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Arsenic Trioxide Uptake by Hexose Permeases in *Saccharomyces cerevisiae**

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Arsenic trioxide is a toxic metalloid and carcinogen that is also used as an anticancer drug, and for this reason it is important to identify the routes of arsenite uptake by cells. In this study the ability of hexose transporters to facilitate arsenic trioxide uptake in *Saccharomyces cerevisiae* was examined. In the absence of glucose, strains with disruption of the arsenite efflux gene *ACR3* accumulated high levels of $^{73}As(OH)_3$. The addition of glucose inhibited uptake by ~80%. Disruption of *FPS1*, the aquaglyceroporin gene, reduced glucose-independent uptake by only about 25%, and the residual uptake was nearly completely inhibited by hexoses, including glucose, galactose, mannose, and fructose but not pentoses or disaccharides. A strain lacking *FPS1*, *ACR3*, and all genes for hexose permeases except for *HXT3*, *HXT6*, *HXT7*, and *GAL2* exhibited hexose-inhibitable $^{73}As(OH)_3$ uptake, whereas a strain lacking all 18 hexose transport-related genes (*HXT1* to *HXT17* and *GAL2*), *FPS1* and *ACR3*, exhibited <10% of wild type $^{73}As(OH)_3$ transport. When *HXT1, HXT3, HXT4, HXT5, HXT7*, or *HXT9* was individually expressed in that strain, hexose-inhibitable $^{73}As(OH)_3$ uptake was restored. In addition, the transport of [^{14}C]glucose was inhibited by $As(OH)_3$. These results clearly demonstrate that hexose permeases catalyze the majority of the transport of the trivalent metalloid arsenic trioxide.

Arsenic trioxide is a carcinogen (1) and is also the active ingredient in the chemotherapeutic drug Trisenox, which is used for the treatment of acute promyelocytic leukemia (2). For trivalent arsenic to be a carcinogen or for Trisenox to be a useful drug, they must first be taken up into cells. Because it is unlikely that transport systems evolved for the uptake of arsenic trioxide, it is most likely taken up by transporters for biological molecules. To predict the routes of uptake, it is necessary to have a clear understanding of the species present in solution at neutral pH. Arsenic trioxide is in equilibrium with the oxyanion arsenite, but, with a pK_a of 9.2, it should be fully protonated and uncharged at neutral pH. We have shown by extended x-ray absorption fine structure spectroscopy that in solution at neutral pH, the predominate species is $As(OH)_3$.[1] Thus, physiologically, As(III) appears to be a polyhydroxylated molecule; therefore, polyol transporters would be reasonable candidates for uptake systems. One class of polyol transporters are the aquaglyceroporins, which are channels for small uncharged solutes, such as glycerol (3). Members of the aquaglyceroporin family from bacteria (GlpF), yeast (Fps1p), and mammals (Aqp9) are $As(OH)_3$ channels (4–6). Disruption of the yeast *FPS1* gene resulted in substantial loss of uptake of $As(OH)_3$ when glucose was present in the assay medium (4).

In this study, we report that hexose permeases are responsible for the majority of $As(OH)_3$ accumulation in *Saccharomyces cerevisiae*. In glucose-free medium, the *fps1*Δ strain exhibits only a 25% reduction in $^{73}As(OH)_3$ uptake compared with its parent. The residual uptake was almost completely inhibited by glucose, with half-maximal inhibition at ~5 mM glucose. Other hexoses, including mannose, fructose, and galactose also inhibited $As(OH)_3$ uptake, whereas neither sucrose nor glycerol had an effect. A strain in which all glucose transporters were disrupted exhibited a low level of $^{73}As(OH)_3$ uptake comparable with that of its parent in the presence of glucose, and transport in that multiply disrupted strain was insensitive to glucose. A similar construct, with *HXT3, HXT6, HXT7*, and *GAL2* remaining, still exhibited glucose-inhibitable $^{73}As(OH)_3$ transport. *HXT1, HXT3, HXT4, HXT5, HXT7*, and *HXT9* were individually expressed from plasmids and shown to restore $^{73}As(OH)_3$ transport. In addition, $As(OH)_3$ inhibited uptake of [^{14}C]glucose in cells expressing all glucose permeases or just *HXT7*. These results clearly demonstrated that glucose carrier proteins catalyze the transport of trivalent arsenic. We propose that hexose permeases may have a role in uptake and toxicity of arsenic trioxide in other eukaryotes, including humans.

MATERIALS AND METHODS

Strains and Plasmids—Escherichia coli strain DH-5α (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(lac-proAB) *F′* (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*) was used for molecular cloning. *S. cerevisiae* strains used in this study are described in Table I. Plasmids YEpKHXT7 carrying the *HXT7* gene and the pTHHXT-series plasmids carrying *the HXT1, HXT3, HXT4, HXT5, HXT9,* and *HXT10* genes (7, 8) were under the control of a truncated and constitutive *HXT7* promoter. The plasmids were transformed into yeast strain HD300.

Media—S. cerevisiae strains were grown at 30 °C in minimal SD medium (9) supplemented with 2% maltose and auxotrophic requirements. Where indicated, 2% galactose or glucose was used as a carbon source. *E. coli* cells were grown in Luria-Bertani medium (9) supplemented with 100 μg/ml ampicillin, as required.

DNA Manipulations—Plasmid purification and *E. coli* transformations were carried out as described by Sambrook *et al.* (10). Transfor-

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[1] A. Ramírez-Solís, R. Mukopadhyay, B. P. Rosen, and T. L. Stemmler, unpublished data.

TABLE I
Strains

S. cerevisiae strains/plasmids	Genotype/description	Source
CEN.PK2-1C	*MATα his3-Δ1,15 leu2–3,112 ura3–52, trp-1–289, Mal2–8, SUC2 hxt17Δ*	(13)
EBY.VW367$^+$	CEN.PK2-1C *hxt13Δ::loxP hxt15Δ::loxP hxt16Δ::loxP hxt14Δ::loxP hxt12Δ::loxP hxt9Δ::loxP hxt11Δ::loxP hxt10Δ::loxP hxt8Δ::loxP hxt514Δ::loxP hxt2Δ::loxP*	(13)
EBY.VW1000	EBY.VW367$^+$ *hxt367Δ::loxP gal2Δ*	(13)
MG102	*MATα ura3–52 his6 leu2–3,112 his3-Δ,200 trp1–901 lys2–801 suc2Δycf1::hisG acr3::URA3*	(11)
HD9	*MATα, ura3–52 his6 leu2–3,112 his3-Δ,200 trp1–901 lys2–801 suc2Δycf1::hisG acr3::URA3 fps1::leu2*	(4)
HD100	CEN.PK2-1C *fps1::leu2 acr3::his3*	This study
HD200	EBY.VW367$^+$ *fps1::leu2 acr3::his3*	This study
HD300	EBY.VW1000 *fps1::leu2 acr3::his3*	This study
YEpHXT7	*HXT7* gene cloned under control of its own truncated and constitutive promoter	(7)
pTHHXT series	*HXT* genes cloned behind a constitutive *HXT7* promoter fragment	(8)

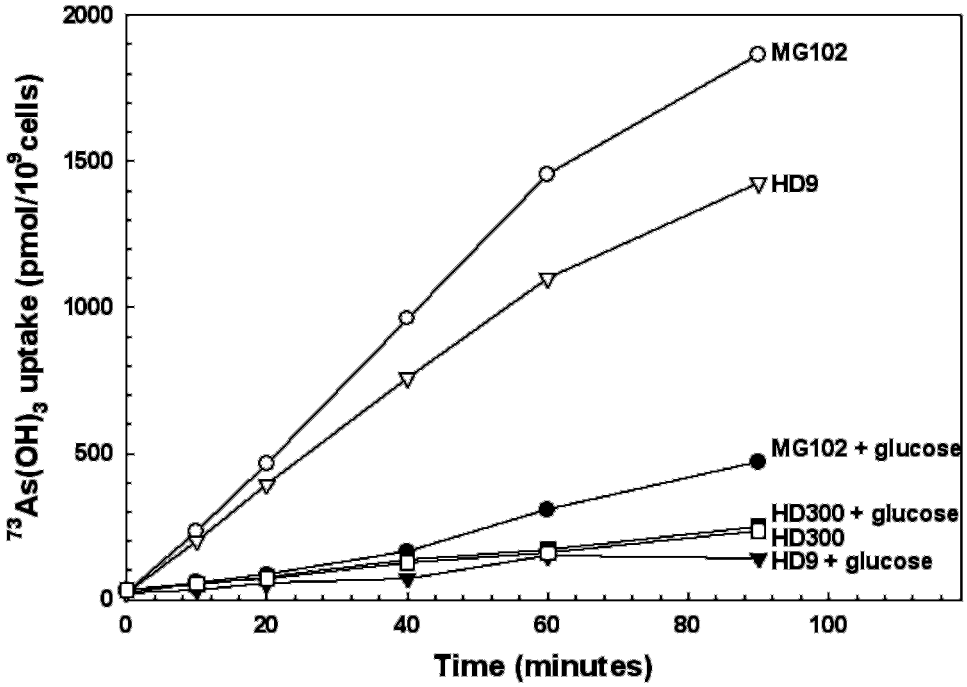

FIG. 1. **Inhibition of arsenic trioxide transport by glucose in *S. cerevisiae*.** $^{73}As(OH)_3$ uptake in *S. cerevisiae* was assayed as described under "Materials and Methods." Shown are MG102 (*acr3*Δ) (●, ○), HD9 (*fps1*Δ *acr3*Δ) (▼, ▽), and HD300 (*hxt1–17*Δ *gal2*Δ *fps1*Δ *acr3*Δ) (■, □) without (○, ▽, □) or with (●, ▼, ■) 0.1 M glucose.

mation of the yeast cells was carried out using a Geno easy-transform kit (Geno Technologies, St. Louis, MO).

Disruption of ACR3 and FPS1—Yeast strain HD100 was constructed from CEN.PK2-1C by a stepwise replacement of 0.8 kbp of *FPS1* with a *LEU2* gene (4) and 1.15 kbp of *ACR3* with a *HIS3* gene (11). HD200 and HD300 were constructed similarly from strains EBY.VW367$^+$ and EBY.VW1000, respectively. Gene disruptions were verified by polymerase chain reaction.

Assays of $^{73}As(OH)_3$ and [^{14}C]Glucose Transport—For the transport assays, most strains were grown to exponential phase at 30 °C in SD medium with 2% galactose. HD300, which cannot grow in hexoses, was grown in 2% maltose. HD300 carrying plasmids with *HXT* genes were grown in 2% glucose. The cells were harvested, washed, and suspended to a density of 5×10^8 cells/ml. *In vivo* assays of $^{73}As(OH)_3$ (Los Alamos National Laboratories) were performed as described by Ghosh *et al.* (11). Uptake of 5 mM D-[1-^{14}C]glucose (PerkinElmer Life Sciences) was assayed as described by Wieczorke *et al.* (12) with minor modifications.

RESULTS

Hexose Permeases Transport $^{73}As(OH)_3$ in S. cerevisiae—The cells of wild type yeast accumulate little arsenic trioxide, but when the *ACR3* gene encoding the arsenite efflux permease is disrupted, they accumulate the metalloid to high levels (11). The combination of the *ACR3* disruption with subsequent disruption of *FPS1*, the gene for the yeast aquaglyceroporin, was shown previously to result in the loss of almost all $As(OH)_3$ uptake (4). However, those transport measurements were performed in the presence of glucose. In the absence of glucose, both MG102 (*acr3*Δ) and HD9 (*acr3*Δ *fps1*Δ) accumulated $^{73}As(OH)_3$ to high levels (Fig. 1). Disruption of *FPS1* reduced uptake by only about 25%, but the addition of glucose inhibited MG102 by ~70–80%, and addition of glucose to HD9 resulted in the loss of nearly all uptake. Thus, in the absence of glucose, Fps1p is responsible for, at most, 20–25% of the uptake of arsenic trioxide. The inhibition by glucose suggested that the majority of $^{73}As(OH)_3$ uptake might be catalyzed by glucose permeases. To examine this possibility, *FPS1* and *ACR3* were disrupted in a strain lacking all known hexose permeases (13). The resulting strain, HD300, exhibited only residual arsenic trioxide uptake that was insensitive to the presence of glucose (Fig. 1). Thus, inhibition must be the result of competitive uptake of $As(OH)_3$ and glucose by one or more hexose permeases.

The effect of glucose concentration on $^{73}As(OH)_3$ was examined in strain HD100 (*acr3*Δ *fps1*Δ), which is isogenic with HD300 but has all of the hexose permeases (Fig. 2*A*). Half-maximal inhibition was observed with ~5 mM glucose. Inhibition was greatest with hexoses, including glucose, galactose, fructose, and mannose (Fig. 2*B*). Neither the disaccharide sucrose nor the triose glycerol inhibited $As(OH)_3$ uptake, al-

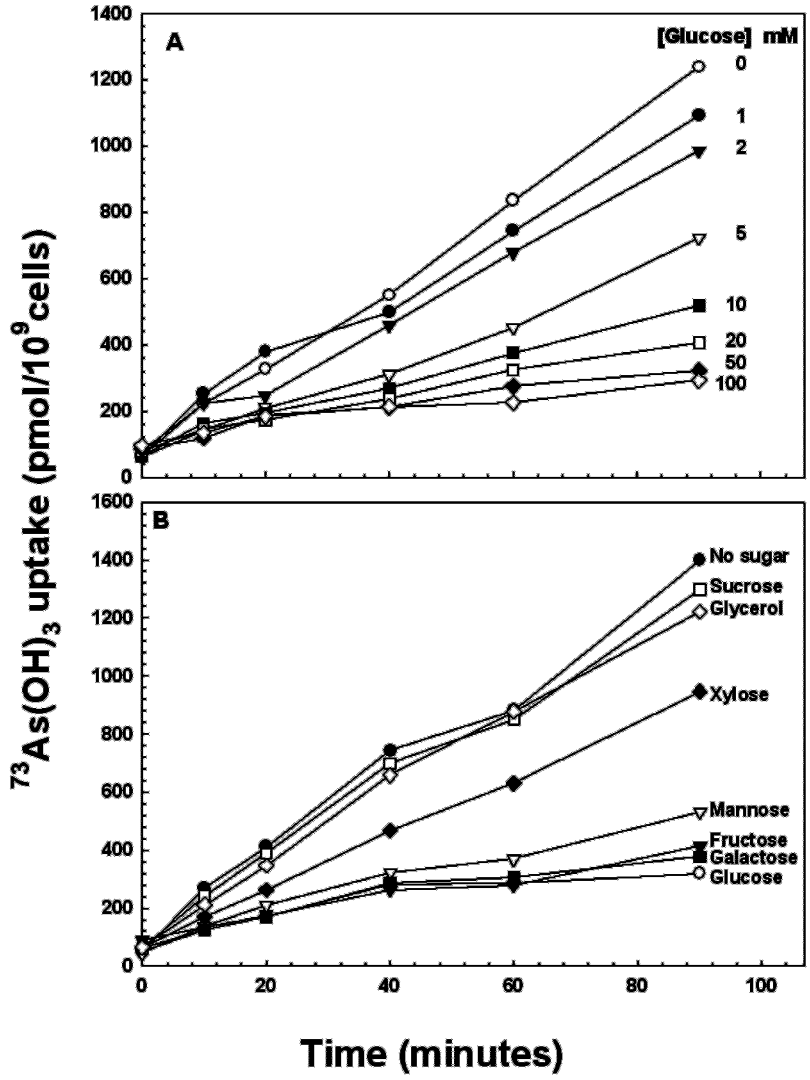

FIG. 2. **Inhibition of $^{73}As(OH)_3$ uptake by glucose and other sugars in HD100 (*acr3*Δ *fps1*Δ).** *A*, concentration dependence of glucose inhibition. Glucose concentrations were: none (○); 1 mM (●); 2 mM (▼); 5 mM (▽); 10 mM (■); 20 mM (□); 50 mM (♦); and 100 mM (◇). *B*, inhibition of $^{73}As(OH)_3$ uptake by various sugars. Each sugar was added at 0.1 M. Symbols used represent: none (●), sucrose (□), glycerol (◇), xylose (♦), mannose (▽), fructose (▼), galactose (■), and glucose (○).

though glycerol was shown to inhibit arsenic trioxide movement through Fps1p (4). A small amount of inhibition was observed with the pentose xylose, but xylose can also be transported by some hexose permeases (8).

Hxt1p, Hxt3p, Hxt4p, Hxt5p, Hxt7p, and Hxt9p Are Arsenic Trioxide Transporters—To deduce which hexose permeases are responsible for glucose-inhibitable arsenite trioxide accumulation, strain HD200 was constructed. HD200 is isogenic with HD100 but has only four hexose permeases: *HXT3, HXT6, HXT7*, and *GAL2*. When grown on galactose as a carbon source, only *HXT6, HXT7*, and *GAL2* are expressed (14). The arsenic trioxide transport properties of HD200 were similar to those of HD100 (data not shown). Half-maximal inhibition by glucose was observed at ~5 mM glucose. The hexoses (glucose, galactose, fructose, and mannose) inhibited $As(OH)_3$ uptake but glycerol did not. Under these growth conditions, *HXT3* is not expressed. Hxt6p and Hxt7p differ in only one residue (14); therefore, it is likely that they have similar transport properties. These results suggest that Hxt6p, Hxt7p, and/or Gal2p transport arsenic trioxide.

The ability of specific hexose transporters to catalyze the uptake of $^{73}As(OH)_3$ was determined by the expression of *HXT1, HXT3, HXT4, HXT5, HXT7, HXT9*, and *HXT10* genes under the control of constitutive promoters in strain HD300, which lacks all hexose transporters. Because HD300 is unable to grow on glucose as a carbon source, the ability of cells bearing plasmids with *HXT* genes to grow on glucose shows that the transporters were all functionally expressed. Expression of *HXT10* in HD300 had no effect on $^{73}As(OH)_3$ transport, suggesting it does not catalyze arsenic trioxide transport (data not shown). In contrast, when *HXT1, HXT3, HXT4, HXT5, HXT7*, or *HXT9* was individually expressed in HD300, $^{73}As(OH)_3$ transport was restored (data not shown).

The arsenic trioxide transport properties of two of these, Hxt7p and Hxt9p, were examined in more detail. When grown in media containing 2% maltose, cultures of HD300 were able to grow at substantially higher concentrations of sodium arsenite than either HD300 YEpHXT7 or HD300 pTHHXT9, indicating that the uptake of arsenic trioxide by those two permeases confers arsenic sensitivity. Transport of $^{73}As(OH)_3$ by Hxt7p was inhibited by glucose (Fig. 3*A*). Of the hexoses, glucose was the most effective inhibitor, suggesting that Hxt7p is more specific as a glucose permease (Fig. 3*B*). Because Hxt7p catalyzes arsenic trioxide transport, most likely the highly related Hxt6p does as well. Hxt9p and Hxt11p are closely related membrane proteins in which sequences differ at only two positions and are about 70% identical to Hxt7p (15). These two proteins may be multidrug uptake systems, because disruption of either *HXT9* or *HXT11* confers resistance to cycloheximide and other drugs, and expression of either from a plasmid increased sensitivity. Although they transport glucose with low affinity, they are not induced by glucose but rather by their drug substrates through the Pdr1p and Pdr3p pleiotropic drug resistance

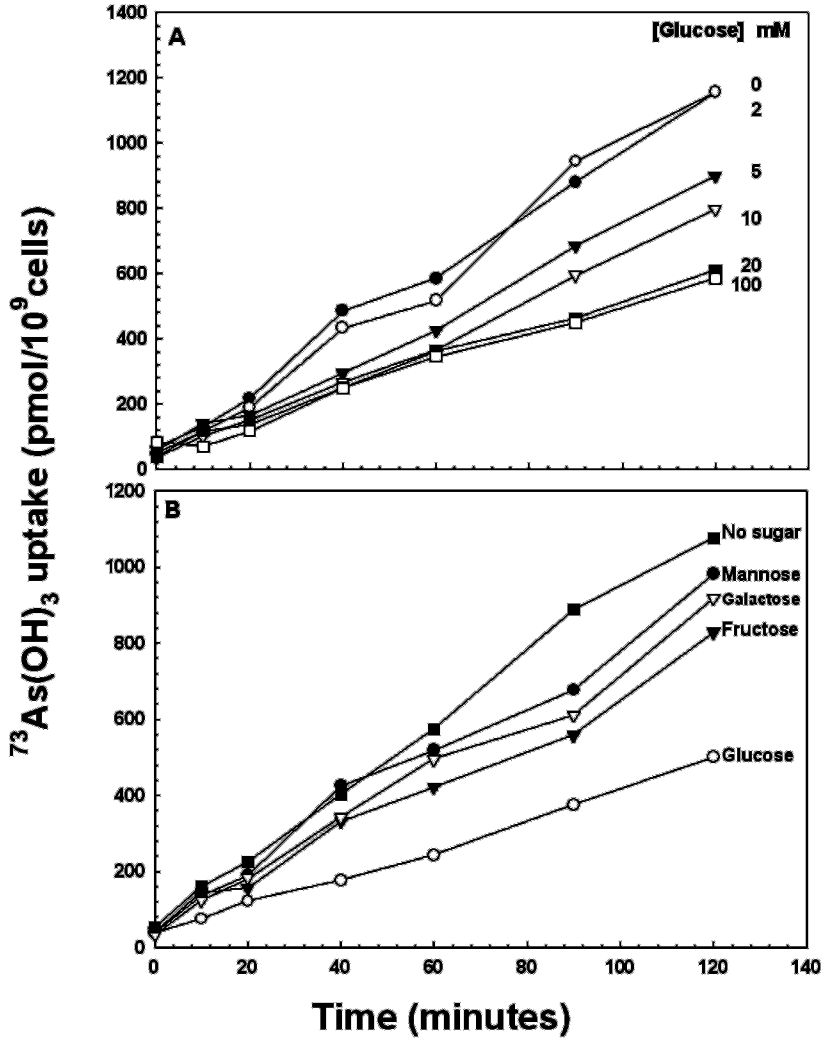

FIG. 3. **Hxt7p catalyzes $^{73}As(OH)_3$ transport.** Transport was assayed in strain HD300 YEpHXT7. *A*, concentration dependence of glucose inhibition. Glucose concentrations were: none (○); 2 mM (●); 5 mM (▼); 10 mM (▽); 20 mM (■); 100 mM (□). *B*, inhibition of $^{73}As(OH)_3$ uptake by various sugars. Each sugar was added at 0.1 M. Symbols used represent: none (■), mannose (●), galactose (▽), fructose (▼), and glucose (○).

transcription factors. To test whether these drug transporters could also transport the anticancer drug arsenic trioxide, *HXT9* was expressed in HD300. Whereas the cells exhibited glucose-sensitive uptake, inhibition required considerably more glucose than either wild type yeast or HD300 YEpHXT7 (compare Fig. 4*A* with Figs. 2*A* and 3*A*). Glucose was a more effective inhibitor than galactose, mannose, or fructose (Fig. 4*B*). Thus, Hxt9p and (by extension) Hxt11p are arsenic trioxide transporters.

Hxt7p is a high affinity glucose transporter with a K_m of 1–2 mM. Hxt9p is a multidrug transporter with only a low affinity for glucose and, physiologically, is probably not involved in glucose utilization (15, 16). Their relative affinities for glucose are reflected in their affinity for arsenic trioxide. Hxt7p is a high affinity, low capacity $As(OH)_3$ uptake system with a K_m of ~2 mM and V_{max} of ~20 nmol/min/10^9 cells (Fig. 5*A*). Hxt9p has a lower affinity but higher capacity, with a K_m of ~10 mM and V_{max} of ~40 nmol/min/10^9 cells (Fig. 5*B*). The differences in relative affinities for glucose and arsenic trioxide explain why glucose appears to be a more effective inhibitor of arsenic trioxide uptake in HD300 YEpHXT7 than in HD300 pTHHXT9. Consistent with these transport properties, cells expressing *HXT7* were more sensitive to growth in the presence of sodium arsenite than those expressing *HXT9* (data not shown).

Glucose Uptake Is Inhibited by $As(OH)_3$—The results above demonstrate that arsenic is transported by hexose permeases. If arsenic trioxide and glucose share a common translocation pathway, glucose uptake should also be inhibited by arsenic trioxide. When uptake of [^{14}C]glucose was assayed in strain HD100, inhibition by arsenic trioxide was observed (Fig. 6). Inhibition was even more pronounced in the cells of HD300 YEpHXT7 (Fig. 6) or HD300 pTHHXT9 (data not shown).

DISCUSSION

Because arsenic trioxide is paradoxically both an environmental toxin and a chemotherapeutic drug, knowledge of the pathways by which the metalloid is brought into cells could be useful both in preventing toxicity and in enhancing drug action. In both prokaryotes and eukaryotes, aquaglyceroporin channels facilitate downhill arsenite trioxide movement into cells (4–6). Here we report that glucose permeases represent the major pathways for arsenic trioxide uptake in *S. cerevisiae*. Under the growth conditions used for this study (basal salts medium with either galactose or maltose as a carbon source), wild type yeast cells use hexose permeases for ~70% of the arsenic trioxide uptake and the aquaglyceroporin Fps1p for ~20%. What catalyzes the residual uptake is not known.

Which hexose permeases catalyze arsenic trioxide uptake? The family of hexose permeases in *S. cerevisiae* is surprisingly large, with 18 hexose transporters (Hxt1p to Hxt17p), Gal2p, as well as two glucose sensors, Snf3p and Rgt2p (14). These secondary carrier proteins, each of which has 12 putative transmembrane segments, belong to the major facilitator

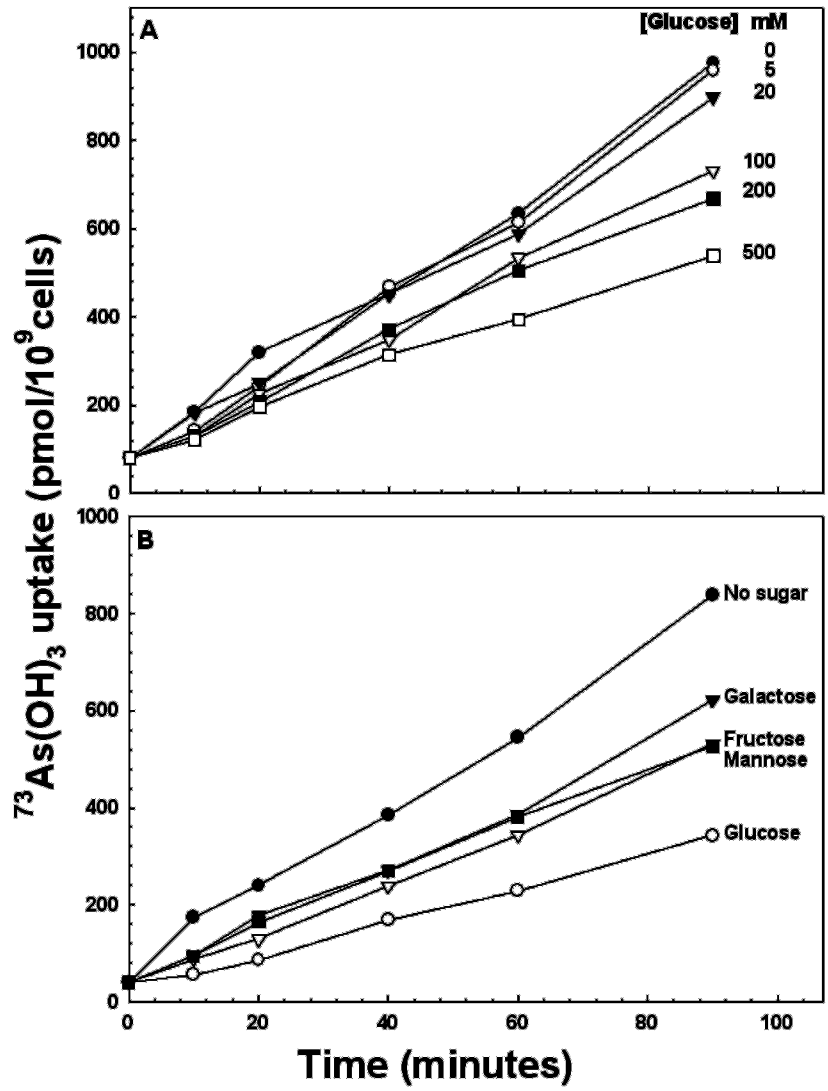

FIG. 4. **Hxt9p catalyzes $^{73}As(OH)_3$ transport.** Transport was assayed in HD300 pTHHXT9. *A*, concentration dependence of glucose inhibition. Glucose concentrations were: none (●); 5 mM (○); 20 mM (▼); 100 mM (▽); 200 mM (■); 500 mM (□). *B*, inhibition of $^{73}As(OH)_3$ uptake by various sugars. Each sugar was added at 0.2 M. Symbols used represent: none (●), galactose (▼), fructose (■), mannose (▽), and glucose (○).

superfamily (17). Hexose transporters can be divided into two categories: low affinity transporters such as Hxt1p, Hxt3p, Hxt9p, and Hxt11p and high affinity transporters, such as Hxt6p, Hxt7p, and Gal2p, where the K_m for glucose is about 1 mM (14). In a strain lacking *FPS1* and the genes for all 18 hexose permeases, there was only residual uptake of arsenic trioxide. The expression of *HXT1, HXT3, HXT4, HXT5, HXT7*, and *HXT9* restored transport. Hxt6p is nearly identical to Hxt7p, and Hxt9p is nearly identical to Hxt11p; therefore, these are likely to catalyze arsenic trioxide uptake as well. Only *HXT10* did not restore transport. Thus, it is possible that most if not all of the hexose permeases are also arsenic trioxide transporters. Moreover, the affinity of Hxt7p and Hxt9p for glucose was reflected in their affinity for arsenic trioxide; Hxt7p has a higher affinity for both glucose and $As(OH)_3$ than does Hxt9p. This indicates that these proteins use the same catalytic mechanism for $As(OH)_3$ as for sugars.

That hexose permeases are capable of transporting arsenic trioxide is surprising. The fact that pentoses and disaccharides do not inhibit uptake indicates specificity. The neutral polyhydroxylated metalloid must have sufficient molecular similarity to substrates of aquaglyceroporins to go through the Fps1p channel, but in some way, $As(OH)_3$ must also mimic a hexose but not other sugars. A search of the Cambridge Structural Data Base identified 109 oxo-bridged As–O–As compounds, including 10 with six-membered $(As–O)_3$ rings. The crystal structure of arsenious oxide, As_4O_6, is also a six-membered $(As–O)_3$ ring with the fourth As(III) coordinated to the three axial oxygens (18). By extrapolation, we predict that arsenic trioxide can form a similar six-membered ring that would have molecular similarity to hexoses but not to pentoses or disaccharides (Fig. 7).

What is the relevance of these findings to human health and disease? Arsenic exposure is associated with increased incidence of diabetes in many countries (19). The mechanism of this diabetogenic effect of arsenic is unknown, but it is conceivable that it is related to inhibition of glucose uptake by arsenic in drinking water. The related organic trivalent arsenical phenylarsine oxide has been shown to bind to and inhibit glucose transport in canine kidney cell lines (20, 21). Phenylarsine oxide has a more cytotoxic effect at low glucose concentrations, which has led to the suggestion that glucose can modulate the effects of arsenic (21). Phenylarsine oxide specifically binds to and inhibits GLUT4 (the major insulin-dependent human glucose permease) from murine 3T3-L1 adipocytes (22, 23). Mouse GLUT4 is 95% identical to human GLUT4, both of which have a conserved Cys-Gly-Cys sequence, and vicinal cysteine pairs are known to react nearly irreversibly with trivalent arsenicals (22). Although Hxt7p is a homologue of GLUT4, the yeast protein does not have the conserved cysteine motif and requires high concentrations of

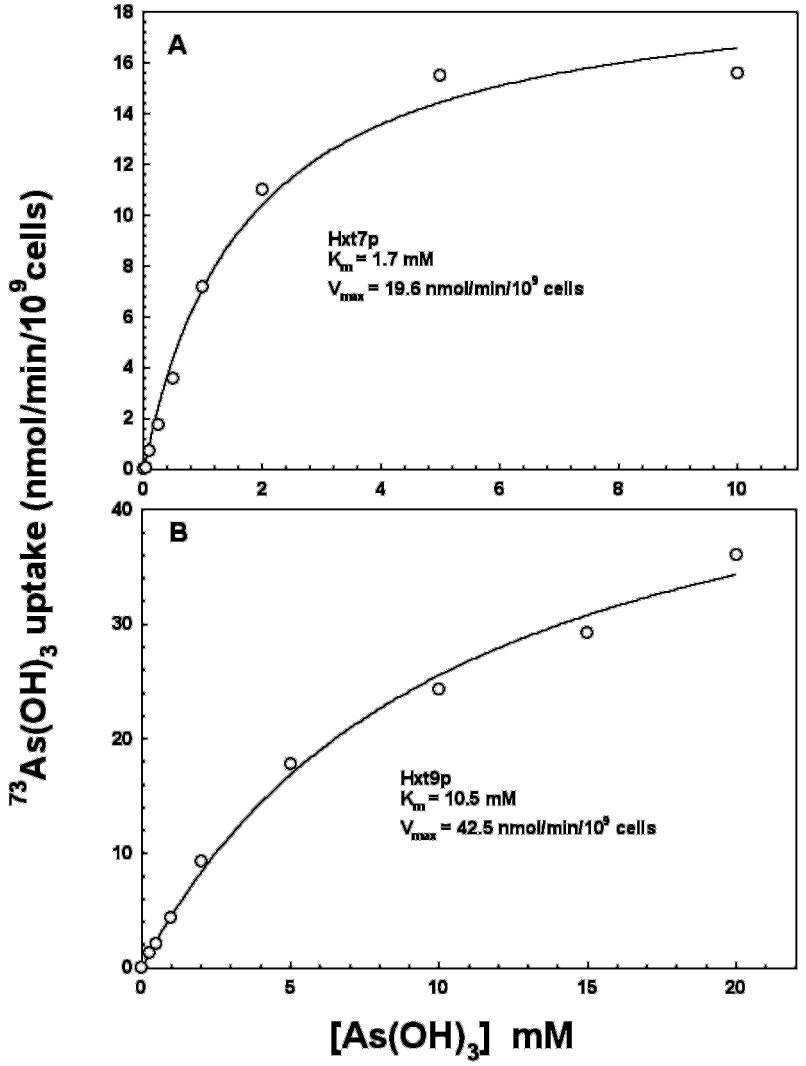

FIG. 5. **Kinetics of $^{73}As(OH)_3$ transport by Hxt7p and Hxt9p.** The rate of transport of $^{73}As(OH)_3$ was assayed in HD300 YEpHXT7 (*A*) or pTHHXT9 (*B*) as a function of the concentration of arsenic trioxide. The *lines* are the best fit to the Michaelis-Menten equation using SigmaPlot version 6.1.

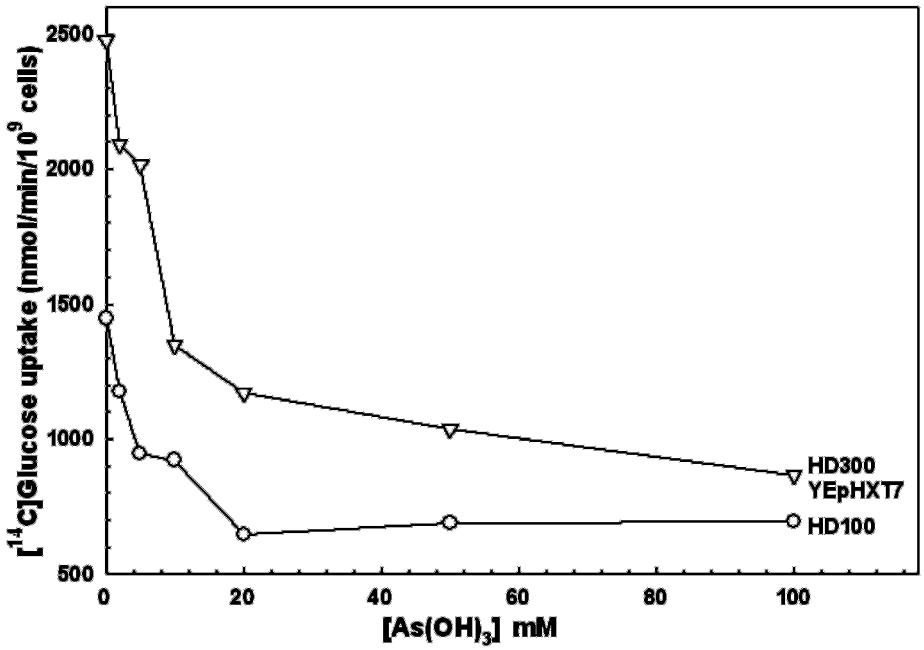

FIG. 6. **Inhibition of [^{14}C]glucose transport by $As(OH)_3$.** Uptake of [^{14}C]glucose was assayed in the presence of the indicated concentrations of $As(OH)_3$, as described under "Materials and Methods." Uptake was measured for 5 s with 5 mM glucose at room temperature. Yeast strains: HD100 (○); HD300 YEpHXT7 (▽).

arsenic trioxide to inhibit glucose uptake. Thus, arsenic trioxide is a competitive inhibitor of yeast glucose transport but an irreversible inhibitor of the mammalian permeases. The irreversible inactivation of glucose uptake could produce the diabetogenic effects of arsenic trioxide in countries such as Taiwan and Bangladesh (19). This suggests that the inci-

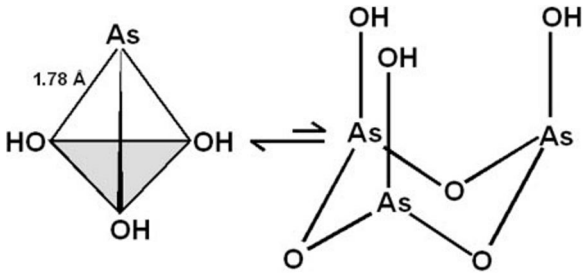

FIG. 7. **Proposed solution structures of arsenic trioxide.** *A*, in solution at neutral pH, the predominant species of arsenic trioxide is $As(OH)_3$ in a trigonal pyramidal structure with three As–O bonds of 1.78 Å. *B*, polymerization of three $As(OH)_3$ is predicted to form a six-membered ring with molecular similarity to a hexose. This is similar to the six-membered ring structure in As_4O_6 (18).

dence of diabetes in those countries could be related to poor nutritional status and might be ameliorated by higher amounts of dietary hexoses.

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