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CYTOCHALASIN B INHIBITION AND TEMPERATURE DEPENDENCE OF 3-*O*-METHYLGLUCOSE TRANSPORT IN FAT CELLS

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Summary

The transport of 3-*O*-methylglucose in white fat cells was measured under equilibrium exchange conditions at 3-*O*-methylglucose concentrations up to 50 mM with a previously described method (Vinten, J., Gliemann, J. and Østerlind, K. (1976) J. Biol. Chem. 251, 794—800). Under these conditions the main part of the transport was inhibitable by cytochalasin B. The inhibition was found to be of competitive type with an inhibition constant of about $2.5 \cdot 10^{-7}$ M, both in the absence and in the presence of insulin (1 μM). The cytochalasin B-insensitive part of the 3-*O*-methylglucose permeability was about $2 \cdot 10^{-9}$ cm $\cdot$ s^{-1}, and was not affected by insulin. As calculated from the maximum transport capacity, the half saturation constant and the volume/surface ratio, the maximum permeability of the fat cell membrane to 3-*O*-methylglucose at 37°C and in the presence of insulin was $4.3 \cdot 10^{-6}$ cm $\cdot$ s^{-1}. From the temperature dependence of the maximum transport capacity in the interval 18—37°C and in the presence of insulin, an Arrhenius activation energy of 14.8 ± 0.44 kcal/mol was found. The corresponding value was 13.9 ± 0.89 in the absence of insulin. The half saturating concentration of 3-*O*-methylglucose was about 6 mM in the temperature interval used, and it was not affected by insulin, although this hormone increased the maximum transport capacity about ten-fold to 1.7 mmol $\cdot$ s^{-1} per l intracellular water at 37°C.

Introduction

Direct measurements of the efflux of [^{14}C]methylglucose from isolated, epididymal rat fat cells have shown that equilibrium exchange of methylglucose takes place by a stereospecific, saturable process [1]. It was further demonstrated that insulin (in a supramaximal concentration) increases the rate of methylglucose exchange by the same factor for a wide range of methylglu-

Abbreviation: Methylglucose, 3-*O*-methylglucose.

cose concentrations, and that the insulin-induced relative increase of incorporation of ^{14}C from [U-^{14}C]glucose into intracellular lipids was of a similar size to the the increase in methylglucose exchange rate, provided a low glucose concentration was used [1].

It has been reported by Czech [2] that a large fraction of the total methylglucose influx at high (20 mM) methylglucose concentrations occurs by a non-saturable and cytochalasin B-insensitive pathway. An unspecific contribution to the methylglucose transport could invalidate inferences on the mechanism of action of insulin based on measurements of the total transport of methylglucose, as it could limit the range of permeabilities which were achievable at different hormone concentrations.

The present study was carried out in order to characterize the basal and maximally insulin-stimulated transport mechanism with respect to unspecific contribution, cytochalasin B inhibition kinetics and temperature sensitivity.

Methods

Preparation of fat cells. Isolated epididymal fat cells were prepared from Wistar rats weighing 110—140 g using the method of Rodbell [3] with some modifications [4]. Crude collagenase from Worthington (type I, lot 45A128) and from Sigma (type I, lot 5c-0337 and type II, lot 15c-6890) was used. [3-^3H]Glucose (12 μCi/mmol) and [^{14}C]methylglucose (59 mCi/mmol) was from Amersham, unlabeled methylglucose and cytochalasin B from Calbiochem. Bovine serum albumin (fraction V) from Sigma was dialysed five times against redistilled water before use. Insulin was from Novo (porcine MC, 27 units/mg) and was used at a concentration of 0.7 or 1 μM. The medium used for incubation of fat cells contained: 130 mM Na^+, 6 mM K^+, 1.2 mM Mg^{2+}, 2.5 mM Ca^{2+}, 128.5 mM Cl^-, 1.2 mM $H_2PO_4^-$, 1.2 mM SO_4^{2-}, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 1% (w/w) albumin. The pH 7.36 was adjusted to 7.40 at 28° C. The resulting pH values at 37 and 18° C were 7.44, respectively; this change in pH, when produced by addition of HCl or NaOH at 28° C, was without effect on the methylglucose exchange rate at 50 mM methylglucose in the presence or in the absence of insulin (data not shown).

Measurement of methylglucose equilibrium exchange. Approximately $2 \cdot 10^6$ fat cells were loaded with unlabeled methylglucose for 30 min before addition of [^{14}C]methylglucose; the equilibration with the tracer, which took place in a total volume of about 500 μl, was allowed to proceed for at least 5 times the expected half time of equilibration; cytochalasin B (when used) was added hereafter. This part of the procedure was carried out at 37° C, since the temperature during the loading of the cells was without effect on the subsequent efflux of [^{14}C]methylglucose (see Results). After concentrating the suspension by centrifugation, the [^{14}C]methylglucose efflux was initiated by resuspending the cells in 10 ml of medium containing unlabeled methylglucose at a concentration identical to that in the loading medium and kept at the appropriate temperature. The change in temperature produced by resuspending the cells was less than 0.4° C. Samples of the suspension were taken during the efflux and the cells recovered by centrifugation through silicone oil as previously

described [1]. The amount of [^{14}C]methylglucose in the cell pellet was determined by liquid scintillation counting; ^{3}H counts in the intracellular lipids incorporated from [3-^{3}H]glucose present during the preparation of the cells served as a measure of the amount of cells per sample and was used to correct for variations in sample size. The amount of [^{14}C]methylglucose in samples taken 1 h after the time of resuspension was determined in quadruplicate; this amount was from about 1% to 5% of the amount present shortly after resuspension, when the half time of [^{14}C]methylglucose efflux was below 5 min. When appropriate, insulin was present from the loading of the cells with methylglucose.

Determination of fat cell size. A 10 μl sample of the cell suspension was withdrawn along with the samples taken during the [^{14}C]methylglucose efflux and placed in a chamber with a siliconized glass cover slide. The diameters of 100 randomly selected cells were measured by means of a microscope equipped with an ocular micrometer with divisions equivalent to 2.5 μm at 400× magnification. The coefficient of variation of the cell diameters within the samples varied from 0.12 to 0.16 (range for 8 samples each consisting of 100 cells from different cell pools).

Calculations. The mean exchange rate constant for methylglucose was determined by means of a non-linear regression of the intracellular amounts of [^{14}C]methylglucose (corrected for variations in sample size) on the time elapsed from the decrease of the specific activity of the extracellular methylglucose produced by resuspending the cells. The model used is based on the assumption that the exchange rate constants are gamma distributed on the cells and that the disappearance of [^{14}C]methylglucose from each individual cell is monoexponential. The function is:

$$a_x = (a_0 - a_\infty) \exp(-r^{-2} \cdot \log_e(1 + r^2\bar{k}t)) + a_\infty ,$$

where a_x is the amount of intracellular radioactivity at time x, r is the coefficient of variation for the gamma distribution, t the time and $\bar{k}$ the mean exchange rate constant, equivalent to the initial, fractional isotopic efflux. In the calculation, r was assigned a constant value of 0.5 [1]. In cases where the half time for the efflux of [^{14}C]methylglucose exceded 30 min, (e.g. due to the presence of inhibitor) an estimate of a_∞ from a parallel curve (without the inhibitor) was entered in the calculation as a fixed value. It should be noted that the efflux curves did not deviate much from a monoexponential decrease, when the efflux was followed for less than 2 half times; under these conditions the rate constant estimated by assuming a monoexponential relationship is less than 10% smaller than the mean rate estimated by assuming a heterogeneity among the exchange rate constants of the cells of 50% [1]. If the efflux is followed for longer periods, however, a larger bias would result from application of the monoexponential model [1].

The relation between the mean exchange rate constant, the methylglucose concentration and the concentration of cytochalasin B was treated as competitive inhibition of a simple, saturable process; a constant term was added to allow for a non-saturable (and non-inhibitable) contribution:

$$\bar{k} = \frac{J}{C_{MG}} = \frac{J_m}{K_t} \cdot \frac{1}{1 + \frac{C_{MG}}{K_t} + \frac{C_{CB}}{K_i}} + \frac{J_{res}}{C_{MG}} \qquad (1)$$

where J is the unidirectional transport of methylglucose across the membrane area of a number of cells with an intracellular distribution space for methylglucose of 1 l (mmol $\cdot$ s^{-1} $\cdot$ l^{-1}); C_{MG} (mM) and C_{CB} (μM) are the concentrations of methylglucose and cytochalasin B; J_m is the maximum transport through a saturable pathway with half saturation constant K_t; K_i is the inhibition constant for cytochalasin B and J_{res} the transport through the non-saturable and non-inhibitable pathway.

The apparent permeability (cm $\cdot$ s^{-1}) was obtained by multiplying the mean exchange rate constant by the V/A ratio, where V (cm^3) is the intracellular distribution space for [^{14}C]methylglucose in a sample of cells with a total surface area A (cm^2). Thus:

$$\bar{k} \cdot \frac{V}{A} = P = P_m \frac{1}{1 + \frac{C_{MG}}{K_t} + \frac{C_{CB}}{K_i}} + P_{res} \tag{2}$$

where P is separated into a part dependent on the concentration of methylglucose and cytochalasin B (with a maximum value P_m), and a residual, concentration independent part, P_{res}.

The relation between the methylglucose concentration and the concentration of cytochalasin B which half-inhibits the cytochalasin B-sensitive part of the mean exchange rate constant was derived as follows. When k' denotes the cytochalasin B-sensitive part of the mean exchange rate constant in the absence of cytochalasin B and k'_{CB} the corresponding quantity in its presence, then (from Eqn. 1):

$$k' = \frac{J_m}{K_t} \cdot \frac{K_i}{K_i\left(1 + \frac{C_{MG}}{K_t}\right)} \tag{1a}$$

and

$$k'_{CB} = \frac{J_m}{K_t} \cdot \frac{K_i}{K_i\left(1 + \frac{C_{MG}}{K_t}\right) + C_{CB}} \tag{1b}$$

Division of the difference between the reciprocal forms of Eqns. 1a and 1b with Eqn. 1a gives (after slight rearrangement):

$$\frac{C_{CB} \cdot k'_{CB}}{k' - k'_{CB}} = K_i\left(1 + \frac{C_{MG}}{K_t}\right) \tag{3}$$

By inspection of Eqn. 1, the right part of Eqn. 3 can be seen to equal the concentration of cytochalasin B which half inhibits the cytochalasin B-sensitive part of the mean exchange rate constant for a fixed concentration of methylglucose.

The V/A ratio was calculated by multiplying the ratio between total volume and surface of the cells of a sample with the intracellular distribution space for [^{14}C]methylglucose expressed as fraction of the total cell volume. The total volume and surface was calculated from the sums of the squares and the cubes

of the cell diameters assuming the cells to be spherical. The intracellular distribution space for [^{14}C]methylglucose was calculated from the intercept of an efflux curve and the [^{14}C]methylglucose concentration of the loading medium. The corresponding total cell volume was calculated from the ^{3}H activity of the samples of the curve and the ^{3}H activity of a known volume of cells; the latter volume was determined by centrifuging part of the suspension in a capillary tube and correcting for extracellular water.

Results

Intracellular distribution space for [^{14}C]methylglucose and cell surface area. The conversion of the mean rate constant for tracer equilibration under equilibrium exchange conditions to an apparent permeability (cm $\cdot$ s^{-1}) or flux (mol $\cdot$ cm^{-2} $\cdot$ s^{-1}) facilitates comparisons with results obtained on other types of cells, because these quantities are measures of a membrane property and not directly dependent on the size or morphology of the cells under study. Therefore, the ratio between the intracellular distribution space for [^{14}C]methylglucose and the cellular surface area was determined. Table I gives an estimate for the cells used in the present study; for comparison, values reported by others are also given. The present value of $1.5 \cdot 10^{-5}$ cm was used throughout to calculate the permeability from the mean exchange rate constant. It should be noted that the intracellular distribution space for [^{14}C]methylglucose has been found to be identical with the intracellular distribution space for $^{3}H_2O$ and [^{14}C]urea in earlier reports [1,8].

The non-saturable part of the total methylglucose transport. The concentration dependence of methylglucose transport measured under equilibrium exchange conditions was determined in a series of eight experiments, one of which is shown in Fig. 1. The left part is a semilogarithmic plot of the amount of tracer remaining in the cells vs. time after resuspension. The right part shows

TABLE I

VOLUME/SURFACE RATIO FOR ISOLATED FAT CELLS

(1) The intracellular distribution space for [^{14}C]methylglucose was determined from the intercept of the efflux curve (exchange conditions), and the mean cell volume and area was calculated from the optical measurement of cell diameters assuming the cells to be spherical (see Methods). The S.D. given is the variation between cell pools, each prepared from 4—6 rats weighing 110—140 g. (2) Read from figure 1 of ref. 5. The distribution space is the difference between the spaces for $^{3}H_2O$ and [^{14}C]inulin in a cell pellet. (3) Calculated from table III of ref. 6 using a value of 6 pg DNA per cell [7]. The distribution space is the difference between the spaces for [^{14}C]urea and [^{3}H]inulin. (4) Intracellular distribution space for [^{14}C]methylglucose given in the text of ref. 2.

Reference	Mean cell volume (pl/cell)		Intracellular distribution space (pl/cell)	Intracellular volume / Surface area (cm $\cdot$ 10^{-5})
(1) Present cells	Mean	87	1.4	1.5
	S.D. (n = 8)	34	0.3	0.5
(2) [5]		100	2.0	1.9
(3) [6]	—		1.8	—
(4) [2]	—		0.5—1.5	—

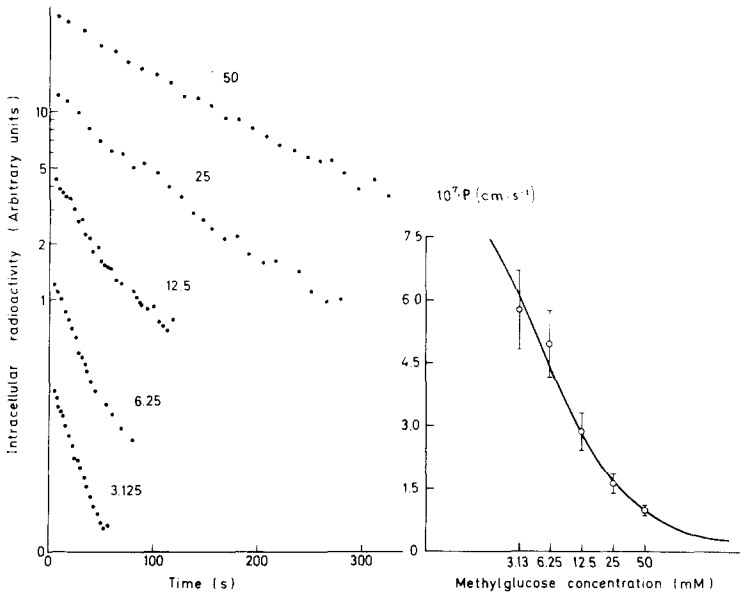

Fig. 1. The relation between methylglucose concentration and methylglucose permeability (P). Left: $2 \cdot 10^6$ fat cells were equilibrated with the indicated concentrations of methylglucose for 30 min at 37°C in the presence of a maximally-stimulating concentration of insulin (0.7 μM). The cell suspension was concentrated, [^{14}C]methylglucose added to a final concentration of 10 μCi/ml in an extracellular volume of about 300 μl and the incubation continued for 20 min. Hereafter, the extracellular volume was reduced to 10—15 μl by brief centrifugation, and the cells were resuspended at time zero in 10 ml of a vigorously stirred medium identical to that used for the initial loading, but kept at 18°C. This decreased the concentration of [^{14}C]methylglucose in the extracellular phase by 600 to 1000-fold. The points represent the [^{14}C]methylglucose content of samples of the cells taken during the efflux and isolated from the medium by centrifugation through silicone oil at the indicated times. Note that the runs are spaced arbitrarily in the direction of the ordinate to obtain visual separation. Right: The permeabilities calculated from the runs shown to the left, plotted against the methylglucose concentration (log scale). The bars represent ± S.E. calculated from the runs shown to the left, and the curve was obtained by a least squares fit of Eqn. 2.

the apparent permeability for [^{14}C]methylglucose calculated from the runs shown to the left and plotted against the logarithm of the total concentration of methylglucose. The curve represent a least squares fit of a model given in *Calculations* (Eqn. 2), composed as the sum of a term with a hyperbolic concentration dependence and a concentration-independent term. The concentration-independent permeability was estimated in the least squares fit, and in agreement with the visual impression from Fig. 1, it was not significantly different from zero. More important, its maximum possible size was about $2.5 \cdot 10^{-8}$ cm · s^{-1}, or about 3% of the permeability extrapolated to zero methylglucose concentration; although this might seem a small fraction, the experiment does not exclude the possibility that up to 30% of the permeability observed at 50 mM is due to transport through a non-saturable pathway. This is not tolerable when it is desired to estimate the maximum transport capacity from a determination of the mean exchange rate at a single methylglucose concentration which is so high that the estimate becomes insensitive to small variations in the half saturation constant.

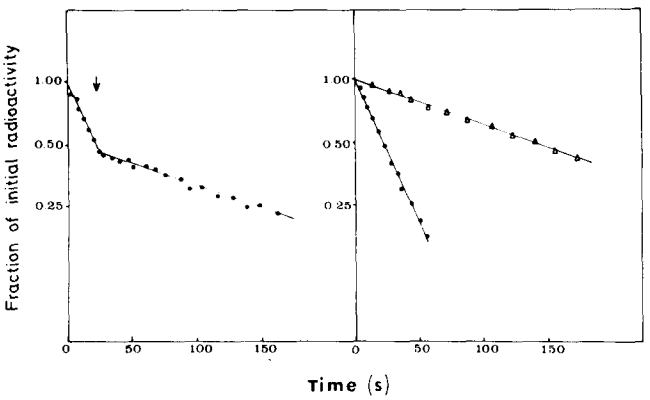

Fig. 2. Onset of action and reversibility of inhibition of methylglucose exchange by cytochalasin B. The experiment was carried out at 37°C, total concentration of methylglucose was 50 mM, insulin was present during loading and washout at a concentration of 1 μM. The points represent the amount of intracellular [^{14}C]methylglucose as fraction of the amount at time zero plotted against time after resuspension of the cells in a medium containing unlabeled methylglucose. Left: The arrow indicates time of addition of cytochalasin B to a final concentration of 10 μM. Right: triangles indicate that 10 μM cytochalasin B was present from 3 min before resuspension. Circles indicate concentration of cytochalasin B (10 μM) from −3 min to zero, at which time the extracellular medium was diluted about 800-fold by the resuspension. The lines were drawn by eye.

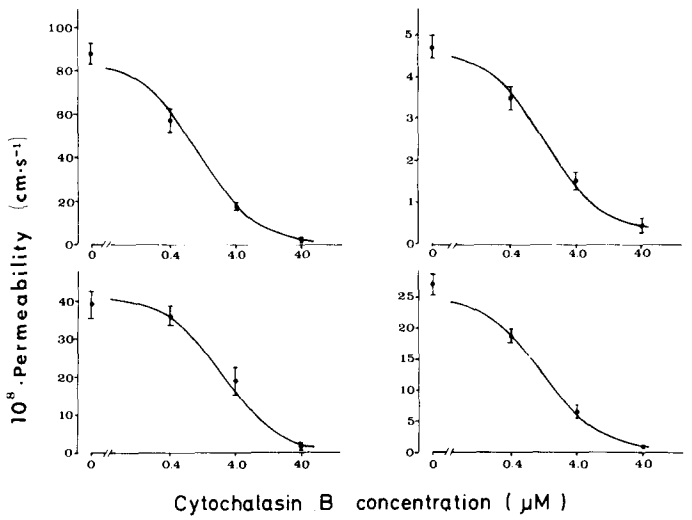

Fig. 3. Dependence of the methylglucose permeability on the concentration of cytochalasin B. The points were obtained from tracer efflux curves as those shown in Fig. 1, left, but at various concentrations of cytochalasin B. The bars represent ± S.E., and the curves are least squares fits of Eqn. 2. Insulin, when present, was used at a concentration of 1 μM. Upper left, 20 mM methylglucose, 37°C, insulin present; upper right, 20 mM methylglucose, 28°C, no insulin; lower left, 50 mM methylglucose, 37°C, insulin present; lower right, 20 mM methylglucose, 18°C, insulin present. The half times for efflux of [^{14}C]-methylglucose were from 12 s to 18 min; the duration of the sampling periods was from 100 s (interval between samples approx. 3 s) to 30 min (interval between samples approx. 1 min).

Inhibition of methylglucose exchange with cytochalasin B. In the search for a tool for estimating the non-saturable part of the methylglucose permeability, the inhibitory properties of cytochalasin B were investigated. Fig. 2, left, shows the effect of addition of this inhibitor during the efflux of [^{14}C]methylglucose under exchange conditions. With the obtainable time resolution, no delay from addition to onset of inhibition can be demonstrated. Similarly, the effect disappears without visible delay when the cell suspension is diluted (Fig. 2, right).

The dependence of the apparent methylglucose permeability on the concentration of cytochalasin B is shown in Fig. 3. The curves represent a least squares fit of a model with a term corresponding to a simple, reversible inhibition of part of the permeability and a constant term corresponding to a residual, cytochalasin B-insensitive part of the permeability. It is seen that insulin changes the permeability about ten-fold without affecting the inhibitory pattern or half-inhibiting concentration of cytochalasin B, which, in turn, seems to increase with the concentration of methylglucose. This finding is further explored in Fig. 4, a plot suitable for distinguishing competitive and non-competitive type of inhibition. As explained in the legend (Fig. 4), the points should be situated around a line intersecting the ordinate at K_i and the abscissa at $-K_t$, the half-saturation constant for methylglucose exchange, when inhibition is of the competitive type. For non-competitive inhibition, there is no correlation between inhibitor concentration and the saturation of the transport mechanism with the transported substance; the points should therefore be situated around a horizontal line. In the present case, there is a clear correlation between the methylglucose concentration and the half-inhibiting concentration for cytochalasin B, demonstrating that the inhibition is of competitive type. K_i is seen to be about $2.5 \cdot 10^{-7}$ M, and the half saturation constant for methylglucose equilibrium exchange is about 6 mM, in agreement with the earlier published value [1], and with other estimates given in the present paper.

The residual permeability is estimated in Fig. 5. The ordinate is the permeability to methylglucose, and the quantity on the abscissa represents the fraction of transport activity not occupied (inhibited) by methylglucose or cytochalasin B, calculated from the concentrations using the kinetic constants given above. The intercept of the lines with the ordinate thus represents the residual permeability for [^{14}C]methylglucose; this parameter could tentatively be taken as a maximum estimate of the permeability of the lipid matrix of the membrane to [^{14}C]methylglucose. It appears from Fig. 5 that a maximum possible value for P_{res} is about $4 \cdot 10^{-9}$ cm $\cdot$ s^{-1}. Due to the high avidity of the transport mechanism in the presence of insulin, a minimum value different from zero can only be determined in its absence. This minimum value is about $1.5\ \ 10^{-9}$ cm $\cdot$ s^{-1}, and a possible influence of insulin on P_{res}, if any, is therefore smaller than its influence on the cytochalasin B-inhibitable part of the permeability. The results do not permit evaluation of the relation between P_{res} and temperature.

Lack of effect of preincubation temperature on distribution space and efflux rate for [^{14}C]methylglucose. Fig. 6 shows that the intracellular distribution space for [^{14}C]methylglucose is the same, whether the loading of the cells with tracer prior to the efflux is carried out at 20 or at 37° C. The distribution space was found to be unaffected by the presence of insulin and to be independent of the

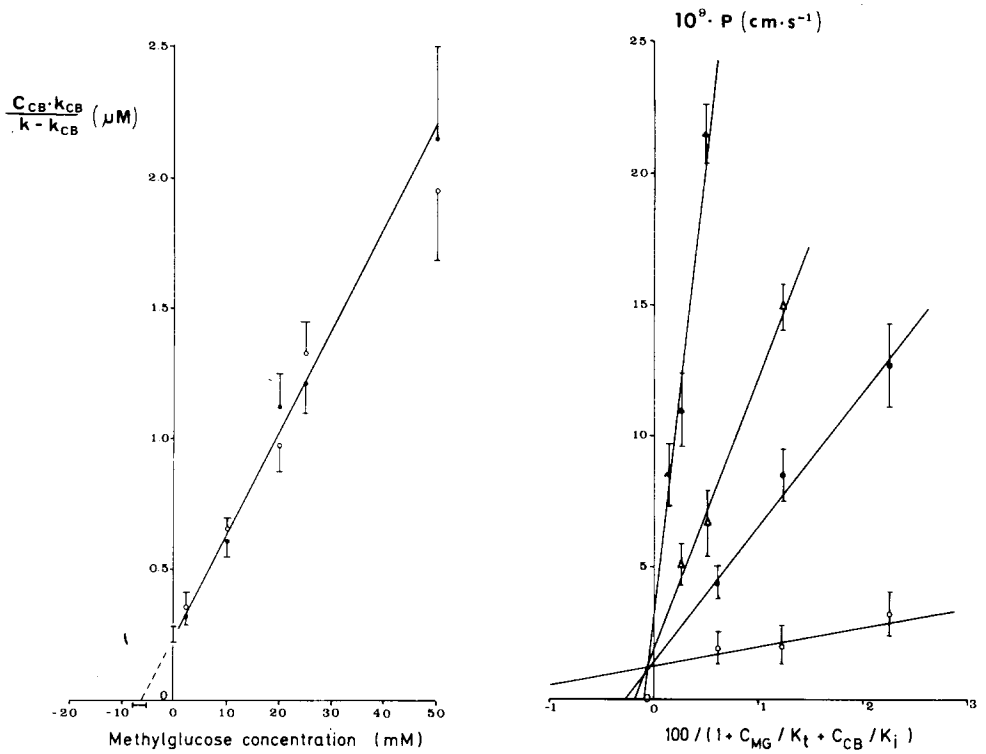

Fig. 4. Type of inhibition produced by cytochalasin B. The concentration of cytochalasin B ($K_{1/2}$) which half-inhibits the efflux rate of [^{14}C]methylglucose under equilibrium exchange conditions is plotted against methylglucose concentration. $K_{1/2}$ was calculated from a pair of efflux curves, one obtained in the absence of cytochalasin B, the other in its presence, using the left part of Eqn. 3:

$$K_{1/2} = \frac{C_{CB} \cdot k'_{CB}}{k' - k'_{CB}} = K_i\left(1 + \frac{C_{MG}}{K_t}\right)$$

where C_{CB} is the concentration of cytochalasin B, k'_{CB} the mean exchange rate constant in the presence of the inhibitor, k' the mean exchange rate constant in its absence, K_i the inhibition constant for cytochalasin B, C_{MG} the methylglucose concentration and K_t the half saturation constant for methylglucose exchange. The mean exchange rate constants were calculated from curves as those shown in Fig. 1, and the rate constant in the presence of inhibitor was from 0.73 to 0.16 of the rate constant in the control experiment. The inhibitor concentrations were 0.2—10 μM, and the half times for [^{14}C]methylglucose efflux were 10 s—5 min. The efflux curves were followed for from approx. 100 s (interval between samples approx. 3 s) to about 20 min (interval between samples approx. 40 s). The rate constants were corrected by subtracting a noninhibitable contribution of $2 \cdot 10^{-4}$ s^{-1}; this correction amounted to 9% of the observed rate in one case, and was otherwise below 5%. The bars represent an approximate S.E. calculated from the S.E. of the rate constants by standard methods [32]. The bars on the ordinate and abscissa indicates the interval (± S.E.) for the estimate of K_i and K_t as obtained from a weighted, linear regression of $K_{1/2}$ on C_{MG}. The filled symbols were obtained in the presence of insulin (1 μM) at 22°C, the open symbols were obtained in its absence and at 37°C.

Fig. 5. Estimation of the residual permeability (P) to methylglucose. The permeabilities (± S.E.) were obtained from tracer efflux curves as those of Fig. 1, and are plotted against a quantity representing the non-occupied fraction of the transport mechanism, calculated from Eqn. 2. The concentrations of methylglucose were 20—50 mM, and the concentrations of cytochalasin B were 10—100 μM. The half time for tracer efflux was from 7.5 min to 86 min, and the efflux was followed for from 0—10 to 0—60 min. Filled symbols, 37°C; open symbols, 18°C. Triangles, 1 μM insulin present; circles, no insulin. The lines were drawn by eye.

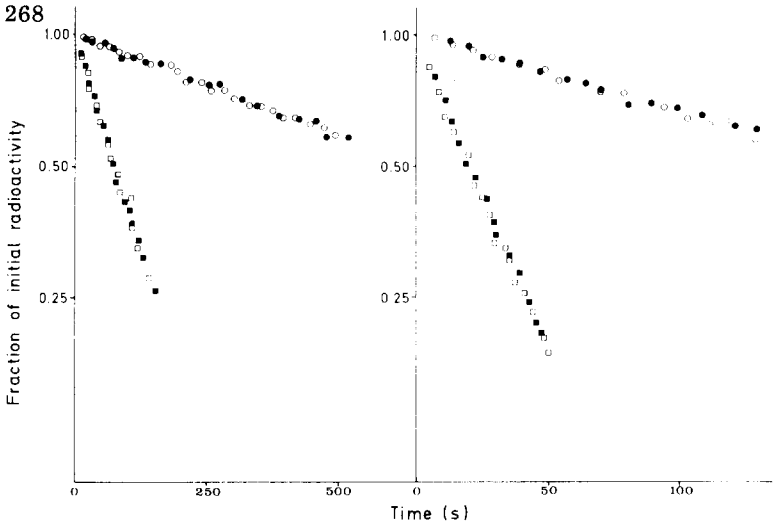

Fig. 6. Insulin effect on [^{14}C]methylglucose efflux under equilibrium exchange conditions at 20 and 37°C. The points represent the amount of intracellular [^{14}C]methylglucose as fraction of the amount at time zero (a common estimate for the eight runs shown) plotted against time after resuspension. The fat cells were equilibrated with [^{14}C]methylglucose (50 mM) for 30 min before resuspension. Insulin (1 μM) was present from the start of the equilibration period when used. Filled symbols, temperature during equilibration 37°C; open symbols, 20°C during equilibration. Circles, no insulin; squares, insulin present. Left, temperature during efflux 20°C; right, temperature during efflux 37°C. Note the difference in time scale.

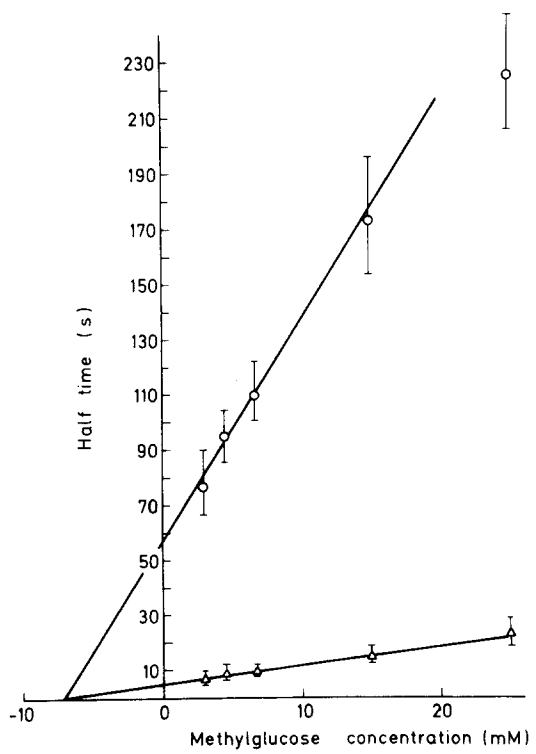

Fig. 7. Half time for [^{14}C]methylglucose efflux at different total concentrations of methylglucose. The half times were calculated from the mean exchange rate constants obtained from tracer efflux curves as those of Fig. 1, and they are plotted against the total methylglucose concentration. It can be derived from Eqn. 1 (see ref. 1 for a derivation) that this plot should produce a straight line with a slope of $\log_e(2)/J_m$ and an intercept of $-K_t$ on the abscissa, when the residual transport is neglected. The temperature was 28°C, the bars represent ± S.E.. Circles, no insulin; triangles, 0.7 μM insulin. Lines were drawn by eye.

TABLE II

ESTIMATES OF J_m AND K_t FOR METHYLGLUCOSE TRANSPORT UNDER EQUILIBRIUM EXCHANGE CONDITIONS AT DIFFERENT TEMPERATURES WITH AND WITHOUT INSULIN

The estimates were obtained from a weighted, linear regression of the reciprocal mean exchange rate on the methylglucose concentration (corresponds to Fig. 7 except for a factor of $\log_e(2)$ on the ordinate). Insulin was used at a concentration of 0.7 μM.

Temperature (°C)	No insulin		Insulin present	
	J_m	K_t	J_m	K_t
	(mmol · s^{-1} · l^{-1})	(mM)	(mmol · s^{-1} · l^{-1})	(mM)
18	0.087	12	0.30	7
	0.065	3	0.35	10
28	0.10	7	0.80	7
	0.13	6	0.71	3
37	0.21	5	1.7	8
	0.47	4	1.6	6

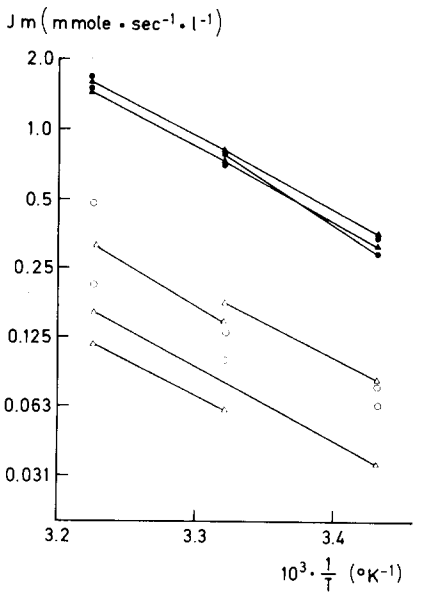

Fig. 8. Arrhenius plot of the maximum transport capacity for methylglucose in the absence and in the presence of insulin. The estimates of J_m were obtained from the concentration dependence of the exchange rate in the absence (○) and in the presence (●) of 0.7 μM insulin (experiments shown in Table II), or from the exchange rate at 50 mM methylglucose in the absence (△) or in the presence (▲) of insulin, using a common K_t value of 6 mM for the calculation: $J_m = \overline{k}_{50} \cdot (C + K_t)$, where $\overline{k}_{50}$ (the exchange rate at 50 mM methylglucose) was corrected for a non-saturable contribution by subtraction of a common value of $1.5 \cdot 10^{-4}$ s^{-1}; this amounts to 25% of the lowest measured value of $\overline{k}_{50}$ (cf. *Calculations* and Fig. 5). The points are connected with lines, when they are obtained from the same cell pool and at the same concentration of insulin (0.7 μM). From a linear regression of log J_m on $1/T$ the value of the Arrhenius activation energy (followed by its S.E.) was found to be 14.8 ± 0.44 kcal/mol in the presence of insulin. In its absence, this quantity was 13.9 ± 0.9 kcal/mol, when estimated by the mean ± S.E. of the slope of the lines. The geometric mean of the relative, insulin-induced increases of J_m is 6.76.

temperature during the efflux. The mean rate constant for equilibrium exchange of intracellular methylglucose is seen to be independent of the temperature during the loading of the cells, but decreases with the temperature during the washout. Furthermore, the relative increase of the exchange rate produced by insulin is not different at the two washout temperatures.

Temperature sensitivity of the kinetic parameters. Fig. 7 shows the effect of insulin on the half time for tracer equilibration under equlibrium exchange conditions at different methylglucose concentrations. The experiment was performed at 28° C. As demonstrated earlier [1], at 37° C the action of insulin is to increase J_m of methylglucose exchange, whereas no detectable change occurs in K_t. Table II gives kinetic parameters calculated from six concentration dependencies, performed at different temperatures but otherwise similar to the experiment shown in Fig. 7. It appears from the table that the estimates of J_m in the presence of insulin are reproducible, and that this parameter is decreased about two-fold by lowering the temperature about 10° C. The estimates of K_t are more variable, and no obvious correlation with temperature is present. In the absence of insulin, the estimates of J_m are lower than the ones obtained in presence of insulin at each temperature, but they constitute a rather variable fraction of the J_m values found in the presence of insulin and their temperature dependence is, therefore, not accurately determined. The estimates of K_t in the absence of insulin are not correlated to the temperature, and they are not different from those obtained in the presence of insulin. Fig. 8 shows a plot of J_m against the reciprocal of absolute temperature; the values are those of Table II, but in order to get a better evaluation of the temperature dependence in the absence of insulin, J_m was also estimated from the exchange rate at a methylglucose concentration of 50 mM, using a K_t value of 6 mM. This approach made it possible to calculate J_m from results on cell samples from the same cell batch, thereby improving the estimate of the temperature sensitivity of J_m in the absence of insulin.

Assuming a linear relation between the logarithm of J_m and the reciprocal of the absolute temperature, the Arrhenius activation energy for methylglucose exchange at complete saturation was 14.8 ± 0.44 kcal/mol in the presence of insulin and 13.9 ± 0.89 kcal/mol in its absence. These values are not significantly different. They are also rather similar to the values given by Hankin and Stein [9] for the activation energy of the maximum exchange capacity for glucose in human erythrocytes (16.3 ± 0.9 kcal/mol). The maximum transport capacity in the absence of insulin was rather variable, and it constituted 14.8% of the capacity in the presence of insulin (geometric mean for the points used for calculating the Arrhenius activation energy).

If the rate of methylglucose translocation by full saturation of the transport mechanism is determined by one reaction, and insulin accelerated this reaction only by decreasing its activation enthalpy, this decrease should amount to about 1.1 kcal/mol *. The outcome of the experiment (an increase of 0.9 kcal/

* With these assumptions, the ratio between the expressions for the maximum rate of methylglucose translocation in the absence and in the presence of insulin written in terms of the absolute reaction-rate theory (see e.g. ref. 29) reduces to:

$$14.8\% = \frac{J_{m,0}}{J_{m,ins}} = \frac{\exp - (\Delta H^{\dagger}/RT)}{\exp - [(\Delta H^{\dagger} - \delta)/RT]} \Leftrightarrow$$

(Footnote continued on next page.)

mol) seems rather unlikely under this assumption, but it is not rejected at the 5% level of significance.

Discussion

Saturable methylglucose transport. It can be calculated from the present and previously published results [1] that the half time for equilibration between the extracellular compartment and the intracellular aqueous space of fat cells with methylglucose in concentrations much below K_t is about 2.5 s, at 37°C when insulin is present in a maximally stimulating concentration. For a non-concentrative transport mechanism, even if it is asymmetric, this figure should be the same whether net influx, equilibrium exchange or net efflux is measured, as long as the fractional saturation of the mechanism is low [10] and the extracellular compartment much greater than the intracellular. Recent net influx experiments on fat cells [11] confirm the half time of 2.5 s, and this does not disagree with the experiments reported by Olefsky [12], as he showed that methylglucose was in equilibrium over the fat cell membrane after 20 s. Methylglucose and glucose have been shown to have almost identical transport characteristics in human erythrocytes [13], and that this could be the case also for fat cells is indicated by the fact that the unidirectional transport of methylglucose at a concentration of 0.55 mM, in the presence of insulin and at 37°C, which can be calculated from the present results ($2.2 \cdot 10^{-16}$ mol $\cdot s^{-1}$ per cell), *, is rather similar to the rate of incorporation of [U-^{14}C]glucose (0.55 mM) into triglycerides found in previous experiments under otherwise similar conditions ($1.3 \cdot 10^{-16}$ mol $\cdot s^{-1}$ per cell, Table I of ref. 14). In light of the preceding considerations, it is surprising that Czech [2,15—17] is estimating the initial rate of methylglucose influx in white fat cells from the intracellular amount of methyglucose present 30 s [2] or 20 s [15] after its addition, and it can be calculated from figure 2 of ref. 2 that the initial rate of (saturable) methylglucose influx at 37°C and in the presence of insulin in his experiments is nearly two orders of magnitude smaller than the one which can be calculated from the present results ($1.2 \cdot 10^{-18}$ mol $\cdot s^{-1}$ per cell vs. $110 \cdot 10^{-18}$ mol $\cdot s^{-1}$ per cell **.

$$RT \cdot \log_e \frac{J_{m,0}}{J_{m,ins}} = \delta\ 1.1 \text{ kcal/mol at } 28°C,$$

the assumptions making other terms cancel out. In this expression, $J_{m,0}$ and $J_{m,ins}$ are the maximum transport capacities in the absence and in the presence of insulin, respectively, $\Delta H^{\dagger}$ the activation enthalpy in the absence of insulin, δ the hypothetical, insulin-induced decrease in this quantity and R and T have their usual meaning. Since the Arrhenius activation energy, E_a, is related to $\Delta H^{\dagger}$ by: $E_a = \Delta H^{\dagger} + RT$ (see e.g. ref. 29), the insulin-induced decrease, δ, should also affect E_a.

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* Calculated as: (J_m/K_t) × intracellular distribution space for methylglucose per cell × methylglucose concentration = 1.7 mmol $\cdot s^{-1} \cdot l^{-1} \cdot$ (6 mM)$^{-1} \cdot$ 1.4 pl $\cdot$ cell$^{-1} \cdot 0.55 \cdot 10^{-3}$ mol $\cdot l^{-1} = 2.2 \cdot 10^{-16}$ mol $\cdot s^{-1}$ per cell.

** In ref. 2, table I, the initial rate of uptake of methylglucose in the absence of insulin at 37°C is given as 6.1 pmol/min per 10^6 cells at a concentration of 0.1 mM of methylglucose. In figure 2 of ref 2, the uptake is about 20 pmol/min per 10^6 cells in the absence of insulin and about 70 pmol/min per 10^6 cells in the presence of insulin (2.4 munits/ml) corresponding to $1.2 \cdot 10^{-18}$ mol $\cdot s^{-1}$ per cell. At a concentration of 0.3 mM methylglucose (corresponding to the elevation of the control value from 6 to 20 pmol $\cdot s^{-1}$ per cell), the present results predict a value of: J_m/K_t × distribution space in one cell · concentration of methylglucose = 1.7/6 · 1.4 pl · 0.3 mM = $110 \cdot 10^{-18}$ mol $\cdot s^{-1}$ per cell. Note that the distribution space for methylglucose per cell in the present paper is within the limits given by Czech (0.5—1.5 pl/cell, [2]), and that it was necessary to estimate the methylglucose concentration because it is not given in the reference [2].

Inhibition of transport with cytochalasin B. Cytochalasin B has been shown to be a potent inhibitor of facilitated diffusion of glucose and methylglucose in many cell types, including fat cells [18,19] and human erythrocytes [20,21]. Both competitive [22] and non-competitive [20,21] type of inhibition have been reported, but most (if not all) of this apparent controversy has been dissipated by the report of Basketter and Widdas [23] who found that cytochalasin B inhibited equilibrium exchange of hexose competitively and net efflux non-competitively in human erythrocytes. A somewhat similar state of affairs might exist for fat cells, where the present results showed competitive-type inhibition of equilibrium exchange of methylglucose, and where Czech [19] has reported a non-competitive type of inhibition in methylglucose uptake experiments. The value of the inhibition constant found in the present paper ($2.5 \cdot 10^{-7}$ M) does not differ much from the values found from inhibition of equilibrium exchange of glucose in the human erythrocyte ($(2.6—4.0) \cdot 10^{-7}$ M [22]) or from values derived from efflux experiments on the same cells ($5 \cdot 10^{-7}$ M [21]). It is interesting that glucose has been found to inhibit the binding of [^{3}H]cytochalasin B to human erythrocyte ghosts [22,24]; unfortunately, this relation could not be demonstrated in fat cell ghosts [19].

In the present paper, the inhibition constant for cytochalasin B was not changed by insulin. This is at variance with the results of Czech, who reported different inhibitory patterns for cytochalasin B inhibition of methylglucose uptake in the absence and in the presence of insulin [17].

The non-inhibitable part of methylglucose transport. The fraction of the methylglucose transport which is mediated by a non-inhibitable pathway is a negligible fraction of the total transport, when moderate concentrations of methylglucose are used. This implies that this pathway is not limiting for the lowering of permeability in the absence of insulin. As judged from the competitive type of interaction between cytochalasin B and methylglucose, the non-inhibitable pathway could well be the same as the pathway which was not saturable by methylglucose. The absolute magnitude of the residual permeability was about $2 \cdot 10^{-9}$ cm $\cdot$ s^{-1}. The glucose permeability of a protein-free lipid bilayer made from a lipid extract of human erythrocyte membranes was found to be $2.3 \cdot 10^{-10}$ cm $\cdot$ s^{-1} at 25°C by Jung [25], so the residual permeability found in the present paper could therefore represent more than diffusion through the lipid matrix of the fat cell membrane.

In the above discussed report from Czech [2], the uptake of methylglucose in the presence of 20 μM cytochalasin (figure 1 of ref. 2) corresponds to a permeability of about $2 \cdot 10^{-9}$ cm $\cdot$ s^{-1}, so the discrepancy between his results and those of the present paper seems to be confined to the cytochalasin B-inhibitable part of the transport.

The temperature dependence of the maximum transport capacity. It has been suggested that the much studied transfer of sugar across the erythrocyte membrane occurs by a conformational change in a protein extended through the membrane and is able to bind sugar reversibly at both membrane surfaces [26—28]. If similar proteins are assumed to mediate the saturable methylglucose transport which is the object of the present study, and if the dissociation of sugar from the protein is never rate limiting, the maximum transport capacity can be written: $J_m = f \cdot C_t$, where C_t is the concentration of the transporting protein and f the frequency of conformational changes leading to

exchange of the sugar molecules bound on opposing membrane surfaces. According to the transition state theory of Eyring [29], as applied to transmembrane transport [30,31], the frequency f can be written: $\kappa \cdot (kT/h) \cdot \exp(-(\Delta G^{\dagger}/RT))$, where κ is the transmission coefficient, k the Boltzmann constant, T the absolute temperature and h is Planck's constant. If κ is assigned its maximum possible value of 1, the preexponential factor has a value of $6.5 \cdot 10^{12}$ s^{-1} at 37°C. The free energy of activation, $\Delta G^{\dagger}$, equals $\Delta H^{\dagger} - T\Delta S^{\dagger}$, the difference between the heat of activation and the change in heat content by transition to the activated complex. $\Delta H^{\dagger}$ is related to the Arrhenius activation energy (E_a) by $\Delta H^{\dagger} + RT = E_a$. Not considering κ, the above formalism shows that insulin could increase the maximum transport capacity by increasing C_t, decreasing $\Delta H^{\dagger}$ or increasing $\Delta S^{\dagger}$ (the entropy of activation) not to mention the possible combinations of changes. Only a few of these possiblities can be ruled out by the present results, as only rather large changes in $\Delta H^{\dagger}$ would have been detected. However, the results lend no support to the idea that different processes could mediate the sugar transfer in the presence and in the absence of insulin.

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