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ASYMMETRY OF GLUCOSE TRANSPORT IN THE YEAST, *KLUYVEROMYCES LACTIS*

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Uptake and efflux of 6-deoxy-D-[^{3}H]glucose and of 2-deoxy-D-[^{14}C]glucose by the yeast *Kluyveromyces lactis* was studied. The tritiated, nonphosphorylatable hexose analogue leaves the cell in the absence and presence of intracellular 2-deoxy-D-glucose 6-phosphate. In energy-rich cells containing pools of hexose 6-phosphate, 2-deoxy-D-glucose is trapped in the cells, for it neither effluxes into glucose-free medium nor exchanges with external, free sugar. In starved, poisoned cells containing negligible amounts of 2-deoxy-D-glucose 6-phosphate, 2-deoxy-D-glucose does leave the cells upon transfer to glucose-free medium. An involvement of analogue structure and availability of metabolites of energy-rich cells in hexose retention is suggested. An internal pool of 6-deoxy-D-glucose does not affect the rate of uptake of 6-deoxy-D-[^{3}H]glucose, nor does internal 2-deoxy-D-[^{14}C]glucose 6-phosphate influence that rate. Hence, transport of glucose by this yeast is probably not regulated by internal pools of glucose 6-phosphate.

Introduction

Kluyveromyces lactis is an oxidative yeast that exhibits a Pasteur effect when grown on glucose-containing medium [1]. In attempting to determine the sites of regulation of the Pasteur effect in this yeast, it was earlier shown that 6-phosphofructokinase (EC 2.7.1.11) of the glucose-grown cells is sensitive to inhibition by ATP [1]. Such allosteric modification of 6-phosphofructokinase is similarly found in the fermentative yeast, *Saccharomyces cerevisiae* [2]. However, citrate enhances the ATP inhibition of 6-phosphofructokinase in *S. cerevisiae* [3], but does not affect the inhibition of the enzyme from *K. lactis* [1].

A second regulatory mechanism of the Pasteur effect in *S. cerevisiae* is thought to be the AMP activation of NAD^+-isocitrate dehydrogenase (EC 1.1.1.41) [4]. With high levels of ATP in the cell, the reduced activity of this enzyme results in the accumulation of citrate which feeds back and augments the ATP inhibition of 6-phosphofructokinase. Regulation of glucose metabolism in *K. lactis* differs from that of *S. cerevisiae* in that AMP activation of NAD^+-isocitrate dehydrogenase is absent [1].

It was earlier proposed that a third means by which the Pasteur effect in yeast is regulated is via the feedback inhibition of the glucose carrier by glucose 6-phosphate which accumulates following the inhibition of 6-phosphofructokinase [5]. More recently, Perea and Gancedo, using a glucosephosphate isomerase-less mutant of *S. cerevisiae*, showed that glucose 6-phosphate does not alter significantly the rate of glucose transport in that yeast, and hence have excluded this metabolite as an effector of the transport system [6]. If other metabolites alter the transport activity in *S. cerevisiae* is not known.

Select data obtained during characterization of the inducible, nonenergy-requiring glucose transport system of *K. lactis* indicated metabolic regulation of the carrier in this yeast [7,8]. For instance, the parabolic double reciprocal plot of exchange of

2-deoxy-D-glucose (2-dGlc) by glucose-grown cells devoid of utilizeable energy and hexose phosphate, is indicative of carrier activation [7]. As well, the reduction in rate of transport of 6-deoxy-D-glucose (6-dGlc) in metabolically-poisoned succinate-grown, i.e., noninduced cells, further suggested an involvement of metabolic control of transport in these cells [8].

The purpose of the present investigation was to determine if glucose 6-phosphate inhibits the glucose transport system in *K. lactis*, and by so doing, to determine if the regulation of glucose metabolism by the two types of yeasts, i.e., oxidative and fermentative, differs at this site. Experiments were conducted using 2-d[^{14}C]Glc and 6-d[^{3}H]Glc, two glucose analogues that compete with glucose for entry into the cell. Following transport of the free sugar through the membrane, 2-d[^{14}C]Glc is phosphorylated by hexokinase to form 2-deoxy-D-[^{14}C]glucose 6-phosphate, (2-d[^{14}C]Glc-6-*P*) [7], whereas 6-d[^{3}H]Glc is unaltered [8]. It is shown that 2-d[^{14}C]Glc-6-*P* does not affect the rate of uptake of 6-d[^{3}H]Glc, and that 2-d[^{14}C]Glc is trapped inside energy-rich cells whereas 6-d[^{3}H]Glc is not.

Methods and Materials

Organism and growth conditions

All experiments were done using *Kluyveromyces lactis* Y123 (NRRL strain Y1118). Growth conditions and harvesting procedures were as earlier described [9,10].

Transport studies

(a) 2-d[^{14}C]Glc. Harvested, mid-logarithmic phase cells were resuspended in 67 mM potassium phosphate buffer (pH 5.4). After equilibrating at 20°C for 10 min, 2-deoxy-D[U-^{14}C]glucose (New England Nuclear; spec. act. 0.077 Ci/mol) was added, and at set time intervals, 0.5 ml samples were withdrawn and added to 5 ml ice-cold 67 mM potassium phosphate buffer (pH 5.4) on a Millipore filter, porosity 0.45 μm. Following filtration, the cells on the filter were washed 3-times with 3 ml buffer as above. The filter was transferred to 0.5 ml water in a test tube, and the sample boiled for 5 min. Preliminary studies revealed that under these conditions, 2-dGlc-6-*P* is not hydrolysed for at least 10 min. After cooling to room temperature and centrifugation for 10 min at 12000 × *g*, one aliquot of the boiled cell free extract was added to a scintillation vial, scintillation fluid [11] added, and the sample counted on a Packard Model 300 CD liquid scintillation counter. Another aliquot was spotted on a thin-layer chromatography plate. In all transport experiments, corrections for label adsorbed to the cell walls and filter were made by subtracting those counts obtained upon incubating cells on ice.

To determine any efflux of label from cells, cells containing labeled analogue were centrifuged, and resuspended in 10-times the original volume of buffer prewarmed to 20°C. Samples were taken at set time intervals, and extracted as above. Efflux of 2-d[^{14}C]Glc from energy-deficient cells was determined by first starving cells for 3 h followed by poisoning with 0.32 mM sodium azide and 2 or 5 mM iodoacetate [7].

(b) 6-d[^{3}H]Glc. Transport of 6-deoxy-D-[G-^{3}H] glucose (New England Nuclear; unlabeled, Sigma) was studied as above. The specific activity of the tritiated analogue used varied between 0.133 and 0.83 Ci/mol.

To preload cells preceding uptake studies, washed cells were incubated with varying concentrations of unlabeled 6-dGlc such that the internal concentration of 6-dGlc was 5-times that of the labeled analogue subsequently added. The rate of uptake of labeled material by these cells as well as by nonpreloaded cells was determined over a 3-min time period. In these experiments as in determinations of 6-d[^{3}H]Glc efflux from cells, washed membrane filters were transferred directly to a liquid scintillation vial and counted as before [7].

Competition studies were done by exposing cells to 6-d[^{3}H]Glc, 5 to 30 mM, 0.195 Ci/mol, and 10 mM 2-dGlc simultaneously, and the rate of transport determined.

(c) Dual label. To determine the rate of uptake of varying concentrations of 6-d[^{3}H]Glc into cells containing a set concentration of 2-d[^{14}C]Glc-6-*P*, the cells were first incubated in 10 mM 2-d[^{14}C]Glc, 0.0038 Ci/mol, for 45 min. Following a rapid wash in ice-cold buffer, 6-d[^{3}H]Glc, 2, 4, 8, and 16 mM (spec. act. 0.83 Ci/mol), was added in buffer prewarmed to 20°C.

The rate of transport of one concentration of

6-d[^{3}H]Glc into cells containing varying concentrations of 2-d[^{14}C]Glc-6-*P*, i.e., 2, 4, 10, 15, and 20 mM, was determined by first incubating cells with 10 mM 2-d[^{14}C]Glc (spec. act. 0.0038 Ci/mol or 0.0077 Ci/mol) for varying periods of time. The specific activity was varied in order that the internal cpm in each aliquot of cells when harvested would be similar. After incubation the cells were washed one time. Buffer, prewarmed to 20°C and containing 40 mM 6-d[^{3}H]Glc, 0.83 Ci/mol, was added. Aliquots of the cells were withdrawn at set time intervals, and counted as above.

The internal level of 6-d[^{3}H]Glc in cells containing 2-d[^{14}C]Glc and 2-d[^{14}C]Glc-6-*P* was determined by first preloading cells with 10 mM 2-d[^{14}C]Glc, 0.064 Ci/mol, at 20°C. At 1 h, cells were washed one time and transferred to prewarmed buffer containing 2 mM iodoacetate, 0.32 mM sodium azide, and 40 mM 6-d[^{3}H]Glc, 0.156 Ci/mol. Labeled products of samples withdrawn at set time intervals were separated upon spotting boiled cell free extracts on thin-layer chromatography plates. Control cells were exposed to inhibitors and 6-d[^{3}H]Glc as above following a 1 h incubation in buffer at 20°C.

In other experiments, cells were loaded as above with 10 mM 2-d[^{14}C]Glc and 20 mM 6-d[^{3}H]Glc, but in the absence of metabolic inhibitors. After internal 6-d[^{3}H]Glc equilibrated with exogenous analogue, cells were transferred to 10-times the original volume of buffer and samples were withdrawn at set time intervals.

Thin-layer chromatography

Boiled cell free extracts were spotted on Silica gel G plates, and developed in *n*-propanol/ammonia/water, (6 : 1 : 3, by vol.). Labeled spots were localized upon detecting unlabeled analogues with ammonium molybdate-perchloric acid spray (0.5 g ammonium molybdate dissolved in 5 ml water, 1.5 ml 25% HCl, 2.5 ml 70% perchloric acid, qs to 50 ml with acetone.) The plates were dried at 45°C for 10 min for color development. Labeled spots were transferred by suction to 1 ml H_2O in a scintillation vial. Labeled sugars were eluted for 4 h at 30°C, scintillation fluid added, and the sample counted.

In vitro phosphorylation of 2-d[^{14}C]Glc

Label from a spot corresponding to unlabeled 2-dGlc was eluted from the silica gel in 0.5 ml H_2O and incubated with 0.1 ml 30 mM ATP, 0.1 ml 30 mM $MgCl_2$, 26 units hexokinase (Sigma: EC 2.7.1.1) and 0.3 ml 100 mM potassium phosphate buffer (pH 7.1). After incubating for 15 min at 30°C, 0.5 ml 5% trichloroacetic acid was added. Following a 15-min incubation on ice, the sample was centrifuged at $12000 \times g$ for 15 min. An aliquot of the supernatant was then spotted on two chromatograms, one of which was developed in the above mentioned solvent, the other in *n*-butanol/acetic acid/water (4 : 1 : 5, by vol.).

Results

Separation of labeled analogues

Between 86% and 96% of label in boiled extracts of cells exposed to 2-d[^{14}C]Glc migrated on thin-layer chromatograms as did authentic 2-dGlc and 2-dGlc-6-*P*. That the labeled spot corresponding to unlabeled 2-dGlc was indeed 2-d[^{14}C]Glc and not another metabolite of the sugar analogue was verified by eluting the label from the silica gel and incubating the eluant under phosphorylating conditions. Upon rechromatographing the extract in two solvent systems, approx. 75% of the counts originally extracted migrated as did 2-dGlc-6-*P*, whereas 25% migrated as did 2-dGlc. It was concluded that the original labeled spot corresponding to authentic 2-dGlc was 2-d[^{14}C]Glc.

Transport studies

The initial approach to the determination of an effect of glucose 6-phosphate on transport of a glucose analogue was to fill induced, glucose-grown cells with 2-d[^{14}C]Glc in order to fill internal pools of 2-d[^{14}C]Glc as well as of 2-d[^{14}C]Glc-6-*P*. Since the glucose transport system of this yeast is a facilitated diffusion system, it was thought that transfer of such loaded cells to a large volume of buffer would result in loss of the free analogue, but retention of the labeled, phosphorylated analogue due to the impermeability of the yeast membrane to hexose phosphates. Comparison of the rate of transport of 6-d[^{3}H]Glc into these cells and into cells not preloaded with 2-d[^{14}C]Glc would indicate any regulatory effect of hexose phosphate

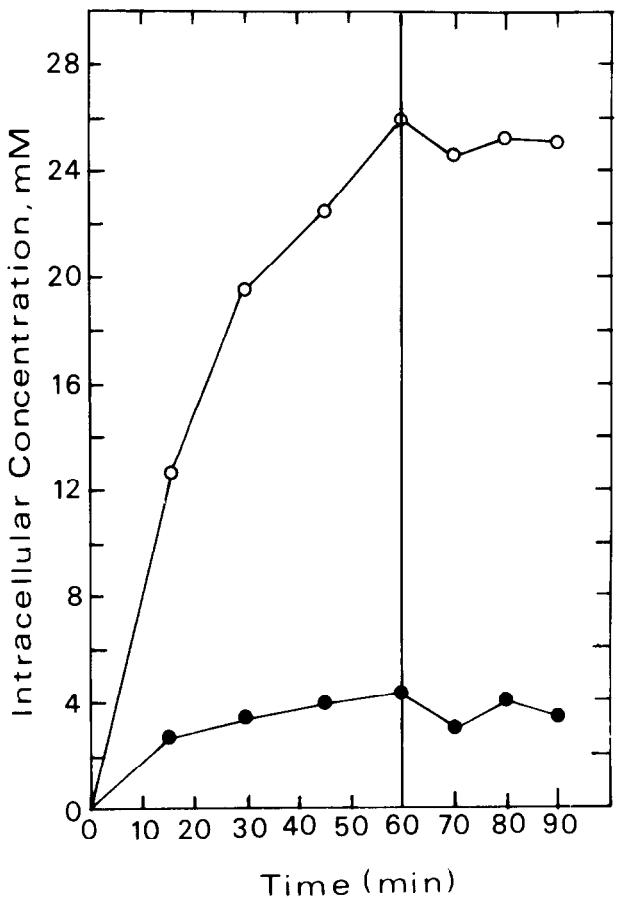

Fig. 1. Distribution of (●) 2-d[^{14}C]Glc and (○) 2-d[^{14}C]Glc-6-*P* in boiled cell free extracts of *Kluyveromyces lactis* exposed to 10 mM 2-d[^{14}C]Glc. At 1 h cells were washed one time and transferred to 10-times the original volume of buffer containing no free analogue. Separation of labeled products was achieved by thin-layer chromatography.

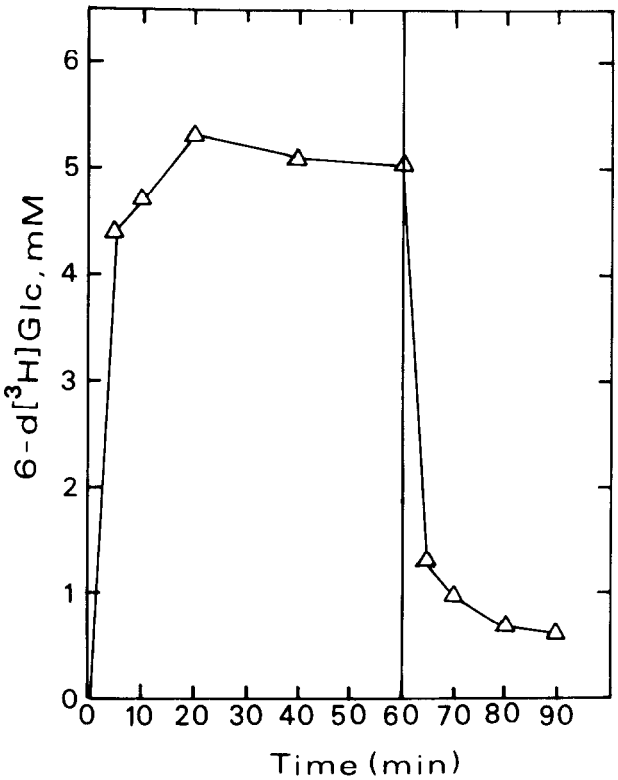

Fig. 2. Intracellular concentration of 6-d[^{3}H]Glc in *Kluyveromyces lactis* exposed to 40 mM 6-d[^{3}H]Glc. At 1 h cells were washed rapidly and transferred to 10-times the original volume of buffer containing no labeled analogue.

on the rate of transport of the free sugar analogue.

Initial investigations revealed that exposure of cells to 2-d[^{14}C]Glc resulted in the filling of pools of 2-d[^{14}C]Glc and of 2-d[^{14}C]Glc-6-*P*, as expected from earlier studies [7]. However, after washing the cells to remove external labeled analogue and transferring the cells to 10-times the initial volume of buffer prewarmed to 20°C, the internal concentration of both labeled molecules remained the same (Fig. 1).

This lack of efflux of free analogue from the cells initially appeared to contradict two earlier findings: (1) that efflux of free 2-d[^{14}C]Glc occurred from starved, poisoned glucose-grown cells [7], and (2) that counterflow of 6-d[^{3}H]Glc from cells exposed to unlabeled glucose occurred [8]. In both of these earlier experiments, however, pools of hexose phosphate in the cells were not filled. An investigation of any efflux of labeled free analogue in the absence of phosphorylated hexose was therefore performed by incubating cells in 6-d[^{3}H]Glc in order to fill the internal pools with the free analogue. Transfer of these cells, as before, to 10-times the original volume of buffer, but containing no glucose analogue, resulted in the rapid loss of labeled analogue from the cells (Fig. 2). Initially, these results suggested a role of the phosphorylated hexose analogue in preventing efflux of the free analogue from the cell. However, it was possible that efflux was instead determined by the molecular structure of the sugar analogue. Hence, cells were loaded with both 2-d[^{14}C]Glc and 6-d[^{3}H]Glc so that pools of the two analogues as

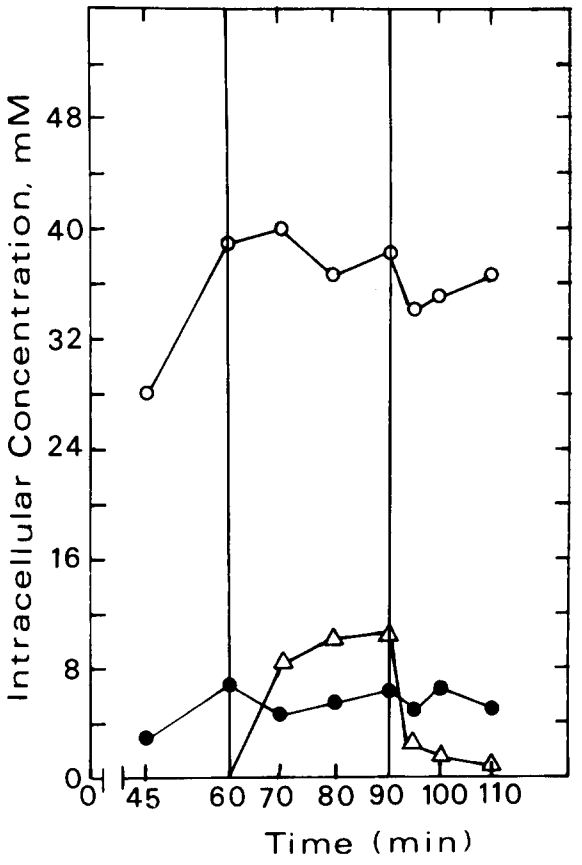

Fig. 3. Distribution of (●) 2-d[^{14}C]Glc and (○) 2-d[^{14}C]Glc-6-*P* in boiled cell free ectracts of *Kluyveromyces lactis* exposed to 10 mM 2-d[^{14}C]Glc. At 1 h cells were washed and incubated with 20 mM 6-d[^{3}H]Glc for 30 min, after which time the cells were transferred to 10-times the original volume of buffer. The intracellular concentration of the 2-deoxy sugars and of (△) 6-d[^{3}H]Glc was determined by thin-layer chromatography.

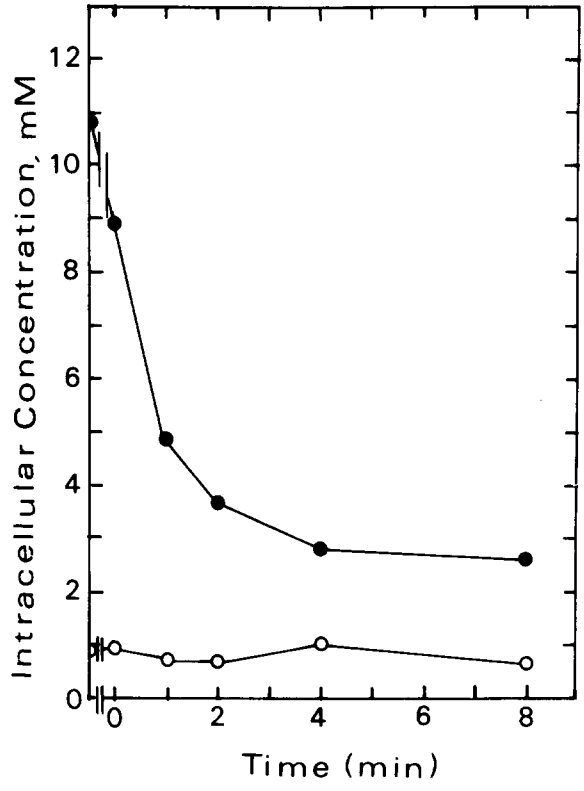

Fig. 4. Efflux of 2-d[^{14}C]Glc from starved, poisoned *Kluyveromyces lactis*. Cells, starved by shaking for 3 h and poisoned with iodoacetate and sodium azide, were exposed to 30 mM 2-d[^{14}C]Glc for 20 min at which time a 0.5-ml sample was taken. Following centrifugation, the cells were resuspended in 10-times the original volume of buffer, and a 5-ml sample taken immediately, i.e., the zero time sample. The intracellular concentrations of (●) 2-d[^{14}C]Glc and (○) 2-d[^{14}C]Glc-6-*P* were determined upon spotting boiled cell free extracts on thin-layer chromatograms.

well as of 2-d[^{14}C]Glc-6-*P* were filled. Transfer of these cells to 10-times the original volume of buffer resulted in the efflux of 6-d[^{3}H]Glc, but not of 2-d[^{14}C]Glc (Fig. 3.). These results suggested an involvement of the C-6 or C-2 hydroxyl group on transport of the substrate from the cell.

Efflux of 2-d[^{14}C]Glc from starved, poisoned cells, i.e., cells devoid of hexose phosphate, was reexamined [7]. In the presence of a low concentration of 2-d[^{14}C]Glc-6-*P*, internal 2-d[^{14}C]Glc does leave the cell (Fig. 4). Hence, efflux of free sugar analogue from the cell is apparently regulated not only by analogue structure, but also by metabolites not found in energy-poor cells.

Another explanation for the selective release of free sugar analogue from the cell may be that two distinct glucose transporters are present in the cell, and that one, specific for glucose and 2-dGlc is inhibited under the conditions employed, whereas, the second one, specific for glucose and 6-dGlc, is not. It has been shown previously that glucose inhibits transport of 2-dGlc [7] and of 6-dGlc [8] into glucose-grown *K. lactis*. It had not been shown, however, that 6-dGlc and 2-dGlc compete for entry into the cell. Competition experiments done at this time revealed that 2-dGlc does competitively inhibit transport of 6-d[^{3}H] Glc into these cells (data not shown).

In order to determine if 2-d[^{14}C]Glc-6-*P* affects the rate of inward diffusion of the free glucose analogue by the cells, cells were exposed to 2-d[^{14}C]Glc for varying periods of times in order to fill the internal pools of 2-d[^{14}C]Glc-6-*P* to levels between 2 and 20 mM. Uptake of 40 mM 6-d[^{3}H]Glc by these cells over a 3-min time period occurred at the same rate as in cells devoid of 2-d[^{14}C]Glc-6-*P*. Similarly, transport of four concentrations of 6-d[^{3}H]Glc by cells containing approx. 25 mM 2-d[^{14}C]Glc-6-*P* occurred at the same velocity over a 3-min interval as found in cells without labeled hexose phosphate. Hence, the phosphorylated hexose does not alter the initial rate of uptake of the free sugar.

In the above experiments, cells contained 2-d[^{14}C]Glc as well as 2-d[^{14}C]Glc-6-*P*. Experiments were conducted to ascertain if the labeled free sugar in the internal pool exchanged with external 6-d[^{3}H]Glc upon exposure to the latter analogue. To do these experiments, cells were first incubated with 10 mM 2-d[^{14}C]Glc for 1 h at which time the internal pool of 2-d[^{14}C]Glc was approx. 4 mM. Next the cells were exposed to 40 mM 6-d[^{3}H]Glc. Chromatographic analyses of cell free extracts obtained during such experiments revealed that counterflow of internal 2-d[^{14}C]Glc did not occur but rather that the cells retained significant levels of the ^{14}C-labeled sugar analogue (Fig. 5A). At equilibration, the internal concentration of 6-d[^{3}H]Glc in control cells, i.e., cells not initially incubated with 2-d[^{14}C]Glc, was greater than that in cells incubated with 2-d[^{14}C]Glc (Fig. 5B). However, at equilibration, the total intracellular concentration of free analogue in the two groups of cells was comparable, suggesting the existence of one pool of free hexose in the cells. In performing this experiment, sodium azide and iodoacetate were added to the cells at 1 h in order to minimize energy production and hence phosphorylation of the 2-d[^{14}C]Glc with subsequent transfer to the hexose phosphate pool. Transfer of cells preloaded with 2-d[^{14}C]Glc to buffer containing 100 mM unlabeled 2-dGlc or to buffer containing 100 mM unlabeled glucose also resulted in retention of internal pools of 2-d[^{14}C]Glc (data not shown).

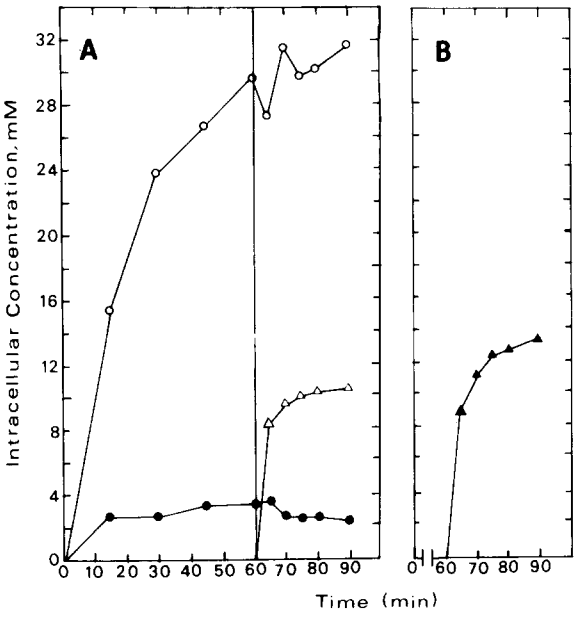

Fig. 5. (A) Intracellular concentration of (●) 2-d[^{14}C]Glc and (○) 2-d[^{14}C]Glc-6-*P* in *Kluyveromyces lactis* incubated with 10 mM 2-d[^{14}C]Glc. At 1 h, 2 mM iodoacetate, 0.32 mM sodium azide, and 40 mM 6-d[^{3}H]Glc were added and the internal concentrations of the ^{14}C-labeled analogues and of (△) 6-d[^{3}H]Glc determined. (B) Control cells were incubated in buffer for 1 h at which time inhibitors and 6-d[^{3}H]Glc were added as above. The intracellular concentration of (▲) 6-d[^{3}H]Glc was determined upon spotting boiled cell free extracts on thin-layer chromatograms.

A double reciprocal plot of the rate of transport of 6-d[^{3}H]Glc by unloaded glucose-grown *K. lactis* as a function of substrate concentration was linear (correlation coefficient 0.98), and revealed that the V_{max} of uptake was 19.5 μmol per g dry weight of cells per min, and the K_m to be 17.4 mM [8]. These values were compared with those obtained using cells preloaded with unlabeled 6-dGlc such that the internal concentration of substrate was 5-times that of external, labeled substrate. The double reciprocal plot here was again linear (correlation coefficient 0.99) and the kinetic constants of transport were similar to those obtained using cells without internal pools of hexose: the V_{max} of transport of labeled substrate into the loaded cells was 25.5 μmol per g dry weight of cells per min, and the K_m was 19 mM.

Discussion

Tritiated 6-dGlc exits from *K. lactis* upon transfer of cells preloaded with the analogue to

glucose-free medium. That such outward diffusion is carrier-mediated is suggested by two earlier findings: (1) counterflow of internal 6-d[^{3}H]Glc, 15 mM, occurred when cells were transferred to medium containing 100 mM glucose, and (2) internal 6-d[^{3}H]Glc exchanged across the membrane against a concentration gradient [8]. Clearly, carrier-mediated efflux of free sugar exists in this yeast as would be expected to occur in a facilitated system.

The indication of a single free sugar pool in these cells suggests that the selective efflux of the C-6 analogue under conditions inhibiting efflux of the C-2 analogue cannot be attributed to inaccessibility of the C-2 analogue to the membrane carrier. Rather, the selective efflux seen here may instead be due to the specificity of the sugar binding sites on the cytoplasmic face of the membrane. It has been found that the inward side of the red blood cell sugar transporter recognizes and binds the C-4 or C-6 end of the glucose molecule, whereas the C-1 end interacts with the transporter on the outside of the membrane [12]. If such a recognition mechanism exists in *K. lactis*, it is suggested that the binding of an analogue containing a C-6 hydroxyl group to the inward side of the carrier results in the inhibition of activity of the transporter, but only in the presence of metabolites of energy metabolism. The involvement of such metabolites on retention of 2-d[^{14}C]Glc is suggested by the experiments using energy-poor cells. The possibility, however, exists that efflux of 2-d[^{14}C]Glc from starved, poisoned cells occurs not due to low levels of energy, hexose phosphate, or possibly other metabolites, but rather due to disrupted cytoplasmic membranes caused by the iodoacetate in the incubation buffer. Spoerl [13] however, has reported that iodoacetate selectively effects intracellular membranes but not external membranes of *S. cerevisiae*. If similar selectivity occurs in *K. lactis* under the conditions employed here is not known at this time. However, the finding that 2-d[^{14}C]Glc remained in cells upon exposure of the cells to 2 mM iodoacetate (Fig. 5A) suggests retention of membrane integrity, at least at low concentrations of inhibitor. In any case, the entrapment of free hexose may serve as an additional means by which cells conserve glucose produced intracellularly. The irreversible inactivation of the glucose transport system in this yeast upon glucose starvation is the other means by which these cells may maintain an internal pool of glucose [14].

In the presence of phosphorylated 2-d[^{14}C]Glc not only is efflux of 2-d[^{14}C]Glc prevented, but exchange of intracellular C-6 hydroxy sugar with exogenous free sugar is also prevented. Hence, in the presence of this intermediate, transport of sugar molecules proceeds unidirectionally only.

In the absence of hexose 6-phosphate, high internal levels of hexose do not alter the rate of inward diffusion of the glucose carrier, as seen by the similar kinetic constants of transport of 6-d-[^{3}H]Glc into cells with pools of unlabeled 6-dGlc and into cells without such pools. Hence, it is unlikely that glucose itself modifies the activity of the glucose transporter. This is also evident in the dual label experiments using cells containing pools of 2-d[^{14}C]Glc and 2-d[^{14}C]Glc-6-*P*. Transport of 6-d[^{3}H]Glc into these cells was not affected by the internal, free sugar analogue. Such experiments also revealed that the hexose 6-phosphate does not alter the inward mobility of the carrier. Hence, in this oxidative yeast, as in *S. cerevisiae*, feedback inhibition of the glucose carrier via accumulated glucose 6-phosphate most likely is not a regulatory event of the Pasteur effect. Rather, it seems that any accumulated glucose 6-phosphate may serve instead to trap free sugar in the cell.

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