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Mechanisms of the Ability of Insulin to Activate the Glucose-Transport System in Rat Adipocytes

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Isolated rat adipocytes were used to assess the mechanisms of the ability of insulin to accelerate glucose transport. Glucose transport was determined by measuring the initial rates of 2-deoxyglucose uptake, and at 24°C insulin increased the $V_{max.}$ of transport from 7.3±1 to 23.1±2nmol/min per 10^6 cells, but the K_m value remained unchanged (2.5, cf. 2.4mM). When the $V_{max.}$ of basal and insulin-stimulated transport was measured as a function of temperature (15–37°C), parallel Arrhenius plots were obtained yielding equal activation energies of approx. 59kJ/mol. Since both processes have equal activation energies the data indicate that insulin increases $V_{max.}$ by increasing the number of available carriers rather than enhancing intrinsic activity of already functioning carriers. Since the ability of insulin to activate glucose transport did not decrease with temperature (whereas plasma-membrane fluidity declines), it is suggested that lateral diffusion of insulin receptors within the plasma-membrane bilayer is not a rate-determining step in insulin action.

One of the major biological actions of insulin is to promote glucose metabolism, and this effect is largely, but not exclusively (Leonards & Landau, 1964; Olefsky, 1975, 1976*a*; Lawrence *et al.*, 1977), due to acceleration of glucose transport. If one assumes that specific glucose-transport structures (carriers?) exist within the plasma membrane, then insulin could accelerate transport by: (1) increasing the transmembrane mobility of glucose through existing transport units, (2) increasing the number of available transport units, (3) increasing the affinity of the system for glucose (decreasing K_m), or (4) any combination of the above. To investigate these possibilities, the glucose-transport step itself must be directly assessed. Thus studies of glucose oxidation (or other aspects of glucose metabolism) alone are not adequate, since glucose oxidation does not always reflect glucose transport, especially at the higher substrate concentrations needed to perform kinetic experiments (Olefsky, 1975). Further, it has been demonstrated that insulin can directly promote various aspects of intracellular glucose metabolism, independently of its ability to augment glucose transport (Leonards & Landau, 1964; Olefsky, 1975, 1976*a*; Lawrence *et al.*, 1977), and this adds to the difficulty in drawing conclusions about glucose transport from studies of subsequent steps of glucose metabolism. However, techniques are now available to measure transport in adipocytes, and the present paper describes studies aimed at examining mechanisms whereby insulin accelerates glucose transport.

Experimental

Materials

Pig monocomponent insulin was generously supplied by Dr. Ronald Chance of Eli Lilly Co. (Indianapolis, IN, U.S.A.). Bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. (Phoenix, AZ, U.S.A.), silicone oil from Arthur Thomas Co. (Philadelphia, PA, U.S.A.), collagenase from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), and $Na^{125}I$, [1-^{14}C]glucose, 2-deoxy[1-^{14}C]glucose, 3-*O*-methyl[1^{14}C]glucose, [^{14}C]inulin, 2-deoxy-[1-^{14}C]glucose 6-phosphate were from New England Nuclear Corp. (Boston, MA, U.S.A.). Cytochalasin B was from Gallard–Schlesinger Chemical Mfg. Corp. (Carol Place, NY, U.S.A.).

Preparation of isolated adipocytes

Male Sprague–Dawley rats were used for all experiments. Control animals weighed 120–160g and were fed *ad lib.* until the morning of the experiment. All studies were begun between 08:00 and 09:00h. Animals were stunned by a blow to the head, decapitated, and epididymal fat-pads were removed.

Isolated fat-cells were prepared by shaking at 37°C for 60min in Krebs–Ringer bicarbonate buffer containing 118mM-NaCl, 5mM-KCl, 1.2mM-KH_2PO_4, 1.2mM-$MgSO_4$, 25.5mM-$NaHCO_3$, 3mg of collagenase/ml and 40mg of albumin/ml by the method of Rodbell (1964). Cells were then filtered through nylon mesh (250μM pore size), centrifuged at 400rev./min (IEC model C1) for 4min, and washed twice in the Krebs–Ringer bicarbonate buffer (Olefsky & Reaven, 1975). Adipocyte counts were performed by a modification of method III of Hirsch & Gallian (1968), in which the cells were fixed in 2% (w/v) OsO_4 in 0.05M-collidine buffer, pH7.4 (made isoosmotic with NaCl), for 24h at 37°C and then taken up in a known volume of 154mM-NaCl for counting in a model ZB Coulter counter with a 400μm aperture. All individual experiments were performed with separate batches of cells on separate days.

Glucose-transport studies

Glucose transport was assessed by described methods (Olefsky, 1975, 1976*b*,*c*). Isolated adipocytes (2×10^5–4×10^5/ml) were incubated with 2-deoxy-D-[1-^{14}C]glucose (sp. radioactivity 2mCi/mmol) at a concentration of 0.1–20mM in the Krebs–Ringer bicarbonate buffer, pH7.4, containing bovine serum albumin (10mg/ml) at 24°C (unless otherwise stated). This assay measures the total uptake of the 2-deoxy-[^{14}C]glucose and is based on the principle that, although 2-deoxyglucose is transported and phosphorylated by the same processes as D-glucose, it cannot be further metabolized (Wick *et al.*, 1957). The incubations were terminated at the end of 3min as originally described by Gammeltoft & Gliemann (1973) by removing 200μl samples from the cell suspension and rapidly centrifuging (10000*g*) the cells in plastic micro-tubes to which 100μl of silicone oil (specific gravity 0.97) had been added. The tubes were centrifuged for 30s in a Beckman microfuge and the assay was considered terminated when centrifugation began. Silicone oil has a specific gravity intermediate between buffer and cells, and therefore, after centrifugation, three layers resulted: cells on top, oil in the middle and buffer on the bottom. The cells were then removed and the radioactivity was determined (Olefsky, 1975). All studies were done in triplicate. In experiments in which the stimulatory effect of insulin on uptake was measured, the cells were preincubated with insulin (25ng/ml) for 90min at the designated temperature. The amount of sugar trapped in the extracellular water space of the cell layers was determined with [^{14}C]inulin by the method of Gliemann *et al.* (1972). Extracellular water space was measured in each experiment, and all sugar-uptake data were corrected for this factor. The percentage of the total amount of sugar available that is trapped in the extracellular water space is a linear function of cell concentration, averaging 0.033± 0.001% at a concentration of 2×10^5cells/ml in cells from normal animals (Olefsky, 1975). The amount of trapped sugar ranges from 2 to 10% of the total sugar uptake, depending on the conditions of incubation. Thus for every 1ml of incubation mixture (2×10^5cells/ml) 330nl of buffer is trapped in the extracellular space of the cell layer after the oil separation. This amounts to 1.65±0.05pl/cell. The total cell volume and intracellular water space (mean±S.E.M., n=12) were 73±4pl/cell and 1.60± 0.11pl/cell respectively.

3-*O*-Methylglucose is another analogue used to assess glucose transport. This sugar is not phosphorylated and thus, theoretically, its uptake is solely a measure of transport (Hatanaka, 1974). Uptake of this sugar was determined by the same procedure used for the 2-deoxyglucose experiments, except for the duration of the assay. Because 3-*O*-methylglucose uptake becomes rapidly curvilinear with time, uptake was measured 10s after addition of the labelled hexose unless otherwise stated.

Measurements of intracellular 2-deoxyglucose and 2-deoxyglucose phosphate

Intracellular concentrations of 2-deoxyglucose and 2-deoxyglucose 6-phosphate were determined by a modification of the method of Tsuboi & Petricciani (1975). (It should be noted that the phosphorylated products of 2-deoxyglucose include 2-deoxyglucose 6-phosphate and 2-deoxy-6-phosphogluconate. Since the former represents the great majority of the phosphorylated products, this sugar is designated as phosphorylated 2-deoxyglucose for the sake of simplicity.) Adipocytes were incubated with 2-deoxy[1-^{14}C]glucose, and, at the indicated time, transport and phosphorylation were stopped by addition of 3ml of iced Krebs–Ringer bicarbonate buffer containing unlabelled 1mM-2-deoxyglucose. The reaction mixture was then centrifuged (3min, 2000rev./min, 4°C) through silicone oil and the infranatants plus oil were discarded. The remaining cell layer was then boiled in water, and the extracted intracellular contents were chromatographed through an ion-exchange column (Dowex 1 X8; Cl^- form) prepared in a 1.5ml pipette containing 1ml of resin. Free 2-deoxyglucose was eluted with 4.5ml of water and 2-deoxyglucose 6-phosphate was eluted with 8.5ml of 0.2M-formic acid/0.5M-ammonium acetate, pH4.9. To monitor recovery, known amounts of 2-deoxy[1-^{14}C]glucose and 2-deoxy[1-^{14}C]glucose 6-phosphate were always chromatographed in parallel. Recovery ranged from 90 to 105%, and, when necessary, the data were corrected for this factor. To calculate intracellular concentrations of the different sugars, the intracellular water volume was determined by simultaneous assessment of the sucrose and

3-*O*-methylglucose spaces. The 3-*O*-methylglucose space (total water) minus the sucrose space (extracellular water) equals the intracellular water space. By knowing the number of cells present, and the amount of 2-deoxyglucose and phosphorylated sugar per cell, the intracellular concentrations of these substances can be calculated.

Results

The results summarized in Figs. 1–3 demonstrate that insulin accelerates glucose transport by increasing the $V_{max.}$ of transport, but does not change the K_m value. The ability of various concentrations of insulin to enhance 2-deoxyglucose uptake, at a single substrate concentration (0.125 mM), is presented in Fig. 1. Insulin leads to a dose-dependent 3-fold increase in initial uptake (3 min).

To examine the kinetics of this effect, 2-deoxyglucose uptake was assessed at increasing substrate (2-deoxyglucose) concentrations, in the basal state and in the presence of a maximally effective insulin concentration (25 ng/ml) (Fig. 2). The data from these experiments (Fig. 2*a*) do not describe a hyperbolic function, and thus do not lend themselves to immediate analysis of apparent $V_{max.}$ and K_m values. The curves indicate a saturable component at substrate concentrations below 5 mM, and a non-saturable linear component at higher substrate concentrations. Previous work has shown that the non-saturable component represents simple inward diffusion of labelled substrate (Renner *et al.*, 1972; Weber, 1972; Hatanaka, 1974; Kletzien & Perdue, 1974; Olefsky, 1975, 1976*b*), and this is demonstrated in Fig. 2(*b*). Cytochalasin B (50 μM) completely inhibits the saturable (facilitated) transport system (Renner *et al.*, 1972; Weber, 1972; Czech *et al.*, 1974; Hatanaka, 1974; Olefsky, 1975). Therefore, in the presence of cytochalasin B, uptake measurements fall on a straight line whose slope is equal to the linear component in the absence of the inhibitor. Thus the original data can be corrected by subtracting the linear component from total uptake at each point (Renner *et al.*, 1972; Weber, 1972; Czech *et al.*, 1974; Kletzien & Perdue, 1974; Olefsky, 1975). When this is done (Fig. 2*b*) the resulting data conform to the Michaelis–Menten equation, and apparent $V_{max.}$ and K_m values can be calculated. This analysis is shown in Fig. 3, which demonstrates that insulin

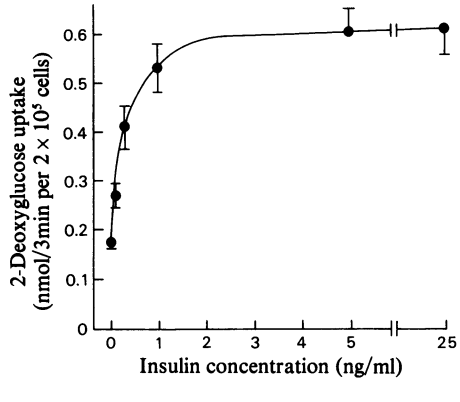

Fig. 1. *Dose-dependent ability of different concentrations of insulin to promote 2-deoxyglucose uptake*
Adipocytes were preincubated with or without the indicated insulin concentrations for 90 min at 24°C. Uptake was then measured after a 3 min incubation with 2-deoxy[^{14}C]glucose (0.125 mM). Data represent the mean (±S.E.M.) of ten separate experiments. In this and all other legends a separate experiment signifies a separate study performed with a different preparation of cells on a different day.

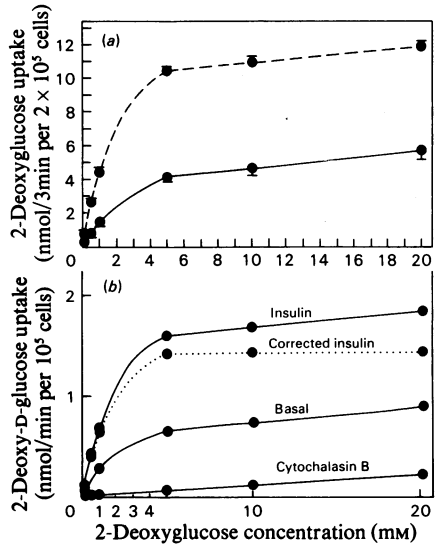

Fig. 2. *Effect of 2-deoxyglucose concentration on sugar uptake in the absence (a) or presence (b) of a maximally effective concentration of cytochalasin B* (50 μM)
(*a*) Studies were carried out in the basal state (——) and in the presence of a maximally effective insulin concentration (25 ng/ml) (– – – –). Data represent the mean (±S.E.M.) of 14 separate experiments. (*b*) Cytochalasin B completely inhibits the facilitated transport system in the presence or absence of insulin, and consequently uptake in the presence of this agent most probably represents simple inward diffusion (see the text). Since the slope of uptake in the presence of cytochalasin B is the same as the slope of the uptake curves in the absence of cytochalasin B at 2-deoxyglucose concentrations above 5 mM, the data can be corrected for this non-saturable process by subtracting the contribution of this linear component from the absolute values (see the text).

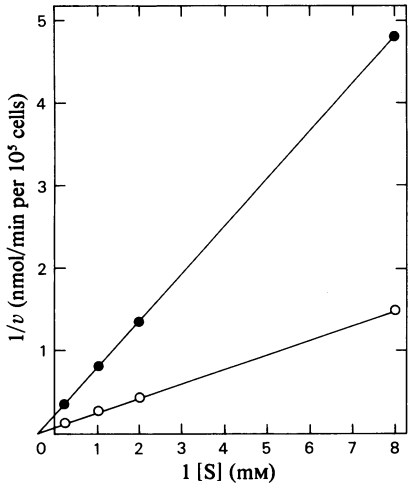

Fig. 3. *Lineweaver–Burk plot of the corrected data from Fig. 2(b)*
The apparent K_m and $V_{max.}$ values calculated from this plot are 2.4mM and 7.3±1 nmol/min per 10^6 cells versus 2.4mM and 23.1±2nmol/min per 10^6 cells in the basal (●) and insulin-stimulated state (○) respectively.

leads to an approximate 3-fold increase in $V_{max.}$, from 7.3±1 to 23.1±2nmol/min per 10^6 cells, with no change in K_m (2.5±0.10 to 2.4±0.12). The magnitude of this effect corresponds quite well to the 3-fold increase in uptake observed at the very low substrate concentration (Fig. 1). Therefore these studies clearly show that insulin accelerates 2-deoxyglucose uptake by increasing $V_{max.}$ without changing K_m.

Validity of 2-deoxyglucose uptake as an assessment of glucose transport

Before analysing the possible mechanisms of this increase in $V_{max.}$, it is important to prove that initial rates of 2-deoxyglucose uptake accurately represent glucose transport. Theoretically, uptake represents the balance between sugar influx and efflux, and to assess initial transport rates it is essential to measure influx under circumstances where efflux is minimal or non-existent. The time course of 2-deoxyglucose uptake at various hexose concentrations is presented in Fig. 4. At the lowest substrate concentration (0.1mM) uptake is linear with time for at least 20min, and the period of linear uptake is inversely related to the 2-deoxyglucose concentration in the medium. However, even at the highest substrate concentration (20mM), uptake remains linear for at least 5min, and this easily exceeds the 3min termination point used in the uptake measurements in Figs. 1 and 2. Thus, since uptake is linear during the 3min

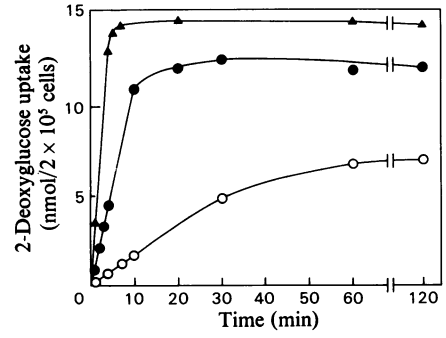

Fig. 4. *Time course of insulin-stimulated (25ng/ml) 2-deoxyglucose uptake*
Studies were performed at 2-deoxyglucose concentrations of 0.1 (○), 2 (●) and 20mM (▲) in the buffer. Data represent the average of three separate experiments.

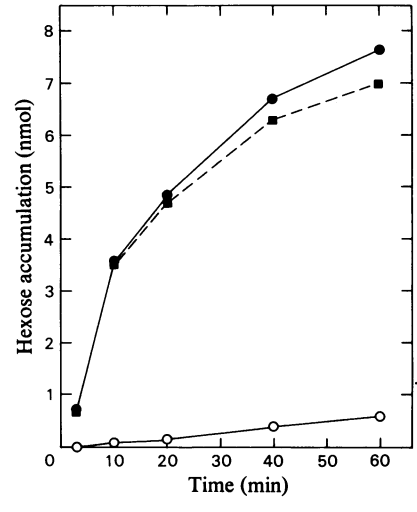

Fig. 5. *Time course of total uptake (●) and intracellular accumulation of phosphorylated 2-deoxyglucose (■) and unphosphorylated 2-deoxyglucose (○)*
Experiments were performed with 1mM-2-deoxyglucose in the buffer. Data represent the mean of four separate experiments.

time interval used, this indicates that true unidirectional flux is being measured, rather than net flux complicated by significant sugar efflux. To prove this point more directly, intracellular concentrations of 2-deoxyglucose and its phosphorylated products (mainly 2-deoxyglucose 6-phosphate, see the Experimental section) were measured. It should be recalled that the 2-deoxy sugar can be phosphorylated, and if

Table 1. *Comparison of extracellular and intracellular hexose concentrations*

For methodological details see the Experimental section. Intracellular hexose concentration was assessed by measurement of intracellular hexose and intracellular water space (3-*O*-methylglucose space minus sucrose space). Intracellular water space was 1.6±0.11 pl/cell (mean ±S.E.M., n=12). All measurements were performed after an incubation period of 3 min at the indicated extracellular 2-deoxyglucose concentration. Data represent the mean (±S.E.M.) of 12 separate experiments.

Concn. of 2-deoxyglucose in the medium (mM)	Intracellular hexose concentration (mM)	
	Basal	Insulin-stimulated (25 ng/ml)
0.1	0*	0
1.0	0	0
5.0	0.16±0.012	0.28±0.010
10.0	0.27±0.021	0.42±0.026
20.0	0.53±0.032	0.68±0.049

* Although no intracellular 2-deoxyglucose was detected under these conditions, it is likely that some was present. However, the concentration of this sugar must be negligible, since intracellular concentrations as low as 0.01 mM are readily measurable with this method (results not shown).

hexokinase activity was rate-limiting during the transport assay, then intracellular 2-deoxyglucose would accumulate and efflux of this sugar would occur. However, as shown in Fig. 5, during the interval of the uptake determination (3 min), intracellular 2-deoxyglucose is undetectable, and essentially all of the intracellular sugar is in the anionic or phosphorylated form. Table 1 demonstrates that similar results are seen when higher extracellular concentrations of 2-deoxyglucose are used; even at the highest substrate concentration used (where transport is greatest and hexokinase activity would be most saturated) the intracellular 2-deoxyglucose concentration is still less than 10% of the extracellular concentration. Therefore, since transport in both directions is concentration-dependent, these data indicate that at 3 min efflux is minimal relative to influx.

Thus 2-deoxyglucose is a convenient analogue with which to assess accurately the glucose-transport step. On the other hand, 3-*O*-methylglucose has not proved as useful for these types of studies. For example, the time course of 3-*O*-methylglucose uptake is shown in Fig. 6. Flux of this sugar is non-linear with time by as early as 15 s in the absence of insulin and even sooner in the presence of insulin. Other workers have found similar results using white adipocytes (Chang & Cuatrecasas, 1974; Livingston & Lockwood, 1974; Chandramouli &

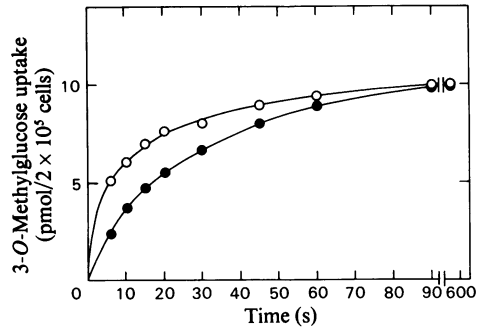

Fig. 6. *Time course of* 3-*O*-*methylglucose uptake* (50 μM) Studies were performed in the absence (●) and presence (○) of a maximally effective insulin concentration (25 ng/ml). Data represent the average of five separate experiments.

Carter, 1977). The final accumulation of 3-*O*-methylglucose within the cells is the same in the presence or absence of insulin, and this represents the equilibrated distribution of this sugar within the intracellular water space. The apparent explanation for the rapid non-linearity of uptake in Fig. 6 is that, unlike 2-deoxyglucose, 3-*O*-methylglucose is not phosphorylated and, consequently, is not 'trapped' within the cell. Therefore, since the adipocyte intracellular water space is comparatively small, the intracellular concentration of this sugar rapidly rises, leading to significant efflux at very early time points. There is additional evidence which suggests that 3-*O*-methylglucose uptake may not completely reflect the D-glucose-transport system. Fig. 3 demonstrates that the K_m for 2-deoxyglucose uptake is approx. 2.5 mM, and Fig. 7 examines the ability of D-glucose and 3-*O*-methylglucose to compete with 2-deoxyglucose for uptake. In these experiments the K_i for D-glucose was 1.9 mM, and that for 3-*O*-methylglucose was 6.1 mM. Thus D-glucose and 2-deoxyglucose share essentially the same affinity for the transport system, whereas the affinity of 3-*O*-methylglucose for this system is lower. Further evidence for this idea has been presented by Czech (1976*a*), who found that adipocytes could take up 3-*O*-methylglucose by a non-saturable but non-diffusion-mediated process, which could be inhibited by cytochalasin B. This finding is confirmed in Fig. 8, which demonstrates a non-saturable (high-K_m) process for 3-*O*-methylglucose uptake even after correction for inward diffusion (uptake in the presence of cytochalasin B). However, even at the early time points used in these experiments (10 s), uptake is non-linear. This means that part of the uptake reflects transmembrane equilibration with the intracellular water space, and this process should be a linear function of the extracellular 3-*O*-methylglucose concentration. Thus this

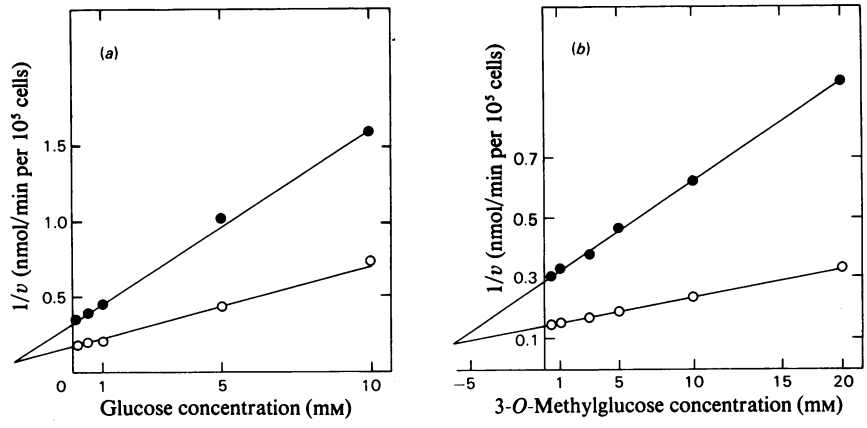

Fig. 7. *Dixon plots of the inhibitory effect of* D-*glucose* (*a*) *and* 3-*O-methylglucose* (*b*) *on* 2-*deoxyglucose uptake*
The concentrations of 2-deoxyglucose used were 1 mM (●) and 3 mM (○). The K_i value is 1.9 when D-glucose is used as the inhibitor and 6.1 when 3-*O*-methylglucose is used as the inhibitor. Data are from a representative experiment. Five similar experiments yielded K_i values for D-glucose with a range of 1.0 to 2.3 mM, and 4.5 to 9.0 mM for 3-*O*-methylglucose. Four experiments analysing Lineweaver–Burk plots in the presence or absence of a single concentration of inhibitor gave K_i values essentially identical with those illustrated.

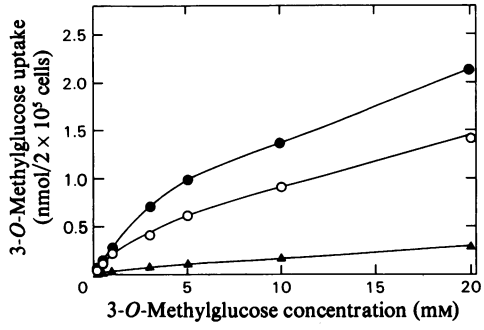

Fig. 8. *Effect of* 3-*O-methylglucose concentration on uptake* (*at* 10*s*) *in the control state* (●) *and in the presence of* 1 *mM-*D-*glucose* (○) *or* 50 *μM-cytochalasin B* (▲)
Control data are uncorrected and it should be noted that the slope of the uptake curve in the presence of cytochalasin B is considerably less than the non-saturable component in the absence of this agent (upper two curves). Data represent the average of two experiments.

could explain at least part of the apparent non-saturable uptake process. Because of the results presented in Figs. 6–8, 3-*O*-methylglucose has not proved to be an accurate probe for quantitative analysis of glucose transport in white adipocytes in our hands. On the other hand, insulin-mediated increases in 3-*O*-methylglucose uptake are readily demonstrated (Fig. 6), and influx measurements of this analogue clearly can be validly used to elucidate certain important mechanistic aspects of adipocyte D-glucose transport (Czech *et al.*, 1974; Czech, 1976*a*,*b*). It should also be emphasized that this discussion does not apply to exchange diffusion or efflux studies, and this latter approach has been successfully used to illustrate several important quantitative aspects of adipocyte glucose transport (Vinten *et al.*, 1976; Vinten, 1976).

Mechanisms of the insulin-mediated increase in the $V_{max.}$ of glucose transport

With the assurance that 2-deoxyglucose uptake accurately reflects D-glucose transport, the ability of insulin to increase the transport $V_{max.}$ was further explored. Given the standard model for the facilitated glucose-transport system (Narahara & Ozand, 1963; Stein, 1967; Lefevre, 1972; Kletzien & Perdue, 1974):

$$S+C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} SC \xrightarrow{k_{+2}} C+S \qquad (1)$$

where S is substrate, C is carrier, k_{+1} and k_{-1} are the association and dissociation rate constants between substrate (glucose) and carrier on the outer surface of the plasma membrane, and k_{+2} is the rate constant for the transmembrane mobility of glucose across the plasma membrane, then:

$$V_{max.} = [C] \cdot k_{+2} \qquad (2)$$

It is apparent then that insulin can increase $V_{max.}$ by either increasing the number of available carriers (C) or by increasing the mobility (k_{+2}) of existing, and already functioning, carriers. The slowest (rate-determining) step in glucose transport is considered

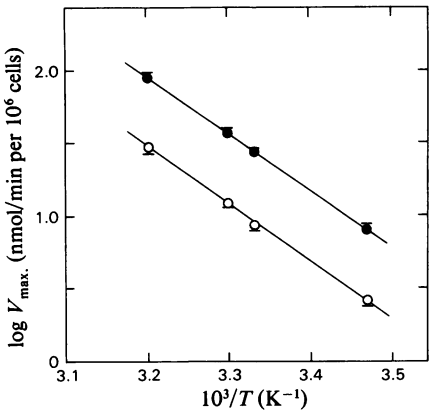

Fig. 9. *Arrhenius plots of basal* (○) *and insulin-activated* (●) *glucose transport*

Cells were preincubated with (25ng/ml) or without insulin for 90min before assessment of transport. Studies were performed at 37, 30, 24 and 15°C. Identical results were obtained whether the temperature during the 90min preincubation with insulin was the same as that during the transport determination, or whether the preincubations were all carried out at 24 or 37°C. Data represent the mean (±S.E.M.) of seven separate experiments.

to be k_{+2}, and thus the temperature-sensitivity of overall glucose transport will be proportional to the temperature-dependence of k_{+2} (Narahara & Ozand, 1963; Stein, 1967; Lefevre, 1972; Kletzien & Perdue, 1974). Consequently, if insulin causes increased k_{+2} values, the activation energies of basal and insulin-stimulated glucose transport will be different; if insulin leads to the conversion of inactive into active transport units (increased C) then the activation energies of basal and insulin-stimulated transport will be the same. To assess these possibilities the $V_{max.}$ of transport was determined at various temperatures, and the data were analysed by the Arrhenius equation (Fig. 9). The relationships are linear, and parallel, and the activation energies for basal and insulin-stimulated glucose transport are the same (59kJ/mol). Thus the data in Fig. 9 demonstrate that insulin has the same ability to increase $V_{max.}$ at all temperatures. Identical results are obtained whether the 90min preincubation with insulin is conducted at the same temperature as indicated for the transport study or whether the preincubations are all performed at 24 or 37°C.

Discussion

The current results demonstrate that insulin augments glucose transport by increasing $V_{max.}$ without changing the K_m value, and this agrees with other reports (Narahara & Ozand, 1963; Denton *et al.*, 1966; Czech *et al.*, 1974; Clark *et al.*, 1975; Olefsky, 1975; Vinten *et al.*, 1976; Halperin *et al.*, 1977). Little information exists, however, as to the mechanisms of this increase in $V_{max.}$. From eqns. (1) and (2), it is apparent that insulin can increase maximal transport capacity by either increasing the number of activated transport units (C), or by increasing the mobility (k_{+2}) of already functioning transport units. In the first formulation glucose-transport units can exist in the plasma membrane in an active and inactive state, and those units active in the absence of insulin account for basal rates of glucose transport. Insulin, then, activates existing but previously inactive carriers, thereby leading to an increase in $V_{max.}$. In the second formulation, all of the existing carriers are functional in the basal state, and insulin increases $V_{max.}$ by enhancing the mobility (k_{+2}) of each of these units. These alternatives can be assessed by determining the energy of activation of basal and insulin-activated glucose transport, since the activation energy of glucose transport is essentially the activation energy of the rate-determining step (presumably k_{+2}, Kletzien & Perdue, 1974). If insulin increases carrier concentration (C), then the activation energies of basal and insulin-stimulated transport will be the same (Narahara & Ozand, 1963; Kletzien & Perdue, 1974). If insulin increases $V_{max.}$ by increasing k_{+2}, then the effect of insulin on this rate constant will be less the higher the temperature, since k_{+2} will already be augmented (and possibly maximally increased) by the higher temperature. However, the ability of insulin to augment $V_{max.}$ is the same at all temperatures, and measurements of activation energy (Fig. 9) revealed equal values (59kJ/mol) in both states, indicating that insulin increases $V_{max.}$ by increasing the number of available glucose-transport units (converting inactive into active carriers). Although it is highly likely that k_{+2} represents the slowest step of glucose transport (Narahara & Ozand, 1963; Stein, 1967; Lefevre, 1972; Kletzien & Perdue, 1974), the present data do not prove this point. However, since $K_m=(k_{-1}+k_{+2})/k_{+1}$, then, since insulin does not change K_m, k_{+2} is either very small relative to k_{-1}, or insulin does not affect k_{+2}. In either case, this would mean that insulin increases $V_{max.}$ by increasing [C]. Further, the above line of reasoning does not require that the rate-determining step be specified. All that is necessary is that some step of glucose transport is rate-determining, and that if insulin enhanced the activity of already functioning glucose-transport units, then it would augment this step. Since the Arrhenius plots are parallel, the argument is still valid that insulin increases the number of carriers, and does not accelerate the rate-determining step of glucose transport (even if that step is not represented by k_{+2}). Similar conclusions have been reached by Vinten (1976), who studied 3-*O*-methylglucose efflux.

The conclusions presented rely on the assumption that measurements of 2-deoxyglucose uptake provide an accurate assessment of glucose transport. The evidence presented strongly supports this contention. For example, at all substrate concentrations, the time course of 2-deoxyglucose uptake was strictly linear over the period of study. This indicates that true influx was measured, rather than net uptake complicated by significant efflux. Further, when intracellular hexoses were assessed (Fig. 5, Table 1), intracellular 2-deoxyglucose was either undetectable, or very low, relative to the buffer 2-deoxyglucose concentration, and essentially identical results have been reported by Chandramouli & Carter (1977). Thus since transport in either direction is concentration-dependent (Olefsky, 1975; Vinten *et al.*, 1976), and since only intracellular 2-deoxyglucose, and not phosphorylated sugars, can be transported out of the cell, sugar efflux must be absent, or minimal relative to influx. For these reasons, 2-deoxyglucose uptake provides an accurate and convenient measure of the adipocyte glucose-transport system under the conditions used.

3-*O*-Methylglucose is another glucose analogue widely used to study glucose transport. However, unlike 2-deoxyglucose, this agent cannot be 'trapped' by phosphorylation, and efflux is significant at very early time points. The time course of uptake of this analogue is rapidly curvilinear (Fig. 6), owing to rapid filling of the relatively small intracellular water space of adipocytes, and initial uptake rates cannot be assessed. Thus rigorous quantitative data, suitable for kinetic analysis, are difficult to obtain by measuring adipocyte 3-*O*-methylglucose uptake, and such studies must await techniques that allow accurate measurement of uptake within 1–2s of the onset of flux.

The results presented may also bear on another aspect of insulin action, although in a more inferential way. According to the mobile-receptor hypothesis, occupied insulin receptors laterally diffuse within the plane of the plasma-membrane lipid bilayer to activate effector units (Jacobs & Cuatrecasas, 1976). With this formulation one might expect that the degree of plasma-membrane fluidity would influence insulin action. However, as the incubation temperatures were lowered from 37 to 15°C, the plasma membrane became much less fluid, and yet the ability of occupied insulin receptors to activate glucose transport was unimpaired (Fig. 9); in other words, the Arrhenius plot for insulin-stimulated transport remained linear. These observations suggest, but do not prove, that although insulin receptors may undergo lateral planar diffusion, this receptor mobility may not be an essential feature of insulin action. Clearly more direct studies will be necessary to substantiate this idea. On the other hand, as a minimal conclusion, one can infer that receptor mobility is not a rate-determining step of the action of insulin on glucose transport.

In summary, these studies show that 2-deoxyglucose uptake provides an accurate measurement of adipocyte glucose transport, and that insulin accelerates glucose transport by increasing the maximal transport capacity through an increase in the number of available transport units. This latter effect most probably involves the conversion of pre-existing but inactive carriers into an active form.

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