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UPTAKE AND PHOSPHORYLATION OF 2-DEOXY-D-GLUCOSE BY WILD-TYPE AND SINGLE-KINASE STRAINS OF *SACCHAROMYCES CEREVISIAE*

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The role of phosphorylation in sugar transport in baker's yeast was studied using 2-deoxy-D-glucose. In wild-type baker's yeast, 2-deoxy-D-glucose is accumulated as a mixture of the free sugar and several derivatives. Pool labeling experiments, designed to determine the temporal order of appearance of labeled 2-deoxy-D-glucose in the intracellular pools, have confirmed previous reports that 2-deoxy-D-glucose first appears in the sugar phosphate pool. Such results are consistent with a transport associated phosphorylation mechanism. Since wild-type yeasts contain three enzymes which could participate in such a process, hexokinase isozymes PI and PII and glucokinase, pool labeling experiments were carried out with single-kinase mutant strains containing only one of these enzymes. Results similar to those for wild-type strains were obtained for all three single-kinase strains, suggesting that if transport associated phosphorylation does occur in baker's yeast, it is not a function of the specific kinase present in the cell. While the results of the pool labeling experiments are consistent with a transport associated phosphorylation mechanism for 2-deoxy-D-glucose, caution is urged in interpreting the results of experiments with whole cells where problems of compartmentation and multiple pools are difficult to assess.

Introduction

The role of phosphorylation in glucose transport in yeast remains unresolved despite numerous studies. The glucose transport system transports nonphosphorylatable glucose analogues by a low affinity, carrier mediated facilitated diffusion process [1–6]. Glucose as well as other phosphorylatable sugar substrates of the glucose transport system (i.e. fructose, mannose and 2-deoxy-D-glucose) are also transported by low affinity carrier mediated facilitated diffusion when their phosphorylation is blocked (i.e. in strains lacking kinases or from inhibition by iodoacetate). However, when these sugars can be phosphorylated, their affinity for transport increases by almost two orders of magnitude [7–9]. The increase in affinity when sugar phosphorylation can occur has lead to two different proposals. One proposes that intermediates of glucose metabolism affect carrier affinity; the other proposes that phosphorylating enzymes participate directly in the transport process. Evidence for the first mechanism, a 3-fold increase in the affinity for transport of nonphosphorylatable substrates in metabolizing cells, is marginal at best [10,11]. On the other hand, evidence for a 'transport-associated phosphorylation' mechanism was reported by Van Steveninck based on pulse labeling experiments using 2-deoxy-D-glucose. Yeast cells incubated with 2-deoxy-D-glucose accumulate a mixture of free sugar and 2-deoxy-D-glucose 6-phosphate. When radioactively labeled 2-deoxy-D-glucose is added to cells equilibrated with unlabeled 2-deoxy-D-glucose, the label was reported to appear in the sugar phosphate pool before it appeared in the free sugar pool [12].

Kotyk and Michaljanicova [13] did not confirm

Van Steveninck's results. They concluded that Van Steveninck had not separated trehalose, a major disaccharide storage product, from the free sugar in his pool analyses. This would have the effect of reducing the specific activity of the free sugar pool relative to the sugar phosphate pool. However, Meredith and Romano [14] repeated the pulse labeling experiments with 2-deoxy-D-glucose avoiding the 'trehalose error' by appropriate chromatographic separations and the use of a petite mutant which does not form trehalose. They confirmed Van Steveninck's claim that the sugar phosphate pool is labeled before the free sugar pool.

The experiments with 2-deoxy-D-glucose show that transport and phosphorylation are kinetically linked. Since wild-type yeast contain three kinases for 2-deoxy-D-glucose phosphorylation (i.e., two hexokinase isozymes, PI and PII, and a glucokinase), it is possible that not all of the kinases are responsible for the apparent linkage between transport and phosphorylation. The availability of single-kinase strains constructed by Lobo and Maitra [15–17] made it possible to determine whether this process is kinase specific. In this paper, we report that pulse labeling experiments with each of these single-kinase strains as well as the parental wild-type strain have produced the same pool labeling order as described by Van Steveninck and Meredith and Romano; thus, whatever the mechanism which accounts for this order of labeling, it is not kinase specific. However, we stress caution in interpreting these results too narrowly because of the uncertainty which subcellular compartmentation may contribute to pool labeling experiments in whole cells.

Materials and Methods

Strains and growth conditions

The strains used in this study are described in Table I. The strains containing only single kinases are designated PI, PII, GK to represent hexokinase isozymes I and II and glucokinase, respectively; a strain lacking all three kinases is designated ZK. These strains and the parental wild-type strain, HSC, were kindly provided by P.K. Maitra and Z. Lobo of the TATA Institute, Bombay, India. The wild-type and single kinase strains were maintained on YPG or YPGal slants (1% Difco yeast extract, 2% Difco peptone and 2% glucose or 2% galactose): Cells used for transport studies were grown for 24–48 h in broth cultures at 30°C in a rotary shaker in 500 ml flasks containing 200 ml of GGY or GGalY medium (1% glycine, 1% glucose or galactose, and 0.1% yeast extract) described by Meredith and Romano [14]. The cells were harvested by centrifugation, washed twice with distilled water and resuspended to 10% (wet weight/volume).

TABLE I

STRAINS USED IN THIS STUDY

Strain	Genotype	Kinase phenotype
L 14	Polyploid, wild type	wild type
HSC	α, haploid, wild type	wild type
P_1T8C	α, *ade*, *HXK1*, *hxk 2*, *glc* 1	PI
$P_2 22D$	α*ade*, *hxk* 1, *HXK* 2, *glc* 1	PII
D308	α, *ade*, *hxk* 1, *hxk* 2, *GLC* 1	GK
D308a	α, *ade*, *hxk* 1, *hxk* 2, *glc* 1	ZK

Transport and pool labeling experiments

Cell incubations were carried out in 15 ml Corex centrifuge tubes at 30°C containing 2% cell suspensions (wet weight/volume) and 1 mM 2-deoxy-D-glucose (12–15 μC/μmol). The suspension was vigorously stirred by a magnetic stirrer. Samples (0.5–1.0 ml) were removed at appropriate intervals and transferred to 5 ml ice cold distilled water over glass fiber filters (Reeve Angel 934 AH). The suspension was filtered by suction and washed with two 5 ml portions of ice cold distilled water. The filters and cells were transferred to 5 ml distilled water in 15 ml centrifuge tubes in a boiling water bath and extracted for 15 min. The boiled water extracts were clarified by centrifugation at $3000 \times g$ for 10 min. Portions of the extracts were analyzed without further treatment for total radioactivity and by thin-layer chromatography to determine the distribution of label among 2-deoxy-D-glucose and its derivatives.

Analysis of 2-deoxy-D-glucose pools

2-Deoxy-D-glucose pool components were identified and their concentrations were determined in extracts from cells incubated for 60 min at 20 or

30°C. 1 ml portions of the cell suspensions (in triplicate) were washed and extracted in 5 ml distilled water as described above. A 1 ml portion of the extract was used to determine the total radioactivity. A 0.5 ml portion of the extract was concentrated by lyophilization and chromatographed by ascending thin-layer chromatography on Silica gel G plates in the following solvent system: *n*-butanol/ethyl acetate/isopropanol/acetic acid/distilled water (35 : 100 : 60 : 35 : 30, v/v) (Fig. 1). The chromatograms were developed twice in the same direction to achieve better resolution [18]. Fluorography of the TLC plates was carried out as described by Bonner and Stedman [19]. The

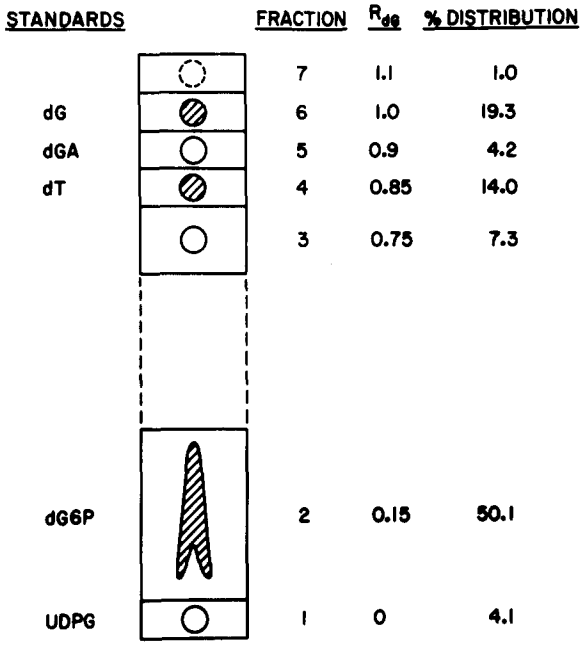

Fig. 1. Fluorogram of boiled yeast cell extract. HSC cells (20 mg wet weight/ml) were incubated with ^{3}H-labeled 2-deoxy-D-glucose (1 mM, 15 μCi/μmol) for 60 min at 30°C. A portion of boiled water extract of washed cells corresponding to 2 μl cell water was chromatographed by ascending thin-layer chromatography on Silica gel G plates. Fluorograms were developed using flash sensitized X-ray film. The radioactivity of each fraction, located from the fluorogram, was determined in the silica gel scraped off the plates. The position of standards are shown to the left of the fluorogram; unlabeled derivatives are presently unidentified. The radioactivity recovered from each fraction (1–7) is presented as percent of the total activity recovered from each lane in the chromatogram. dG, 2-deoxy-D-glucose; dG6P, 2-deoxy-D-glucose 6-phosphate; dGA, 2-deoxygluconate; dT, 2-deoxytrehalose.

plates were coated with 7% (w/v) PPO in diethyl ether and stored for 1 week at −80°C with flash-sensitized X-ray film. The position of radioactive pool components were identified by placing the thin-layer plates over the fluorograms on a light box. The silica powder over the fluorogram spots was removed by aspiration into a Swinney microfiltration apparatus holding a glass fiber filter. The trapped silica powder and filter were transferred to Amersham ACS counting fluid for counting with a Packard liquid scintillation spectrometer. The cellular concentration of each of the pool components (μmol/μl cell water) was calculated from the concentration of the extract (μl extract/μl yeast cell water), the amount of radioactivity recovered (cpm/μl extract) and the specific activity of the labeled sugar (μmol/cpm). One gram of wet yeast was assumed to contain 0.5 g yeast cell water [2].

Pool labeling experiments

Cells used for pool labeling experiments were incubated in parallel with cells used to determine 2-deoxy-D-glucose pools. The cells were incubated at 20 or 30°C for 60 min with 1 mM unlabeled 2-deoxy-D-glucose. Labeling was begun by the addition of an analytically insignificant concentration of labeled 2-deoxy-D-glucose. At intervals, usually beginning 5 s after addition of label, 0.5 ml portions of cell suspension were removed, washed and extracted as described above. The extracts were analyzed by TLC as described above. The specific activity of each pool (cpm/μmol) was determined from the radioactivity found in the pool labeling experiment (cpm/μl yeast cell water) divided by the concentration of the component determined as described in the previous section (μmol/μl cell water).

Retention of external medium by washed cells in pool labeling experiments

Because a high cell density (20 mg wet cells/ml) and high level of radioactivity (approx. $5 \cdot 10^6$ cpm/ml) were used in pool labeling experiments, a significant retention of external medium by washed cells was observed. The concentration of 2-deoxy-D-glucose in the extracts which resulted from retention of external medium by the washed cells and filters was determined from the retention of

radiolabeled 1 mM L-glucose by comparable amounts of cells (i.e. 0.5 ml 2% cell suspensions). About 0.4 μl of medium is retained despite two washes with 5 ml ice cold distilled water; this represents 0.1% retention of the external medium. A similar retention was observed with cells incubated with 2-deoxy-D-glucose for 5 min at 0°C. Therefore, all calculations of the specific activity of the 2-deoxy-D-glucose pool have been corrected for 0.4 μl contamination per 10 mg (wet weight) of cells.

Chromatographic standards

Standards for identification of 2-deoxy-D-glucose derivatives were prepared from 2-deoxy-D-[6-^{3}H]glucose: (a) 2-deoxy-D-glucose 6-phosphate was prepared by hexokinase treatment with ATP [20] and (b) 2-deoxygluconate by treatment with glucose oxidase [21]. 2-Deoxytrehalose was extracted from yeast cells incubated with 2-deoxy-D-[6-^{3}H]glucose according to the procedure of Farkas et al. [22].

Materials

2-Deoxy-D-[6-^{3}H]glucose was prepared by Mr. James Balschi of our Chemistry Department; 2-deoxy-D-[U-^{14}C]glucose was purchased from Amersham. Silica gel G thin-layer plates were purchased from Analtech, Newark, DE. Enzymes and ATP were purchased from Sigma Chemical Co., St. Louis, MO. All other chemical and biochemical reagents were of the highest commercial grades available.

Results

The 2-deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate pools

The number and size of the intracellular pools formed when cells are incubated with 2-deoxy-D-glucose was determined by TLC and fluorography of cell extracts. Hot water extracts were prepared from cells incubated with labeled 1 mM 2-deoxy-D-glucose for 60 min at 30°C. A representative fluorogram obtained from wild-type, HSC cells is shown in Fig. 1. The radioactivity is distributed among seven spots. Nearly 50% is present in non-phosphorylated derivatives of which free 2-deoxy-D-glucose makes up about 40% and D-trehalose constitutes about 30%. The intracellular concentration of each pool component is determined from the radioactivity recovered from the chromatogram, the amount of extract chromatographed and the specific activity of the sugar used.

Kinetics of pool labeling in wild-type cells

Pool labeling was initiated by the addition of labeled 2-deoxy-D-[6-^{3}H]glucose to a parallel suspensions incubated with unlabeled 2-deoxy-D-glucose for 60 min at 20 or 30°C. The time course of the appearance of radioactivity in the chromatogram at the position corresponding to each pool component is presented in Table II. Only the 2-deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate fractions are labeled significantly between 5 and 45 s. The activities of the other fractions are small and quite variable, therefore, only the specific activities of the 2-deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate pools are considered in subsequent experiments. The specific activity of each pool was calculated from the cpm of radioactive 2-deoxy-D-glucose or 2-deoxy-D-glucose 6-phosphate in 1 ml of boiled water extract of cells to which label was added at 60 min ('pulsed cells') divided by the size (μmol/ml boiled extract) of the 2-deoxy-D-glucose or 2-deoxy-D-glucose 6-phosphate pool, respectively. The specific activity of the 2-deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate pools, as a function of labeling time, for the experiment described in Table II are presented in Table III (Expt. 1) and Fig. 2A. The plot of the specific activities of the 2-deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate pools presented in Fig. 2A shows that the uncorrected specific activity of the 2-deoxy-D-glucose pool is higher than that of the 2-deoxy-D-glucose 6-phosphate pool for the first 30 s, however, by 45 s, there is a reversal of the relative specific activities of these two pools. A similar 'crossing over' of uncorrected specific activities of the 2-deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate pools is also observed in a duplicate experiment (Table III, Expt. 2 and Fig. 2B), however, in this case 'crossing over' occurs by 15 s. The 'crossing over' disappears, however, when the specific activity of the 2-deoxy-D-glucose pool is corrected for the amount of external medium retained by the washed cells (see Methods). The corrected 2-deoxy-D-glucose

TABLE II

DISTRIBUTION OF LABEL AMONG 2-DEOXY-D-GLUCOSE AND ITS DERIVATIVES

'Pool' components were determined in boiled extract from cells incubated (20 mg wet weight/ml) at 30°C for 60 min with 1 mM labeled 2-deoxy-D-glucose (2-deoxy-D-[6-^{3}H]glucose; 15 μCi/μmol). Samples corresponding to 2 μl cell water were chromatographed by TLC on Silica gel G plates. The radioactivity was measured in the silica gel scraped from the TLC plates according to the fractionation scheme of Fig. 1. The radioactivity of the 'pulse' samples was determined in a similar manner in extracts of cells incubated in parallel suspensions with unlabeled 2-deoxy-D-glucose. Labeling was begun by the addition of labeled 2-deoxy-D-glucose (15 μCi/μmol) at 60 min. Samples corresponding to 1 μl cell water were chromatographed and assayed as described above.

Chromatogram Fraction No.	Pool		Pulse							
	cpm	% Distribution	cpm				% Distribution			
			5	15	30	45	5	15	30	45
7	311	1.3	9	9	6	10	2.2	0.9	0.6	0.5
6 dG	4620	19.3	146	326	301	469	36.1	33.1	29.0	23.0
5	1006	4.2	7	18	18	78	1.8	1.8	1.7	3.8
4	3351	14.0	4	18	6	5	1.0	1.8	0.6	0.2
3	2035	8.5	6	13	6	10	1.5	1.3	0.6	0.5
2 dGP	12208	51.0	210	556	659	1364	51.9	56.4	63.5	66.8
1	479	2.0	23	46	42	106	5.7	4.6	4.1	5.2
Totals	24010	100	405	986	1038	2042	100	100	100	100

specific activity (designated ΔdG in Table III and Fig. 2) is lower than that of the 2-deoxy-D-glucose 6-phosphate specific activity at all time points. Thus, the sugar phosphate pool appears to be labeled before the free 2-deoxy-D-glucose pool, confirming the results of Van Steveninck [12] and Meredith and Romano [14] with other wild-type (i.e. multiple kinase) strains. It was, therefore, of

TABLE III

KINETICS OF POOL LABELING IN HSC CELLS

The radioactivity of cell extracts from cells incubated for 1 h at 30°C with 1 mM labeled 2-deoxy-D-glucose (15 μCi/μmol) for 1 h were analyzed as described in Table II. The concentration of the 2-deoxy-D-glucose 6-phosphate and 2-deoxy-D-glucose pools was determined from the radioactivity of TLC fractions 2 and 6 (see Fig. 1), respectively, of a sample of boiled water extract corresponding to 2 μl of cell water. The specific activity of the pulse samples was determined from the radioactivity of the corresponding TLC fractions of extracts from cells incubated with unlabeled 1 mM 2-deoxy-D-glucose for 1 h before the addition of label. The time of exposure to label is indicated. dG, 2-deoxy-D-glucose; dGP, 2-deoxy-D-glucose 6-phosphate.

Expt. No.	Sugar derivative	Pool (nmol/μl)	Pulse (cpm/nmol)			
			Seconds			
			5	15	30	45
1	dG (uncorrected)	1.1	169	368	403	509
	ΔdG (corrected)		30	170	187	294
	dGP	2.8	92	273	355	559
	dGP/ΔdG		3.1	1.6	1.9	1.9
2	dG	1.0	348	464	616	868
	ΔdG		89	140	224	347
	dGP	5.0	98	239	492	660
	dGP/ΔdG		1.1	1.7	2.2	1.9

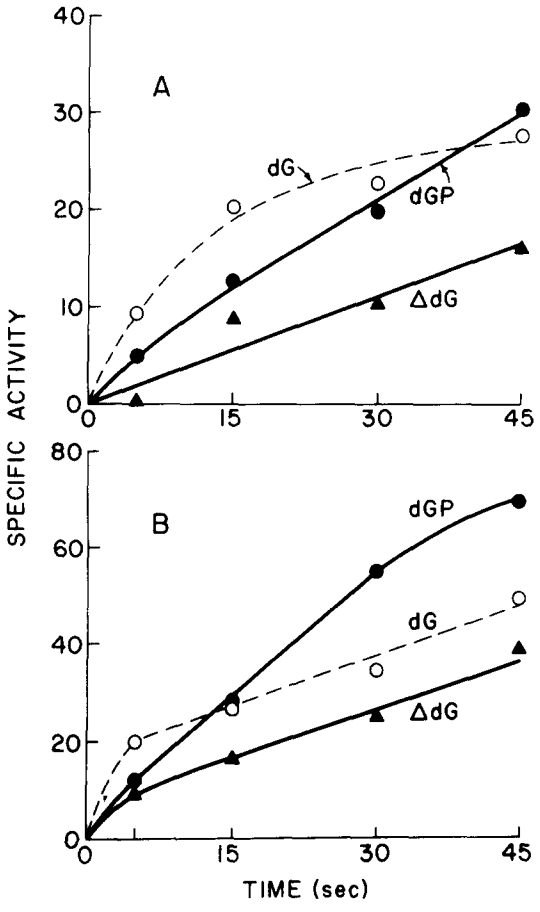

Fig. 2. Pool labeling kinetics in HSC cells. The specific activity in arbitrary units of the free sugar and sugar phosphate pools presented in Table III are presented in graphic form. The specific activity of the free sugar pool is shown without correction for extracellular contamination (dG) and after correction (ΔdG). A and B represent Expts. 1 and 2 of Table III, respectively. dG, 2-deoxy-D-glucose; dGP, 2-deoxy-D-glucose 6-phosphate.

interest to determine whether similar results would be obtained with the Maitra and Lobo single-kinase strains [15–17,20].

Kinetics of pool labeling with single-kinase strains

Results essentially identical to those of wild-type cells were obtained in pool labeling experiments with the GK (Table IV), PI (Table V) and PII (Table VI) strains. In all cases, use of the uncorrected 2-deoxy-D-glucose specific activity suggested that the free sugar pool is labeled before the sugar phosphate pool. However, after correction, ΔdG is lower than that of 2-deoxy-D-glucose 6-phosphate. The single exception (Expt. 1 with PI cells; Table V) in which the specific activity of ΔdG is still higher than that of deoxy-D-glucose 6-phosphate (dGP) at 5 s (i.e. the dGP/ΔdG ratio

TABLE IV

KINETICS OF POOL LABELING IN GK CELLS

Conditions and analyses as described in Table III.

Expt. No.	Sugar derivative	Pool (nmol/μl)	Pulse (cpm/nmol)			
			Seconds			
			5	15	30	45
1	dG	0.5	1076	1680	3473	4829
	ΔdG		334	1394	2605	3977
	dGP	2.4	567	2369	4428	6363
	dGP/ΔdG		1.7	1.7	1.7	1.6
2	dG	1.5	223	305	465	527
	ΔdG		94	178	319	361
	dGP	6.9	179	334	542	938
	dGP/ΔdG		1.9	1.9	1.7	2.6
3	dG	1.0	187	478	656	767
	ΔdG		31	373	558	688
	dGP	2.3	313	745	1394	2547
	dGP/ΔdG		10.0	2.0	2.5	3.7

TABLE V

KINETICS OF POOL LABELING IN PI CELLS

Conditions and analysis as described in Table III.

Expt. No.	Sugar derivative	Pool (nmol/μl)	Pulse (cpm/nmol)		
			Seconds		
			5	15	30
1	dG	1.5	553	385	378
	ΔdG		495	279	277
	dGP	10.5	99	279	664
	dGP/ΔdG		0.2	1.0	2.4
2	dG	1.7	170	185	219
	ΔdG		42	61	92
	dGP	13.3	71	179	354
	dGP/ΔdG		1.7	2.9	3.9

TABLE VI

KINETICS OF POOL LABELING PII CELLS

Conditions and analysis as described in Table III.

Sugar derivative	Pool (nmol/μl)	Pulse (cpm/nmol)		
		Seconds		
		5	30	45
dG	0.6	438	590	1023
ΔdG		217	365	790
dGP	2.3	260	1016	2482
dGP/ΔdG		1.2	2.8	3.1

is below 1.0) is suspect since the 5 s uptake is anomalously high. The similarity between single-kinase and wild-type cells indicate that the apparent order of pool labeling is not a function of the specific kinase present in the cells.

Discussion

In this study we have used a pool labeling technique to determine the apparent order of labeling of intracellular pools when labeled 2-deoxy-D-glucose is added to cells previously equilibrated with this sugar. Using this technique Van Steveninck [12] and Meredith and Romano [14] reported that the sugar phosphate pool is labeled before the free sugar pool. On this basis they supported a transport-associated phosphorylation mechanism for glucose. We have confirmed their experimental results with this sugar; furthermore, we obtained similar results with wild-type and single-kinase strains. However, interpretation of pool labeling experiments in whole yeast cells must be made with caution.

Van Steveninck introduced the pool labeling technique to study the mechanism of sugar transport in yeast based on the success of the use of this procedure to study glucose transport in purified bacterial membrane vesicles by Kaback [23]. Van Steveninck studied the transport of glucose [12,24], galactose [25, 26] and α-methyl-D-glucopyranoside [27]; in all cases he reported that the sugar phosphate pool is labeled before the free sugar pool. On this basis he concluded that transport associated phosphorylation is the general mechanism for all the yeast sugar transport systems. However, subsequent studies cast doubt on Van Steveninck's conclusions. In Van Steveninck's original study he reported that cells equilibrated with 2-deoxy-D-glucose contained only two pools, unmodified 2-deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate. However, subsequent studies of 2-deoxy-D-glucose metabolism in yeast by Biely, Bauer and coworkers [28–31] showed that 2-deoxy-D-glucose is transformed to many derivatives including 2-deoxytrehalose, 2-deoxygluconate, 2-deoxy-D-glucose 6-phosphate, 2-deoxy-D-glucose 1,6-diphosphate and UDPdGlc. Failure to recognize these derivatives could have affected Van Steveninck's results. Kotyk and Michaljanicova [13] then showed that trehalose was a major component formed by glucose, galactose and α-methyl-D-glucoside. When the trehalose pool was separated from the free sugar and sugar phosphate pool, they reported that the free sugar pool was labeled before the sugar phosphate pool. Thus, they attributed the difference between their conclusions and Van Steveninck's to his failure to separate trehalose (or 2-deoxytrehalose) and free sugar. Kotyk and Michaljanicova's observations on galactose confirmed earlier results of pool labeling experiments by Kuo and Cirillo [32] with mutants lacking UDP:galactose-1-phosphate uridylyltransferase. In these cells, in which galactose can be phosphory-

lated but not converted to any other derivative, pool labeling experiments showed that the free sugar pool is labeled before the sugar phosphate pool. Jaspers and Van Steveninck [21] rejected Kotyk and Michaljanicova's suggestion that his results were the result of incomplete separation of free sugar and trehalose or 2-deoxytrehalose. In rebuttal, Jaspers and Van Steveninck [21] presented results of new experiments on 2-deoxy-D-glucose uptake by a different yeast species, *Saccharomyces fragilis*, which produces several 2dG derivatives including 2-deoxy-trehalose. Pulse labeling experiments with this strain produced results similar to those they previously obtained with *Saccharomyces cerevisiae*; the sugar phosphate pool is labeled before the free sugar pool even though 2-deoxytrehalose was clearly separated from 2-deoxy-D-glucose. This study is noteworthy because in some individual experiments the opposite order of labeling was observed. The authors presented convincing experimental and theoretical evidence to show that when the reverse order was observed, it was due to an incomplete removal of the extracellular sugar from the filters containing the cells. A similar source of error was observed in some experiments in the present study. Contamination with extracellular sugar has a characteristic effect on the relative rates of increase of the specific activity of the free sugar and sugar phosphate pools. The specific activity of the free sugar pool is initially higher than that of the sugar phosphate pool but subsequently falls below that of sugar phosphate (Fig. 2) although the specific activity of the free sugar is still far from equilibrium. By contrast, the specific activity of the sugar phosphate pool increases continuously from zero time and crosses over that of the free sugar. This 'crossing over' of the sugar phosphate specific activity while the free sugar activity is still far below the equilibrium value (i.e. less than 10–20%) is diagnostic of the presence of more than one free sugar pool which are labeled with markedly different kinetics. It is noteworthy that the pool labeling experiments described by Kotyk and Michaljanicova [13] also exhibit two component kinetics for the labeling of the free sugar pool as well as 'crossing over' between the free glucose and glucose 6-phosphate specific activity curves (their Table IV and Fig. 6). Their pool labeling data for glucose and galactose show that by their first time point (i.e., 9 s) the specific activity of the free sugar pool is about 40% of its maximum value and does not change from 9 s to 5 min of labeling. Complete equilibration is achieved at a slower rate between 5 min and 1 h of incubation. The two pools which these labeling kinetics suggest exist, either represent extracellular and intracellular free sugar, respectively, or the presence of intracellular compartmentation of multiple sugar pools. The significance of subcellular compartmentation of metabolites in eukaryotic cells is now well recognized. The existence of separate cytoplasmic and vacuolar pools is yeast for sugars, amino acids and inorganic ions is now well documented. Spoerl [33] was the first to present evidence for the subcellular compartmentation of sugar based on multiphasic efflux kinetics for non-metabolized sugars. He presented indirect evidence that the 'slow compartment' is the cell vacuole. We have observed similar multiphasic efflux kinetics of glucose in yeast strains lacking all three kinases (phenotype ZK) (unpublished data). Direct evidence for the existence of separate cytoplasmic and vacuolar pools has been demonstrated for basic amino acids [34,35] and for K^+, Mg^{2+} and inorganic phosphate [36]. It is very probable that 2-deoxy-D-glucose and its metabolites are also distributed between the cytoplasm and the vacuole. Such compartmentation complicates the simple interpretation of pool labeling experiments. An example of the effect of subcellular compartmentation on pool labeling kinetics was illustrated in a recent study by Visser et al. [37] in blood stream forms of *Trypanosoma brucei* in which sugar metabolites are distributed between a cytologically distinct organelle, the glycosome, and the cytoplasm. Sufficient appreciation for the problem of subcellular compartmentation has not been expressed by previous investigators using pool labeling techniques to study sugar transport in yeast.

Even if problems of extracellular contamination or subcellular compartmentation were discounted, the observation that the sugar phosphate pool is labeled before the free sugar pool does not necessarily mean that the yeast glucose transport system is a 'transport-associated phosphorylation' process homologous to the bacterial PTS. Evidence for a membrane carrier which mediates facilitated diffu-

sion of glucose and its analogues under nonphosphorylating conditions is overwhelming [1–14]. Evidence that glucose phosphorylation in baker's yeast is mediated only by soluble kinases is equally well established [38,39]. Glucose transport and phosphorylation must, therefore, involve the activity of the carrier coupled with the kinases. The pool labeling experiments do not allow an unambiguous interpretation of the nature of this coupling. Meredith and Romano [14] point out that while the results are compatible with a transport-associated phosphorylation process, they do not exclude intracellular phosphorylation following facilitated diffusion if the kinases occur in sufficient excess to phosphorylate the sugar as fast as it enters the cell.

These extreme alternatives suggest very different mechanisms for the effect that sugar metabolism has on transport affinity of metabolizable sugars. In the absence of a kinase-carrier association, transport affinity would have to be mediated by a metabolite of glucose metabolism. Support for such a mechanism was presented by Serrano and DelaFuente [10]. They presented kinetic evidence that the glucose carrier exists in a high- and low-affinity state. They propose that the transition between the high- and low-affinity states of the carrier is regulated by an intermediate of sugar metabolism as proposed earlier by Sols [6].

If, on the other hand, the kinases are complexed to the carriers, a functional analogue of the bacterial PTS would exist. Such a hypothetical complex provides an attractive mechanism for the influence of kinase activity on carrier affinity. Yeast hexokinase undergoes remarkable conformational changes upon substrate binding [40–42]. Such conformational changes could be transduced from the kinase to the carrier with a concomitant effect on its affinity. Regulation could also involve the effects of metabolites on carrier-kinase association and dissociation. The ability of 'soluble' enzymes to become attached to membranes is now well documented [43]. The attachment of brain hexokinase to mitochondria [44] and glycolytic enzymes to erythrocyte membrane proteins [45,46] are but two examples. The distribution of soluble proteins between a membrane bound and free form has led to their description as 'ambiguous' (i.e. in two places) [47]. Since we have found that the pool labeling results are the same for all three single-kinase strains, it would be necessary to propose that both hexokinase isozymes and glucokinase are capable of forming a functional complex. Meredith and Romano [14] have cited the marked correlation between the relative affinity of various sugars as transport substrates and as substrates of hexokinase in support of a direct role of the kinases in the transport process. On the other hand, the similarity in substrate specificity of the sugar carrier and the sugar kinases might reflect a common evolutionary origin. Saier [48] has proposed that sugar kinases and sugar carriers are evolutionary products of a duplicated kinase gene. Support for this attractive hypothesis must await the final isolation and appropriate characterization of the sugar carrier.

It is clear from the foregoing speculations that the question of the role of transport associated phosphorylation in the yeast glucose transport system has not been answered by this study. However, we feel that the results of our study and those of Meredith and Romano [14] support Van Steveninck's proposal that some form of a transport-associated phosphorylation mechanism is a possibility for glucose transport. It is clear that an unambiguous conclusion cannot be reached by studies with whole cells. It is imperative, therefore, that future studies be carried out with membrane vesicles [49,50] and reconstituted liposomes [51,52].

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