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EFFECTS OF THIOL REAGENTS ON GLUCOSE TRANSPORT IN THYMOCYTES

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Rat thymocytes were incubated with 3-*O*-[^{14}C]methyl D-glucose for 1 h and diluted 100× and the efflux was followed for 1 h. In control cells, about half of the methyl glucose efflux was rapid ($t_{1/2} \approx 3$ min) and about half was slow ($t_{1/2} \simeq 40$ min). The fast and slow compartments represent active and quiescent cells, respectively. A physiological mixture of amino acids present during the loading period dramatically increased the amount of methyl glucose exiting rapidly at the expence of that exiting slowly. Further studies revealed that cysteine was entirely responsible for this action. Cysteine (0.06 mM), glutathione (0.5 mM) and dithiothreitol (0.02 mM) added after completion of fast-phase exit, stimulated subsequent exit about 3–4-fold with no detectable delay. This action was inhibited by catalase and mimicked by 0.04 mM H_2O_2 and by 0.03 mM *N*-ethylmaleimide. It did not require extracellular or intracellular Ca^{2+}. These effects are analogous to those seen in adipocytes, implicating sulfhydryl groups in glucose transport regulation [12]. Sulfhydryl oxidation may be a late event in the chain of events leading to glucose transport stimulation by physiological agents.

Introduction

Rat thymocyte populations contain cells in two (or more) distinct regulatory states [1–4]. Active or stimulated cells equilibrate methyl glucose rapidly ($t_{1/2}$ of 1–3 min) and quiescent cells equilibrate methyl glucose slowly ($t_{1/2}$ of 40 min). Stimulatory agents (concanavalin A, A-23187, croton oil, phenazine methosulfate, anoxia and arsenate [1–3,5–7]) cause varying portions of the quiescent cells to transport sugar like active cells. In a study to determine whether the inhibitory effect of glucocorticoids [7,8] might depend on availability of glucose and amino acids, we observed that a physiological amino acid mixture stimulates about two-thirds of the quiescent cells. This effect turned out to be attributable entirely to cysteine.

Czech and coworkers [9–13] observed glucose transport in rat adipocytes to be stimulated by thiols, and showed this effect to be mediated by H_2O_2. Peroxide appeared to be generated in a cyclic reaction wherein copper, contaminating the medium, was oxidized by molecular oxygen and reduced by the thiol. The effect was mimicked by H_2O_2 and antagonized by catalase. Stimulation by H_2O_2 was not addtive with that by insulin. They proposed that oxidation of a sulfhydryl on the carrier or a regulatory protein may be a signal mediating transport stimulation by insulin. They observed that virtually any sulfhydryl reagent at low concentration stimulates glucose transport in adipocytes. Mukherjee and Lynn [14] and May and de Haën [15] eventually demonstrated that insulin stimulates peroxide formation in adipocytes. The present studies demonstrate analogous effects of thiols and thiol reagents in thymocytes.

Methods

Preparation of cells

Rat thymocytes suspensions were prepared essen-

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tially as described earlier [1,2,5,16]. Cells (freed from minced thymus by crushing in a loose fitting Dounce homogenizer) were passed through nylon net, centrifuged through dense medium, labeled with [^{3}H]isoleucine, centrifuged and resuspended in medium at a cytocrit appropriate for the experiment (about 7% cells). In the early experiments, the dense medium was 7% Ficoll dissolved in incubation medium. In later experiments, three layers of Percoll-containing medium (p = 1.09, 1.08, 1.05) were used. Erythrocytes sedimented to the bottom and thymocytes accumulated between the middle and top layer.

Incubation medium

Buffered balanced salt solution (medium) was prepared from isotonic salt solutions (0.31 osM/l) in the proportions: 114 NaCl, 5 KCl; 2 $CaCl_2$, 1 $MgSO_4$, 2 $NaPO_4$ (pH 7.3), 6 sodium Hepes (pH 7.3). Sodium β-hydroxybutyrate (1 mM) and sodium lactate (5 mM) were added for fuel. Bovine serum albumin (0.2%) was added and the pH of the final solution checked at 35°C (pH being 7.2–7.3).

Transport tests

Cell suspensions (50 μl) were incubated with 1 μCi/ml 3-*O*-[^{14}C]methyl D-glucose with 0.1 mM unlabeled methyl glucose for 1 h, then diluted with 5 ml medium to initiate methyl glucose exit from the cells. Samples (0.5–0.7 ml) were taken at intervals and assayed for cellular radioactivity as described earlier [1,2,5,16]. In essence, the sample was squirted into 4 ml ice-cold medium (containing 1 μM cytochalasin B) layered on 9 ml medium (made dense with sucrose) in a conical centrifuge tube. After centrifugation, the upper fluid was aspirated and the walls rinsed in stages. After a final aspiration, the cells were suspended and transferred to a counting vial. The cellular ^{3}H, which was largely incorporated into acid-precipitable constituents [16], did not exit the cells and served as an internal sampling control. It also alerted us to cytolysis when this occurred. Data are expressed as [^{14}C]methyl glucose in cells relative to that in the first sample of control cells after dilution (0.2 min, 0.3 min or 10 min). These were plotted against time after dilution.

Materials

Ficoll and Percoll were obtained from Pharmacia, Uppsala Sweden; L-[4,5(n)-^{3}H]isoleucine (1000 mCi/mg) and 3-*O*-[^{14}C]methyl D-glucose (0.25 mCi/mg) were obtained from New England Nuclear. ACS counting fluid was obtained from Amersham, Arlington Heights, IL.

Results

Effects of amino acids on methyl glucose transport

In control rat thymocytes about 1/3 of the methyl glucose space equilibrates rapidly and about two-thirds equilibrates slowly [1,4]. When cells are loaded for 1 h, the slow compartment is incompleteley equi-

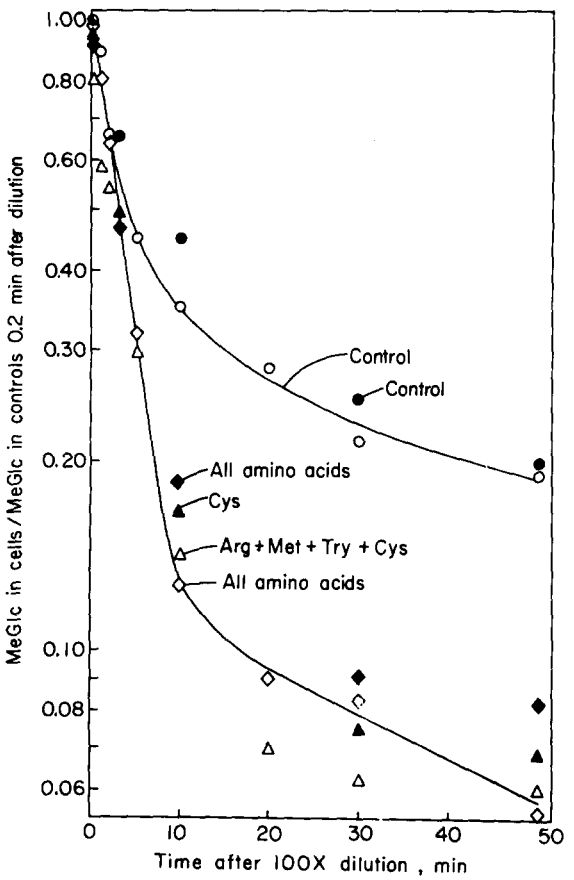


Fig. 1. Effects of amino acids on methyl glucose efflux. The amino acids were present during the 1 h methyl glucose loading periods. This is a composite of two experiments, the first (○, ◇, △) comparing amino acid groups with the physiological mixture, the second (●, ◆, ▲) comparing individual amino acids with the physiological mixture. Several other groups (basic amino acids, dicarboxylic amino acids, aliphatic amino acids, aromatic amino acids) were without effect.

librated, so about half of the equilibrated methyl glucose is in each compartment. As shown in Fig. 1, a physiological amino acid mixture present during the 1-h methyl glucose loading period caused 85% of accumulated methyl glucose to exit rapidly and 15% to exit slowly. The amino acid mixture stimulated about two-thirds of the quiescent cells.

Various amino acids were tested in combination and individually to determine which were responsible for this dramatic conversion of quiescent cells to rapidly transporting cells. Only cysteine and cysteine-containing combinations increased the fraction of methyl glucose exiting rapidly at the expense of that exiting slowly.

Effects of thiols and related agents on methyl glucose efflux from quiescent thymocytes

The stimulatory effect of cysteine appeared to be an example of the hydrogen peroxide-mediated stimulation of glucose transport by thiols as reported by Czech and coworkers [9–13]. As seen in Fig. 2, glutathione and dithiothreitol (at optimal doses) stimulated as effectively as cysteine. These thiols stimulated methyl glucose efflux from quiescent cells

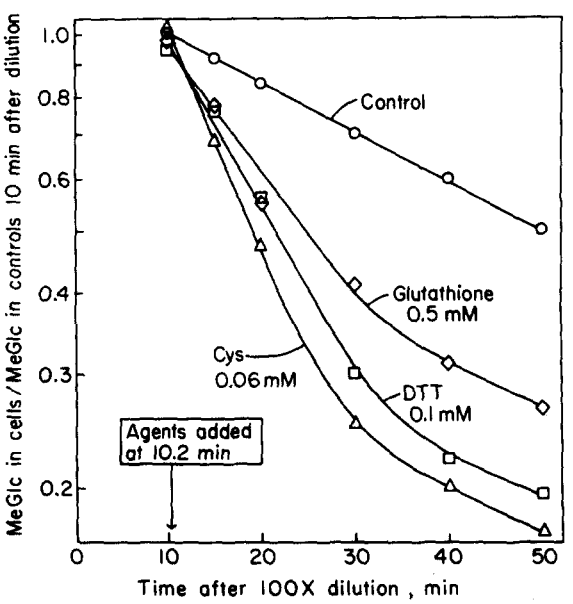


Fig. 2. Effects of thiol compounds on methyl glucose efflux. The thiol compounds were added at 10.2 min, i.e. as quickly as was convenient after the first sample was takem 10 min following the 100X dilution.

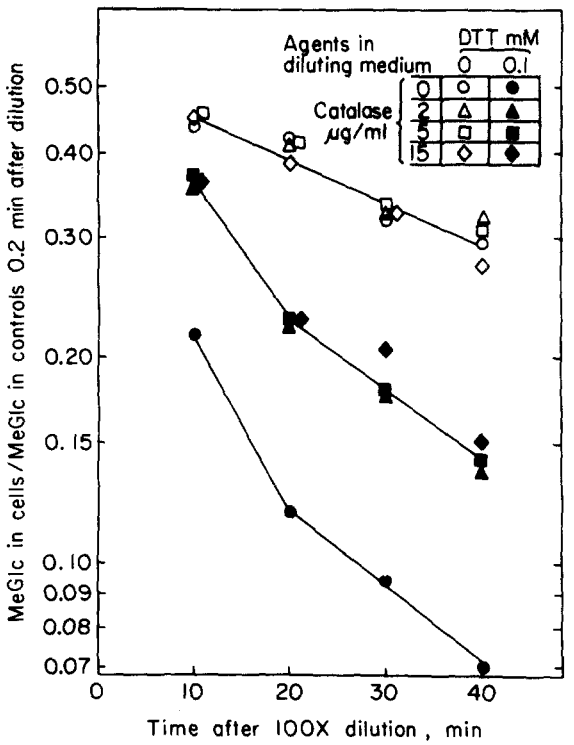


Fig. 3. Effects of catalase on glucose transport stimulation by dithiothreitol (DTT). Catalase and dithiothreitol (DTT) were added to the diluting medium 1 min prior to the 100X dilution of the methyl glucose loaded cells.

without any apparent delay. The stimulation occurred in the absence of albumin (data not shown). Fig. 3 shows that catalase interfered with stimulation by dithiothreitol. Peroxidase with ethanol had a similar action (not shown).

As shown in Fig. 4, low concentrations of hydrogen peroxide and *N*-ethylmaleimide also stimulated methyl glucose efflux from quiescent thymocytes without delay. These stimulants were as effective as thiols. Their effects were hardly dose dependent over the range of doses used. Potent effects were also seen at lower doses (Figs. 5 and 6).

Lack of Ca^{2+} requirement

The stimulatory effects of concanavalin A and A23187 require extracellular Ca^{2+} [5]. If the thiol agents were mimicking an early event of concanavalin A stimulation, then they might also be Ca^{2+} dependent. We found, however, that stimulation by dithio-

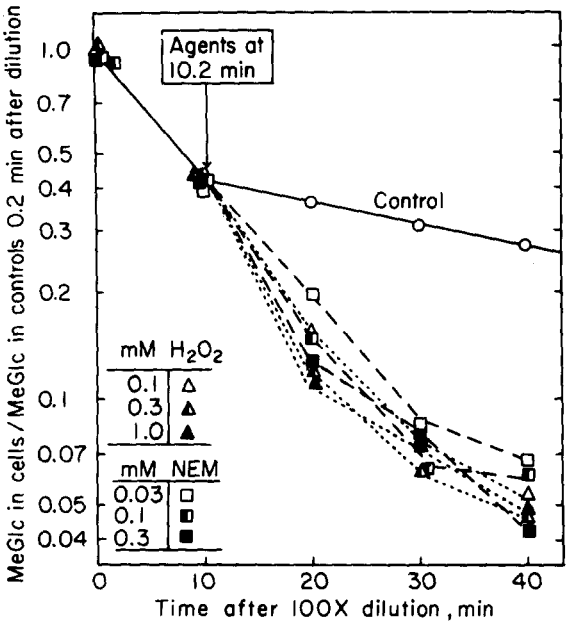
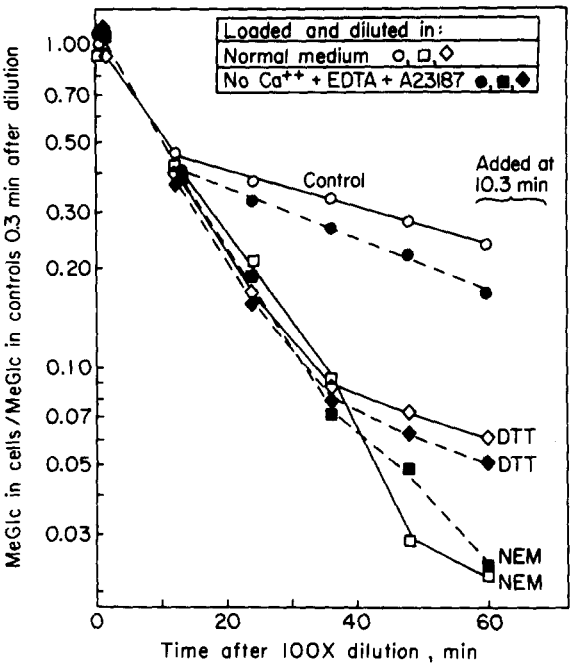


Fig. 4. Effects of hydrogen peroxide and *N*-ethylmaleimide (NEM) on methyl glucose efflux. These agents were added at the indicated concentrations at 10.2 min, i.e. as quickly as was convenient after the second sample was taken 10 min following the 100X dilution.

threitol, *N*-ethylmaleimide and H_2O_2 was as effective in Ca^{2+}-free medium (containing EDTA equivalent to 1/4 of the Mg^{2+}) as it was in normal medium (data not shown). To test whether intracellular Ca^{2+} stores might be required for stimulation, the stimulatory agents were added to cells which were both loaded (1 h with methyl glucose) and diluted in Ca^{2+}-free medium containing both EDTA (equivalent to 1/4 of the Mg^{2+}) and A23187 (1 μg/ml in the loading medium and 0.5 μg/ml in the diluting medium). The data in Figs. 5a and 5b show that all stimulatory agents (except A23187) were as effective in the Ca^{2+}-depleted cells as in cells loaded and diluted in normal medium. If stimulation by thiol agents is analogous to stimulation by concanavalin A and A23187, then the thiol agents must act at some step beyond the Ca^{2+}-dependent step.



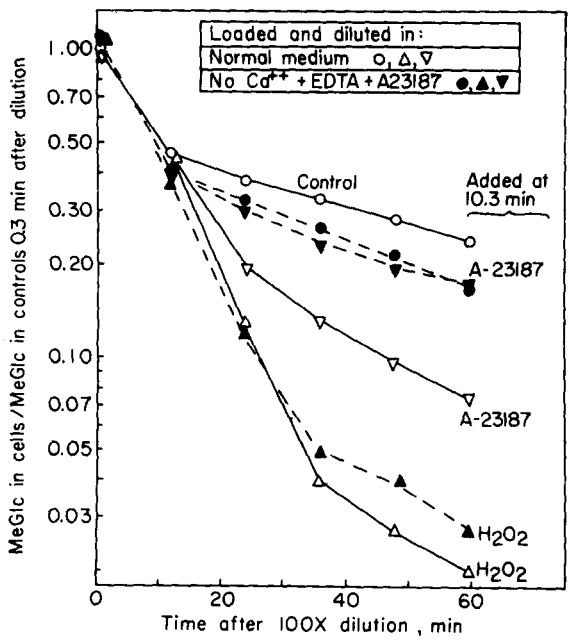


Fig. 5. Effects of Ca^{2+} deprivation on glucose transport stimulation. Half of the cell preparation (●, ◆, ■, ▲, ▼) was suspended in 10 ml Ca^{2+} free medium, centrifuged and resuspended in 0.4 ml Ca^{2+} free medium containing EDTA equivalent to 1/4 of the Mg^{2+} and 1 μg/ml of A23187. They were loaded 1 h with methyl glucose in this medium and diluted 100X with a medium differing only in that the A23187 concentration was 0.5 μg/ml. The other half (○, ◇, □, △, ▽) was similarly handled in normal medium. The agents (0.016 mM dithiothreitol (DTT) (◇, ◆), 0.024 mM *N*-ethylmaleimide (NEM) (□, ■), 0.04 mM H_2O_2 (△, ▲) and 0.6 μg/ml A23187 (▽, ▼)) were added just after the second sample was taken.

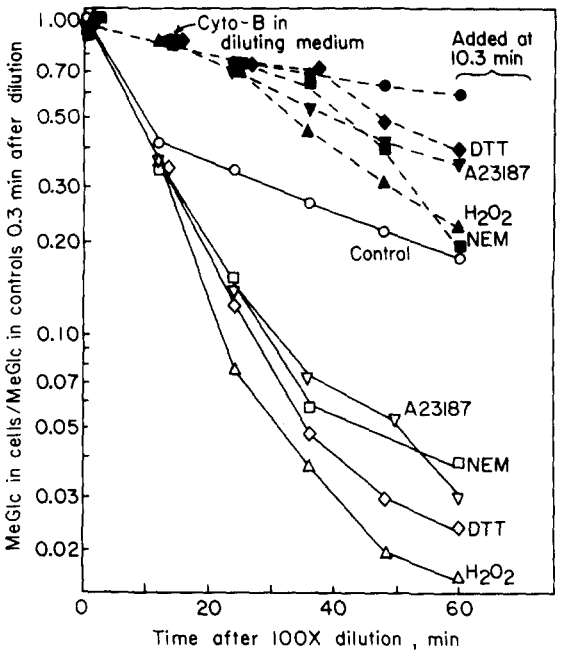


Fig. 6. Effect of cytochalasin B on glucose transport stimulation. Cytochalasin B (0.7 μM) was present in the dilution medium as indicated (filled symbols) or absent (open symbols). Other agents (0.016 mM dithiothreitol (◇, ◆), 0.024 mM *N*-ethylmaleimide (NEM) (□, ■), 0.04 mM H_2O_2 (△, ▲) and 0.6 μg/ml A23187 (▽, ▼)) were added just after the second sample was taken.

Inhibition by cytochalasin B

Since some of the stimulatory agents are cytotoxic, it was necessary to check whether they enhanced sugar exit by increasing passive membrane permeability. Fig. 6 shows that cytochalasin B present in the diluting medium strongly inhibited efflux from both control cells and cells treated with the stimulating agents. It did not block stimulation, since efflux from cells stimulated in the presence of cytochalasin was faster than that from cells simply diluted with the cytochalasin medium. Cytochalasin simply inhibited efflux from stimulated cells as it did efflux from unstimulated cells. It appears, therefore, that efflux from stimulated cells is carrier mediated.

Discussion

These studies demonstrated analogies between thymocytes and adipocytes as regards the effects of thiols and related agents on glucose transport. Cysteine stimulated methyl glucose efflux from rat thymocytes, and this action was shared by other thiol containing compounds (glutathione, dithiothreitol). Several non thiol reducing agents (bisulfite, ascorbic acid, hydroquinone) also stimulated efflux at concentrations between 0.3 mM and 1 mM (data not shown), possibly by the same mechanism. Stimulation by thiol compounds was antagonized by catalase and mimicked by hydrogen peroxide, consistent with peroxide mediation of thiol stimulation as proposed by Czech and coworkers [9–13] for adipocytes. If thiol stimulation depends on copper contamination of the system, albumin was not the source of the contamination, since thiol stimulation occurred in albumin free medium. Low concentrations of *N*-ethylmaleimide stimulated thymocyte glucose transport more dramatically than was the case with adipocytes [10,12]. Thiol compounds, hydrogen peroxide and *N*-ethylmaleimide stimulated glucose transport of quiescent thymocytes to a similar degree. Stimulation was abrupt, suggesting that the target(s) of these agents may be the glucose carrier or a protein the function of which is to regulate the carrier.

Czech and his colleagues [9–13] have provided evidence that H_2O_2 stimulates glucose transport by the same final mechanism as does insulin. Mukherjee and Lynn [14] and May and de Haen [15] have provided evidence that insulin increases H_2O_2 production in adipocytes. Cushman and Wardzala [17] and Suzuki and Kono [18] have provided convincing evidence that insulin enhances glucose transport by triggering translocation of glucose carrier from an intracellular organelle to the membrane. Taken together, these findings suggest that H_2O_2 is a mediating signal in triggering this translocation or at least that exogenous H_2O_2 triggers the same translocation. In view of these findings, it is interesting that we could not demonstrate a role of Ca^{2+} in stimulation by dithiothreitol, H_2O_2 or *N*-ethylmaleimide. The amounts of A23187 and EDTA used in these tests (with no Ca^{2+} in the medium) should have largely depleted intracellular Ca^{2+} stores and should have abolished any physiological control of Ca^{2+} movements, yet stimulation was unaffected. It appears, therefore, that Ca^{2+} is neither an intermediary signal nor a permissive cofactor in the action of H_2O_2 to stimulate glucose transport, presumably by translocation of the carrier

to the membrane. Conceivably, alteration of sulfhydryls on a protein of the system which moves the carrier to the membrane is a signal sufficient to induce the movement. The role of Ca^{2+} in glucose transport stimulation by insulin [19], concanavalin A and A23187 [5] is likely at an early step in the sequence of events leading to stimulation, perhaps leading to H_2O_2 production.

Appendix

The studies of glucocorticoid action, from which the above experiments diverged, revealed a few points of interest. As seen in Fig. A1, dexamethasone (10^{-6} M) present during the 1 h methyl glucose loading period, did not alter the amount of methyl glucose accumulated, but it slightly increased the fraction exiting slowly and increased the half-time of slow phase exit from its usual 40 min to 60 min. Dexamethasone apparently caused some cells to be quiescent cells. These effects were essentially similar with 2 mM glucose present during drug exposure.

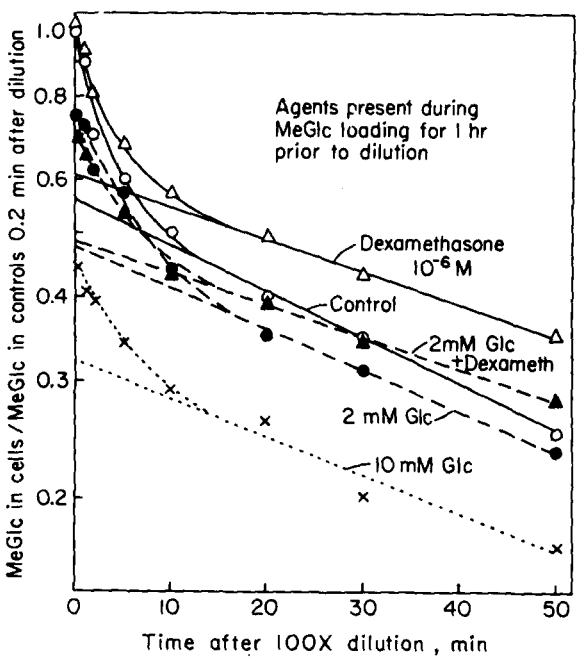


Fig. A1. Effects of dexamethasone and glucose on compartmental distribution of methyl glucose. Dexamethasone (△, ▲) was present at 10^{-6} M in the loading medium. Glucose was present at 2 mM (●, ▲) or 10 mM (X) in the loading medium.

Glucose (2 mM) present during the loading period reduced methylglucose accumulation in active cells by 40% and reduced that in quiescent cells by 15%. The high glucose concentration (10 mM) reduced methyl glucose accumulation in active cells by 73% and that in quiescent cells by 43%. These may be kinetic effects of glucose [20–22]. Glucose inhibits early methylglucose entry approximately according to the rule, $(dS_i/dt)/[S_o] = F_s/(1 + [G_o]/K_{go})$, and reduces the methyl glucose equilibrium space according to the rule, $[S_i]/[S_o] = (1 + [G_i]/R_g)/(1 + [G_o]/R_g)$, where S_o is external methyl glucose, S_i is internal methyl glucose, G_o is external glucose, G_i is internal glucose, F_s is methyl glucose V/K_m, K_{go} is the glucose entry K_m, and R_g is the glucose counterflow constant [20]. Since 1 h is at least 15 active-cell half times, the rate effect of glucose would be negligible; so the pronounced reduction of methyl glucose in active cells would be due to the counterflow action and would demonstrate that steady-state $[G_i]$ is much less than $[G_o]$, hence that transport limits glucose utilization in active cells despite their very rapid transport. The lesser effects of glucose on 1 h quiescent cell methyl glucose is consistent with the lesser carrier affinity seen in this cell as compared to the active cell (2); though the situation is complicated by the fact that 1 h is only 1.5 half times, so both rate effects and equilibrium effects would be involved. According to the counterflow equation, if 2 mM glucose reduces methyl glucose equilibrium space by 40%, then R_g of active cells must be less than 3 mM. Moreover, the $[G_i]/[G_o]$ ratio of active cells could not be more than the $[S_i]/[S_o]$ ratio; so, with 10 mM $[G_o]$, $[G_i]$ must be less than 2.7 mM. Glucose may exert regulatory affects [23] in addition to kinetic effects.

When this experiment was repeated with a complete amino acid mixture present during the methyl glucose loading period, about 85% of methyl glucose excited rapidly, indicating that about two-thirds of the quiescent cells were stimulated (as in Fig. 1). However, in this background of stimulation, the effects of dexamethasone and glucose were as seen in Fig. A1. Among other things, this shows that transport limits glucose utilization in stimulated cells as in endogenously active cells, suggesting that stimulation may enhance glycolytic activity as well as transport. It also suggests that glucocorticoids reduce the amount

of carrier available to be activated by H_2O_2, analogous to the proposal of Zyskowski and Munck [7].

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