

		Net D-Glucose Uptake at 1 min (nmol/mg of Protein)	
	Inhibitor	Control	Insulin
Expt. 1	None	1.6	6.1
	10 mM NaN_3	2.1	2.2
Expt. 2	95% O_2/5% CO_2	2.7	6.8
	95% N_2/5% CO_2	1.4	5.4

[a] In experiment 1 whole adipocytes were preincubated for 30 min at 37 °C in Krebs-Ringer phosphate buffer with or without NaN_3 and then one-half of each set of cells was exposed to insulin (50 μU/mL) for 15 min more. Disruption of cells, isolation of microsomes, and measurement of glucose uptake were done as described under Materials and Methods. In experiment 2, whole adipocytes were preincubated in Krebs-Ringer phosphate buffer containing 1 mM D-glucose under the indicated gas phase; addition of insulin and subsequent steps were as in experiment 1. Only the difference between D- and L-glucose uptake (net D-glucose) is shown. Results are the means of duplicate determinations.

TABLE II: Effect of Preincubation of Fat Cells with Glucose Analogues on Insulin Stimulation of Glucose Uptake.[a]

	Net D-Glucose Uptake at 1 min (nmol/mg of Protein)	
Preincubation	Control	Insulin
Control	2.88 ± 0.29	9.06 ± 0.18
3-*O*-Methylglucose, 5 mM	3.39 ± 0.23	10.48 ± 0.23
2-Deoxyglucose, 5 mM	4.44 ± 0.36	4.63 ± 0.56
2-Deoxyglucose, 5 mM, and Na pyruvate, 5 mM	3.08 ± 0.27	6.98 ± 0.47

[a] Conditions are as in Table I, experiment 1. Data are given as the mean ± standard error of triplicate determinations.

no significant stimulation by insulin was observed in vesicles prepared from azide-treated cells.

Cells incubated anaerobically, but in the presence of glucose, responded normally to insulin (Table I, experiment 2). When cells were incubated with 1 mM D-glucose for 30 min under an atmosphere of 95% N_2/5% CO_2, and insulin was then added anaerobically, the response of vesicles prepared from these cells was similar to that of vesicles prepared from control cells incubated under 95% O_2/5% CO_2. This accords with earlier work in muscle (Morgan et al., 1965; Morgan and Neely, 1972) showing that anaerobiasis enhances cellular response to insulin.

It should be noted that, in the usual aerobic incubations, it made no difference whether or not an exogenous energy supply in the form of glucose was provided to the intact cells. In the absence of metabolizable substrates in the incubation medium, the adipocyte presumably must rely upon oxidation either of its plentiful supply of fatty acids or of the meager store of glycogen for continued generation of ATP and other high-energy phosphates; in aerobic incubations, ATP levels are well maintained in the absence of an external energy source (see below). However, adipocytes incubated in the presence of the glucose analogue, 2-deoxyglucose, but without added glucose in the medium, showed complete loss of the insulin response,

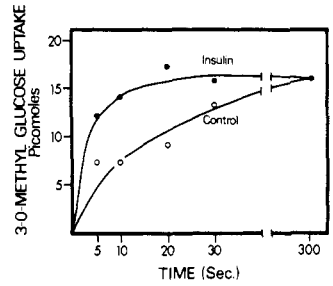

FIGURE 1: Insulin stimulation of 3-*O*-methylglucose uptake in fat cells. Isolated adipocytes were incubated in the presence (●) or absence (○) of insulin (100 μU/mL) for 15 min at 37 °C in Krebs-Ringer phosphate buffer (pH 7.4) containing 2% albumin. Uptake of 3-*O*-[^{3}H]methylglucose (0.1 mM) was determined in 0.2-mL aliquots of cells as described under Materials and Methods; [^{14}C]sucrose (0.1 mM) in the incubation was used to correct for sugar trapped in the extracellular fluid. Results are the mean of quadruplicate determinations and are expressed as picomoles per 10^5 cells.

comparable to that seen with azide treatment (Table II). 2-Deoxyglucose is a substrate for hexokinase but does not undergo significant metabolism beyond the stage of 2-deoxyglucose 6-phosphate (Kipnis and Cori, 1959, 1960). In contrast, 3-*O*-methylglucose, an inhibitor of glucose transport which is not phosphorylated, had no effect on the ability of cells to respond to insulin (Table II).

The ability of 2-deoxyglucose to block the insulin effect was readily reproducible, being seen in 19 out of 20 separate experiments, whereas 3-*O*-methylglucose was never observed to inhibit insulin stimulation of glucose transport. Furthermore, this effect could not be explained by competitive inhibition of glucose uptake by small amounts of 2-deoxyglucose present in the membrane vesicles, since much higher concentrations of this sugar are required to inhibit glucose transport in the vesicle system (Carter et al., 1972). Provision of metabolic energy in the form of sodium pyruvate partially reversed the 2-deoxyglucose inhibition (Table II).

Both NaN_3 and 2-deoxyglucose effectively lower intracellular levels of ATP in isolated adipocytes. Control (zero time) levels of ATP vary considerably between different preparations of cells; in eight separate experiments, levels ranging between 36 and 86 nmol/g dry weight of fat cells were observed. Typically these levels rise during the first 30 min of incubation at 37 °C, even in the absence of utilizable substrate in the medium. In four separate experiments, NaN_3 was observed to depress intracellular levels of ATP to 10–20% of those observed in control cells. 2-Deoxyglucose, in a similar number of experiments, lowered ATP levels to 20–40% of control values.

Thus, inhibition of intracellular metabolism, reflected in lowered levels of intracellular ATP, was associated with a failure of the cell to respond to insulin. It should be emphasized that, since the response was determined by a direct measure of glucose transport in isolated vesicles, these results clearly indicate inability of insulin to stimulate glucose transport across the cell membrane and not just depressed metabolism of the sugar once it enters the cell.

Studies in Whole Cells. The availability of a method (Gliemann et al., 1972) which permits direct assessment of transport in intact adipocytes (Livingston and Lockwood, 1974) has permitted us to extend our studies to whole cells. Like Livingston and Lockwood (1974), we found that 3-*O*-methylglucose equilibrated rapidly with the small intracellular aqueous space present in the fat cell (Figure 1); even with measurements as early as 5 and 10 s, the shortest practical time points with this method, linear uptake was not observed. As

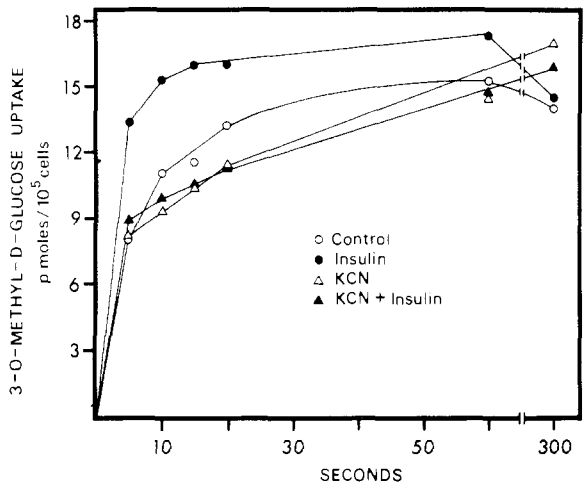

FIGURE 2: Effect of KCN on insulin stimulation of 3-*O*-methylglucose uptake. Isolated adipocytes (6×10^5 per mL) were incubated in the presence or absence of KCN (0.5 mM) for 30 min at 37 °C; one-half of each group of cells was then exposed to insulin (100 μU/mL) for 15 min at 37 °C. 3-*O*-Methylglucose uptake was determined at 24 °C as described under Materials and Methods.

TABLE III: Effect of Cyanide on Insulin Stimulation of 3-*O*-Methylglucose Uptake.[a]

Preincubation Conditions	3-*O*-Methylglucose Uptake (pmol/10^5 cells per 5 s)	
	Control	Insulin
Expt. 1		
Control	7.91	13.47
KCN, 0.1 mM	8.06	13.91
Expt. 2		
Control	8.07	18.28
KCN, 0.5 mM	8.32	8.84

[a] Conditions are the same as in Figure 1 except only the 5-s uptake value is given. The results shown are the means of quadruplicate values.

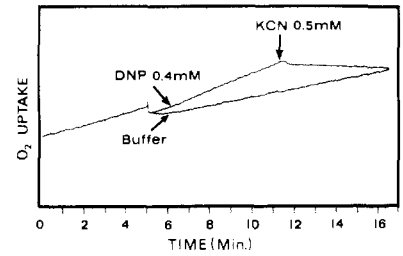

FIGURE 3: Effect of energy inhibitors on oxygen uptake by isolated fat cells. Isolated fat cells (10^6 per mL) in Krebs–Ringer phosphate buffer (pH 7.4) containing 2% albumin were incubated at 37 °C with constant stirring in an oxygen electrode chamber. After a 10-min period to establish basal uptake, 2,4-dinitrophenol (0.4 mM) was added and the incubation continued for 10 min, after which KCN (0.5 mM) was added. The breaks in the curves are artefacts due to the additions. A separate control experiment in which only buffer was added is superimposed on the tracing.

others have noted (Livingston and Lockwood, 1974), uptake of 2-deoxyglucose was linear over a considerably longer period of time and showed a brisk response to insulin (data not shown). However, we have shown (Chandramouli and Carter, 1977) that in adipose tissue as in muscle (Kipnis and Cori, 1959) the bulk of the intracellular 2-deoxyglucose is present as phosphorylated metabolites. Under normal conditions in adipose tissue, where transport has been shown to be the rate-limiting step in glucose metabolism (Crofford and Renold, 1965), 2-deoxyglucose may provide a good measure of rates of transport. However, we have noted that under experimental conditions in which phosphorylation becomes rate limiting, as for example incubation of the adipocytes with metabolic inhibitors, apparent rates of uptake of 2-deoxyglucose decline dramatically and reach equilibrium with approximately the same time course as 3-*O*-methylglucose. We have, therefore, routinely used 3-*O*-methylglucose to assess sugar transport, since only transport is measured with this sugar under all experimental conditions. The addition of [^{14}C]sucrose provides a correction factor for the amount of sugar present in the small volume of extracellular fluid trapped in the fat cake. While the rapid equilibration of 3-*O*-methylglucose prevented precise estimates of initial rates of transport, a consistent and reproducible effect of insulin was observed (Figure 1).

It should be noted that, when direct measurements of sugar transport are made, only about a two-fold stimulation is seen in the presence of insulin. This coincides with the experience of Livingston and Lockwood (1974) and Czech (1975, 1976) and is also seen when measurements are made in brown fat cells (Czech et al., 1974a) which contain a much larger intracellular water volume. Yet many laboratories, including our own, routinely find five- to tenfold stimulation by insulin of CO_2 production from glucose in intact adipocytes. The difference presumably reflects direct stimulation by insulin of the intracellular metabolism of glucose beyond the step of transport.

Figure 2 shows the effect of preincubating the isolated fat cells with KCN prior to exposure to insulin and the measurement of 3-*O*-methylglucose uptake. There was complete loss of the stimulatory effect of insulin upon transport but no change in the basal uptake or the final equilibrium level reached (300 s). Thus, neither the equilibrium glucose "space" nor basal transport was affected by cyanide, but the ability of insulin to stimulate transport was completely blocked.

Preincubation of the fat cells with 0.1 mM KCN had essentially no effect on insulin stimulation of sugar transport, whereas 0.5 mM KCN completely blocked response to the hormone (Table III). Since the shortest time point measured (5 s) most closely approached the initial rate of transport, this was chosen to assess the effect of the inhibitor on insulin stimulation. For these experiments, cells were counted in a hemocytometer and all values are corrected to 10^5 cells. It is clear that with sufficient inhibition of oxidative metabolism (i.e., 0.5 mM KCN) response to insulin was abolished. At 0.1 mM KCN the insulin response was intact despite depression of the ATP level to 30% of the control value (ATP data not shown).

In four similar experiments done at either 0.5 or 1.0 mM KCN, no insulin response was observed. In these experiments (data not shown), ATP levels were reduced to 10–27% of control values in the inhibitor-treated cells. Different preparations of cells show varying sensitivity to the effects of cyanide; for this reason, we were unable to determine an exact level of intracellular ATP required for the response to insulin.

Cyanide blocks mitochondrial electron flow and oxygen uptake. 2,4-Dinitrophenol, on the other hand, which uncouples phosphorylation from oxidation, should enhance the rate of electron flow down the respiratory chain. This appears to be true for isolated fat cells as well as other tissues. Figure 3 shows the change in oxygen uptake when a suspension of isolated fat cells was exposed sequentially to dinitrophenol followed by KCN. As expected, oxygen uptake was accelerated slightly in

TABLE IV: Effect of 2,4-Dinitrophenol (DNP) on Insulin Stimulation of 3-*O*-Methylglucose Uptake.[a]

Preincubation Conditions	3-*O*-Methylglucose Uptake (pmol/10^5 cells per 5 s)	
	Control	Insulin
Expt. 1		
Control	8.81	19.05
DNP, 0.1 mM	5.45	11.40
Expt. 2		
Control	7.46	15.46
DNP, 0.2 mM	5.49	4.80

[a] Methods are the same as described in Table III.

TABLE V: Effect of ATP on Insulin-Stimulated Glucose Metabolism and 3-*O*-Methylglucose Transport in Intact Adipocytes.[a]

Addition	$^{14}CO_2$ Production (cpm/10^5 cells per h)		3-*O*-Methylglucose Uptake (pmol/10^5 cells per 5 s)	
	Control	Insulin	Control	Insulin
None	10 390	27 393	12.4	17.4
ATP, 5×10^{-5} M	5 043	12 557	10.7	16.3

[a] To measure the effect of ATP on glucose metabolism, isolated fat cells in Krebs–Ringer bicarbonate buffer containing 2% bovine albumin and 0.5 mM D-[U-^{14}C]glucose (1 μCi/μmol) were incubated for 1 h at 37 °C in the presence or absence of ATP and insulin (100 μU/mL). The reaction was stopped with 5% trichloroacetic acid, $^{14}CO_2$ was collected in Hyamine hydroxide, and radioactivity was determined. For 3-*O*-methylglucose transport, isolated fat cells were preincubated for 30 min at 37 °C in the presence or absence of insulin (100 μU/mL), then ATP was added to one-half the cells, and incubation was continued for a further 10 min at 24 °C. Uptake of 3-*O*-methylglucose was determined as before. Results are the means of triplicate determinations.

the presence of the uncoupler but completely inhibited by cyanide. If the energy necessary for insulin action could be obtained from oxidative electron flow rather than high-energy phosphate bonds, it might be expected that dinitrophenol would not block the action of the hormone. However, as shown in Table IV, the uncoupling agent was as effective in blocking the cellular response to insulin as was cyanide. In two other experiments done with 0.5 and 1.0 mM dinitrophenol no insulin response was observed; in these experiments (data not shown), ATP levels were reduced to 18 and 5% of control values. Unlike cyanide, a small but significant depression was observed in basal transport rates for 3-*O*-methylglucose in dinitrophenol-treated cells.

Chang and Cuatrecasas (1974) have recently reported that low concentrations of ATP added to the incubation medium of isolated rat fat cells effectively block the action of insulin upon glucose transport. This effect of ATP is reportedly prevented by an ATP analogue such as β,γ-methyleneadenosine 5′-triphosphate which is not itself capable of acting as a phosphate donor. Since this experiment has direct relevance to our own findings, we have attempted to duplicate it. Addition of ATP to the external medium in low concentrations inhibited insulin-stimulated conversion of glucose to CO_2 (Table V); there was also some suppression of basal glucose metabolism. However, when transport was measured directly by 3-*O*-methylglucose uptake (Table V), no effect of ATP on insulin

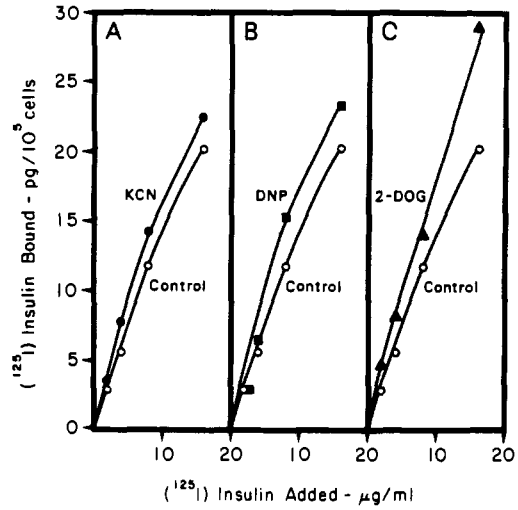

FIGURE 4: Effect of energy inhibitors on ^{125}I-labeled insulin binding to isolated fat cells. Isolated fat cells were incubated at 37 °C for 30 min in Krebs–Ringer phosphate buffer (pH 7.4) containing 1% albumin only (control, ○) or in the presence of 1 mM KCN (A, ●), 0.5 mM 2,4-dinitrophenol (B, ■), or 20 mM 2-deoxyglucose (C, ▲); then binding of ^{125}I-labeled insulin was determined as described under Materials and Methods. "Nonspecific" binding has been subtracted. The concentrations of inhibitors chosen have been shown in separate experiments to inhibit the cellular response to insulin. Results are the mean of duplicate determinations.

stimulation was observed. In a total of four experiments (data not shown), insulin stimulation of 3-*O*-methylglucose transport was not inhibited significantly by the presence of 5×10^{-5} M ATP.

Effect of Energy Inhibitors on Insulin Binding. Energy poisons consistently blocked insulin action while having little effect on basal rates of transport; this could be easily explained by a requirement for energy in the binding of insulin to its specific "receptor" or cell surface recognition site. That this was not the case is shown in Figure 4; after preincubation of fat cells with cyanide, dinitrophenol, or 2-deoxyglucose, there was no change in the specific binding of ^{125}I-labeled insulin to isolated adipocytes. Similar results were observed in two other experiments with each inhibitor. The significance of the modest increase in binding observed after incubation with 2-deoxyglucose is not clear at present.

Discussion

The fundamental mechanism by which insulin exerts its multiple metabolic effects remains unknown. It is well established that under certain circumstances insulin can lower intracellular levels of cyclic AMP (Butcher et al., 1966; Jungas, 1966), either by direct inhibition of adenylate cyclase (Illiano and Cuatrecasas, 1972; Hepp, 1972; Kuo et al., 1973), or by stimulation of phosphodiesterase (Loten and Sneyd, 1970; Manganiello and Vaughan, 1973); this action may explain certain effects of the hormone, such as its ability to inhibit lipolysis (adipose tissue) and gluconeogenesis (liver). To date, cyclic nucleotides have not been convincingly related to other actions such as stimulation of glucose transport. Insulin's well-known ability to stimulate glycogen synthesis has been investigated by Larner et al. (1974); they have reported the presence in muscle of an insulin-dependent inhibitor of glycogen synthase (protein) kinase, in part explaining the glycogenic action of the hormone, but the nature of this apparently unique inhibitor and its possible relation to other hormonal effects remain obscure (Larner et al., 1975).

Recently, the possibility that insulin might be directly in-

volved in phosphorylation–dephosphorylation reactions has been raised. Benjamin and Singer (1974) have demonstrated insulin-stimulated phosphorylation of a specific soluble protein in rat adipose tissue. Chang and Cuatrecasas (1974) have suggested a relation between ATP and insulin action, based on their observation that low concentrations of ATP added to incubations of isolated adipose cells block the ability of insulin to stimulate glucose transport. Further, they have demonstrated (Chang et al., 1974) that two specific membrane proteins are phosphorylated by [γ-^{32}P]ATP added to whole cell incubations or to broken cell preparations; the phosphorylation, carried out by an endogenous protein kinase, is completely dependent on cyclic AMP.

While we have confirmed (Table V) the observations of Chang and Cuatrecasas (1974) on the effect of exogenous ATP on insulin-stimulated glucose metabolism, we have been unable to demonstrate a true "block" to insulin action at the level of glucose transport (Table V). It should be noted that Chang and Cuatrecasas (1974) relied primarily on measurements of [^{14}C]glucose conversion to $^{14}CO_2$ to assess insulin action; they report only a single experiment in which transport was measured directly, and we have been unable to confirm this observation in four separate experiments. Our results with exogenous ATP are most compatible with a block in glucose metabolism, since insulin-stimulated conversion of glucose to CO_2 is clearly depressed in the presence of ATP whereas 3-*O*-methylglucose transport, in the presence or absence of insulin, is unchanged (Table V). These experiments emphasize again the importance of separating measurements of glucose transport from measurements of glucose metabolism in assessing the effects of insulin.

Despite some argument (Davidson et al., 1972; Katzen and Vlahakes, 1973), present evidence (Cuatrecasas, 1969; Suzuki et al., 1972) strongly suggests that most, if not all, of insulin's effects are initiated at the external surface of the cell. There is now extensive evidence (Cuatrecasas, 1974) for the presence of specific "receptors" on the outer surface of insulin-responsive cells, and interaction with these receptors appears to be a necessary prerequisite to hormonal action. This generalization is now accepted for most polypeptide hormones. Except for the demonstration in adipocytes (Cuatrecasas and Illiano, 1971) that desialylation of the membrane blocks insulin action without inhibiting binding, little is known of the changes subsequent to binding which are presumably induced in the cell surface structure that lead rapidly to an increase in glucose transport and the other manifestations of insulin action. Czech et al. (1974b) have proposed that insulin stimulates glucose transport primarily by inducing the oxidation of specific sulfhydryl groups; thus, the "insulin stimulated" state is viewed as one in which a critical disulfide bond is formed, presumably within the glucose transport system. The hypothesis is based on evidence that: (1) sulfhydryl blocking agents like *N*-ethylmaleimide block the action of insulin, (2) insulin "protects" against this effect of *N*-ethylmaleimide, and (3) once in the "insulin-stimulated" state *N*-ethylmaleimide blocks the return to the basal rate of transport. The present experiments bear only indirectly on this hypothesis. The ability of both oxidation-inhibiting (cyanide) and oxidation-stimulating (dinitrophenol) agents to block insulin action suggests that the "redox" state of the cell is not the sole determinant of insulin action. In particular, the ability of dinitrophenol to block insulin action suggests that oxidative electron flow is not sufficient by itself to permit insulin action; high-energy phosphate bonds must be required at some step. Furthermore, experiments with sulfhydryl-binding agents must be interpreted with caution, since we have shown in the case of the red cell membrane (Carter, 1973) that sulfhydryl-binding agents can have major effects on membrane structure. Despite this, the possibility that sulfhydryl–disulfide interchange may play an important role in the mechanism of insulin action remains a plausible and intriguing hypothesis.

The present studies were undertaken to explain our inability to induce insulin effects in an isolated membrane system from fat cells. After showing that the isolated membranes formed vesicles which were capable of transporting glucose (Carter and Martin, 1969b), presumably into an enclosed intravesicular space, we were able to demonstrate that membranes prepared from suitably insulin-exposed cells retained the effect of the hormone (Martin and Carter, 1970; Carter et al., 1972). We showed that this represented true enhancement of glucose transport and not a change in glucose "space" (Martin and Carter, 1970), a critical demonstration since our techniques did not permit accurate assessment of initial rates of transport. This finding indicated that whatever change occurs in the plasma membrane upon exposure to the hormone is stable and persists through cell disruption, differential centrifugation, etc. Covalent bonding of some type seemed the most likely but not the only possible explanation.

Despite multiple attempts, we have been unable to duplicate these results by exposing isolated membranes to insulin, although such membrane preparations are well known to bind insulin to the specific "receptor" (Cuatrecasas, 1974). It seemed logical to assume that in some way the cellular response was dependent upon the presence of intact intracellular metabolism. The present studies verify this assumption. Agents that block intracellular energy metabolism, such as cyanide and dinitrophenol, are capable of inhibiting the cellular response to insulin. This is true whether glucose transport is measured in isolated membranes or whole cells. The present studies suggest the specific site (or sites) affected by inhibition of oxidative phosphorylation. Theoretically, the insulin "receptor", the unknown "transducer" of the signal, or the glucose transport system itself could be blocked. Insulin binding is unchanged in energy-poisoned cells (Figure 4), so this first step in insulin action is clearly independent of the energy "set" of the cell. Glucose transport itself appears independent of energy; all evidence to date suggests that glucose transport in adipose tissue, like that in muscle, involves facilitated diffusion down a chemical gradient and that energy is not involved. In the present experiments, basal glucose transport was not changed in either vesicles or whole cells after inhibition of energy metabolism. It appears most likely that energy is required in the unknown steps modulating the response to insulin within the plasma membrane.

The nature of the required energy source is not entirely clear, but certain inferences can be drawn. In bacteria, where the coupling of energy to transport has been extensively studied, "active" transport can be coupled directly to respiratory electron flow or can utilize high-energy phosphate bonds (Klein and Boyer, 1972), depending on the particular bacterium, transport system, and conditions of cell growth. The present studies suggest strongly that, for insulin to act upon mammalian fat cells, high-energy phosphate bonds must serve as the energy source. Thus, anaerobiasis did not inhibit the response to insulin when glucose was present in the medium, although presumably respiratory electron flow was blocked. Furthermore, dinitrophenol, which enhanced respiration while blocking high-energy phosphate bond generation (Figure 3 and Table IV), completely inhibited the cellular response to insulin. It thus seems likely that in some manner high-energy phos-

phate bonds, and not oxidative electron flow per se, are involved in insulin stimulation of glucose transport.

Since these studies have been completed, Kono et al. (1976) have also shown that uncouplers of oxidative phosphorylation are capable of inhibiting both insulin stimulation of glucose transport and insulin inhibition of lipolysis in adipose tissue.

While we have used measurement of cellular ATP levels to indicate the extent of inhibition of oxidative phosphorylation, there is, at the moment, no compelling reason to assume that this nucleotide is the direct source of energy for the insulin-induced change. Phosphoenolpyruvate, GTP, or other nucleotides could be as well the direct energy donor.

In studying the means by which insulin stimulates glucose transport, it is essential to utilize an assay system that gives a direct assessment of sugar transport per se and not of transport coupled to metabolism. While it is true that under normal circumstances transport is the rate-limiting step in glucose metabolism in both muscle (Park et al., 1961) and adipose tissue (Crofford and Renold, 1965), any experimental manipulations that affect intracellular energy generation will complicate the interpretation of measurements based on glucose metabolism. For example, it is clear that drastically lowering intracellular ATP levels, as in our experiments, will block glucose metabolism, yet it appears to have no significant effect on basal glucose transport. As Czech et al. (1973) have pointed out, and we have confirmed, even the use of a non-metabolized glucose analogue such as 2-deoxyglucose, which can be phosphorylated, could lead to a false presumption of changes in rate of transport when in fact rates of phosphorylation have been affected.

There are now available methods for studying glucose transport directly, unrelated to metabolism, both in whole cells and in isolated membranes. These should permit more studies directed specifically at the link between insulin–receptor interaction and stimulation of glucose transport. It now appears clear that this link is energy dependent.

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Covalent Structure of Collagen: Amino Acid Sequence of Cyanogen Bromide Peptides from the Amino-Terminal Segment of Type III Collagen of Human Liver†

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ABSTRACT: Human liver type III collagen was prepared by limited pepsin digestion, differential salt precipitation, and carboxymethylcellulose chromatography. Cyanogen bromide digestion of purified type III collagen chains yielded nine distinct peptides. Three peptides, α1(III)-CB3, α1(III)-CB7, and α1(III)-CB6, were isolated by carboxymethylcellulose chromatography and Sephadex G-50 SF gel filtration. Automated Edman degradation together with selective hydroxylamine cleavage and chymotrypsin and trypsin digestion enabled determination of their complete amino acid sequence. Compared with type I collagen, the data show tentative homology of α1(III)-CB3 with α1(I)-CB1, α1(I)-CB2, and α1(I)-CB4; α1(III)-CB7 with α1(I)-CB5; and α1(III)-CB6 with the amino-terminal portion of α1(I)-CB8. Close interspecies homology was found between the sequences presented here with 90 residues of α1(III)-CB3 and 26 of α1(III)-CB8 of calf aorta. The present study establishes the amino acid sequence of 229 residues near the amino terminus or nearly one-quarter of the type III collagen chains. The disaccharide, Glc-Gal, was convalently bound to hydroxylysine at a position corresponding to the same location in the α1(I) chain.

Collagen exists as a triple-stranded helix of three α chains, each containing over 1000 amino acid residues (Gallop et al., 1972; Traub and Piez, 1971). The most extensively studied so far is type I collagen consisting of two α1(I) chains and one α2 chain. A composite amino acid sequence of α1(I) constructed from CNBr peptides of chick, calf, and rat collagen has been tabulated (Hulmes et al., 1973; Gallop and Paz, 1975; Piez, 1977; Fietzek and Kuhn, 1976).

Recently, a genetically distinct collagen, type III, has been identified and found to exist with type I collagen in most soft connective tissues, such as human skin (Chung and Miller, 1974; Chung et al., 1974; Epstein, 1974), aorta (Fietzek and Rauterberg, 1975; Trelstad, 1974), lung (Hance et al., 1975; Seyer et al., 1976a), liver (Gay et al., 1975; Rojkind and Martinez-Palomo, 1976), and hypertrophic scar (Seyer et al., 1976b). Nine cyanogen bromide peptides from type III collagen of human skin and lung have been isolated and characterized (Chung et al., 1974; Seyer et al., 1976a). Partial amino acid sequences of three of these, α1(III)-CB4, α1(III)-CB5, and α1(III)-CB6, have been determined, which has enabled identification of their location within the α1(III) chains (Fietzek and Rauterberg, 1975). They were suggested to be homologous to α1(I)-CB3, α1(I)-CB7, and α1(I)-CB8, respectively.

This report represents a complete sequence analysis of three CNBr peptides, α1(III)-CB3, α1(III)-CB7, and α1(III)-CB6, which most probably comprise the amino-terminal one-quarter of human type III chains. The alignment of the three peptides within the collagen chain was tentatively suggested to be 3-7-6 by homology with the known α1(I) sequence. The α1(III)-CB7 was homologous to α1(I)-CB5 and contained a similar single Glc-Gal-Hyl residue at position 103 of the collagen polypeptide chain.

Materials and Methods

Preparation of Type III Collagen. Human cirrhotic liver was obtained from adult males after autopsy. Hepatic blood vessels were removed as much as possible and all subsequent operations were performed at 4 °C. The tissue was pulverized with a mechanical meat grinder, followed by brief homogenization in a Waring blender. The homogenate was subsequently extracted five times with 0.05 M Tris-HCl (pH 7.4), followed by five successive washes with distilled water in order to remove as much soluble noncollagenous material as possible.

Hepatic collagen was extracted by limited pepsin digestion (Chung and Miller, 1974) using 1 g of enzyme per 50 g wet weight at pH 2.8 (0.5 M acetic acid adjusted with formic acid). All operations were performed at 4 °C as previously described (Seyer et al., 1976a). Three successive pepsin digestions for 72 h enabled solubilization of nearly 80% of the total liver collagen. The collagens present in the three extracts were precipitated by dialysis against 0.01 M Na_2HPO_4 and harvested by centrifugation. The precipitate was redissolved in 0.5 M acetic acid and precipitated again by the addition of NaCl to a final concentration of 1 M. This precipitate was resolu-

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