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# REGULATION OF *N*-ACETYLGLUCOSAMINE UPTAKE IN YEAST

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## Summary

Various yeasts have been investigated for their ability to grow on *N*-acetylglucosamine as the sole carbon source and only those which are associated with the disease, candidiasis, gave positive results. The yeasts unable to grow on *N*-acetylglucosamine lacked the capacity to transport the aminosugar across the cell membrane. In pathogenic yeasts, two systems of different affinity for substrate were found to operate in the uptake of *N*-acetylglucosamine. In glucose-grown cells a constitutive, low affinity uptake system was present, but upon addition of inducer, a specific high affinity uptake system was synthesized. Experiments with the inhibitors of macromolecule synthesis suggested that the synthesis of RNA and protein is necessary for induction whereas the synthesis of DNA is not.

In glucose-grown *Candida albicans* cells which are devoid of *N*-acetylglucosamine enters into the cells as phosphorylated form using a constitutive uptake system. Uranyl acetate (0.01 mM) which binds to cell membrane-associated polyphosphates, inhibited completely the inducible uptake of *N*-acetylglucosamine. Labelling experiments, designed to determine the temporal sequence of appearance of *N*-acetylglucosamine in intracellular free sugar and sugar-phosphate pools, indicated that *N*-acetylglucosamine first appeared in the cells as phosphorylated form. Similar results were obtained with *Saccharomyces cerevisiae* 3059 and some other yeasts which are devoid of *N*-acetylglucosamine kinase in both uninduced and induced conditions. These results are consistent with the model of van Steveninck that involves phosphorylation during transport. Furthermore, inhibitors of energy metabolism (arsenate, azide and cyanide), proton conductor (*m*-chlorocarbonylcyanide phenylhydrazine) and

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Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; *m*-CCCP, *m*-chlorocarbonylcyanide phenylhydrazine; $DDA^+$, dibenzyl diammonium ion; $TPB^-$, tetraphenylboron; ECT, epichlorohydrin triethanolamine.

dibenzyl diammonium ion (membrane permeable cation) inhibited the inducible *N*-acetylglucosamine uptake in *C. albicans*.

## Introduction

Candidiasis is a disease caused by *Candida albicans* and some other yeasts [1,2]. Infection occurs mainly in mucous membranes which are particularly rich in aminosugars. Since the first and foremost requirement for any pathogenic microorganism is its ability to grow on the site of infection, it is tempting to test whether the pathogenic yeasts possess some mechanism(s) to control aminosugar metabolism that other non-pathogenic yeasts do not. Our results indicate that only pathogenic yeasts are able to grow on GlcNAc medium. Furthermore, it was found that in addition to a constitutive uptake system already present, a high affinity uptake system for GlcNAc was induced by GlcNAc.

In the yeast *Saccharomyces cerevisiae* several transport systems for sugars have been described: a constitutive system common for glucose, fructose and mannose [3] and three inducible systems for galactose [4], α-methylglucoside [5] and maltose [6], respectively. But there are conflicting reports on the mechanism of sugar transport. The results from van Steveninck's laboratory on transport of glucose [7], 2-deoxyglucose [8,9], α-methylglucoside [10] and galactose [11] indicated transport-associated phosphorylation of actively transported sugars in yeast. However, the interpretation of these experiments was questioned by Kotyk and Michaljanikova [12] and Brocklehurst et al. [13]. Under similar experimental conditions these authors reached different results. The results were interpreted as indicating facilitated diffusion of the free sugar, with subsequent intracellular phosphorylation by cytoplasmic sugar kinases. In the present studies these contradictions were resolved by using *C. albicans* (GlcNAc kinase plus) and *S. cerevisiae* (GlcNAc kinase minus) to study the mechanism of aminosugar transport. All the pathogenic yeasts tested so far (except for *C. albicans*) lack the capacity to synthesize GlcNAc kinase even when they can use GlcNAc as sole carbon source and induce high affinity uptake system for its transport. An inducible kinase for GlcNAc in *C. albicans* might constitute an important regulatory difference between these yeasts [14–16]. Other enzymes involved in *N*-acetylglucosamine metabolism namely *N*-acetylglucosamine 2-epimerase (EC 5.1.3.-) [17], *N*-acetylglucosamine kinase (EC 2.7.1.59) [14] and glucosamine-6-phosphate deaminase (EC 5.3.1.10) [18, 19] are also induced in presence of GlcNAc. Moreover, a specific GlcNAc binding protein which appears to be involved in GlcNAc uptake is also inducible (Singh, B., Biswas, M. and Datta, A., unpublished data). This inducible pathway provides an ideal eukaryotic system to study the various events involved in gene expression.

In the present paper, we report the results of some experiments on the uptake and phosphorylation of GlcNAc in *C. albicans* and *S. cerevisiae*, in which the ambiguity introduced by kinase is removed. Our results indicate that there is no intracellular concentration of the free sugar, and the sugar phosphate is the first species to be detected in labelling experiments.

## Materials and Methods

*Materials.* Agar, peptone and yeast extract were from Difco. The following chemicals were obtained from Sigma Chemical Company, St. Louis, U.S.A.: *N*-acetyl-D-glucosamine, bovine serum albumin, ECT-cellulose, ammonium biborate, *p*-dimethylaminobenzaldehyde, sodium azide, sodium arsenate, cycloheximide, ethidium bromide, cordycepin, mitomycin C, *m*-chlorocarbonylcyanide phenylhydrazine (*m*-CCCP), dibenzyl diammonium ion ($DDA^+$), tetraphenylboron ($TPB^-$), PPO and POPOP; hydroxyurea was purchased from Calbiochem, Los Angeles, U.S.A. *N*-[$^3$H]Acetyl-D-glucosamine (686 Ci/mol) was purchased from Radiochemical Centre, Amersham; [$^3$H]uridine, [$^3$H]-thymidine and [$^3$H]lysine were obtained from Bhabha Atomic Research Centre, Bombay, India. All other reagents were of analytical grade.

*Organisms and culture conditions. S. cerevisiae* 211 (*Val*$^-$, *Ile*$^-$) was a gift from Prof. Ilse Muller, Institute für Entwicklungsphysiologie, Köln, F.R.G. All other strains used in this study were obtained from National Chemical Laboratory, Pune, India. Composition of growth medium and conditions for growth and induction were same as described previously for *C. albicans* [14].

*Measurement of N-acetylglucosamine uptake.* For uptake studies cell suspensions in a final volume of 5 ml (0.24 mg cell protein) were incubated in a water-bath shaker at 30°C. After incubation for 5 min, *N*-[$^3$H]acetyl-D-glucosamine was added at a final concentration of either 0.05 mM (1 $\mu$Ci/ml) to study high affinity uptake, System I (inducible), or 5 mM (5 $\mu$Ci/ml) to study low affinity uptake, System II (constitutive). At various times, 0.5 ml aliquot was taken out, diluted with 5 ml water and immediately filtered on discs of glassfiber filter paper (GF/C, Whatman). The cells retained on filter paper were immediately washed with 10—15 ml water, dried and placed in 10 ml toluene-based liquid scintillation cocktail. Radioactivity was measured in a Packard Tricarb liquid scintillation spectrophotometer model 3380. The controls were made either by omission of the yeast cells or by previously boiling the cells or by incubating at 0°C instead of 30°C. All methods gave identical results. The control values varied from 1 to 2% of the actual uptake and have been substrated to all the presented data. Total protein in the cells was determined in the trichloroacetic acid precipitate of cells by the method of Lowry et al. [20] using bovine serum albumin as standard.

*Separation of intracellular GlcNAc and GlcNAc-6-P.* The radioactivity present in GlcNAc and its phosphorylated derivative was extracted from the filters by boiling in water for 30 min. Analysis was done by thin-layer chromatography (TLC) on silica gel plate and ECT-cellulose plate according to the method of Lewis and Smith [21]. Solvent systems used were acetone/water (90 : 10) for TLC on silica gel plate and ethanol/0.1 M ammonium biborate (60 : 40) for TLC on ECT-cellulose plate. GlcNAc and GlcNAc-6-*P* spots containing radioactivity were scratched, contents transferred to vials and radioactivity present was measured in presence of toluene-based scintillation fluid. About 95—97% of the total intracellular radioactivity was always found in GlcNAc and GlcNAc-6-*P*; we could not recover the other 3—5% from the plate. Furthermore, by using the method of $Ba(OH)_2$-$ZnSO_4$ precipitation to precipitate specifically the phosphorylated derivative of *N*-acetylglucosamine [22],

only less than 5% of the total radioactivity was recovered in the supernatant, suggesting the remainder to be in phosphorylated form. For calculating the label in GlcNAc and GlcNAc-6-*P* necessary corrections for quenching and background counts were made.

## Results and Discussion

*Growth and uptake of N-acetyl-D-glucosamine in various yeasts*

To answer the question whether there is any correlation between the disease-causing ability of yeast and its aminosugar metabolism, we have surveyed various pathogenic and non-pathogenic yeasts for their ability to grow on GlcNAc medium. As shown in Fig. 1, all the strains of yeast could utilize glucose for their growth, however, only pathogenic ones could utilize GlcNAc. *S. cerevisiae* 3059 is the only exception to this and grew well on both the glucose and the GlcNAc media. However, though normally it is non-pathogenic, sometimes becomes pathogenic in favourable conditions and has occasionally been isolated as an etiological agent of pulmonary disease of brewers [23]. Other species of Saccharomyces genera, e.g. *S. cerevisiae* 211, *S. cerevisiae ellipsoideus* and *S. fragilis* failed to grow on GlcNAc.

Inability of non-pathogenic yeasts to grow on GlcNAc could be due to the defect either in uptake system or in any subsequent step in the metabolism of GlcNAc. Since transport is the first step at which control of metabolism could operate, we have surveyed all the yeasts for their ability to transport GlcNAc in glucose-grown cells and in cells exposed to GlcNAc either in growing or non-growing conditions. The results are presented in the insets of Fig. 1. It is interesting to note that all those yeasts which have capacity to grow on GlcNAc can take up GlcNAc (0.05 mM) only after the cells are exposed to GlcNAc (induced cells). All the yeasts grown on glucose and non-pathogenic yeasts even after exposing to GlcNAc medium failed to accumulate GlcNAc (0.05 mM) inside the cells (Fig. 1).

*Induction of uptake system*

In case of pathogenic yeasts, GlcNAc uptake appears to be inducible. As shown in Fig. 2, there is a gradual increase in the uptake capacity of *C. albicans* cells after incubating the washed cells in induction medium (12.5 mM GlcNAc and 22 mM $KH_2PO_4$). After 3 h of induction when GlcNAc is metabolically exhausted from the medium, a gradual decrease in GlcNAc uptake capacity starts. Moreover, the decay rate was comparable to that of other enzymes of the inducible GlcNAc pathway as reported earlier [14,17,19]. This result suggests that the presence of *N*-acetylglucosamine is essential for induced synthesis of the uptake system.

To study the specificity of the inducer for the induction of GlcNAc uptake, cells of $KH_2PO_4$ solution were incubated for 3 h with the following aminosugars: *N*-acetylmannosamine, *N*-acetylgalactosamine and glucosamine. The capacity of *N*-acetylmannosamine as an inducer for GlcNAc uptake was same as that of GlcNAc, however, both *N*-acetylgalactosamine and glucosamine failed to induce the uptake system (data not shown).

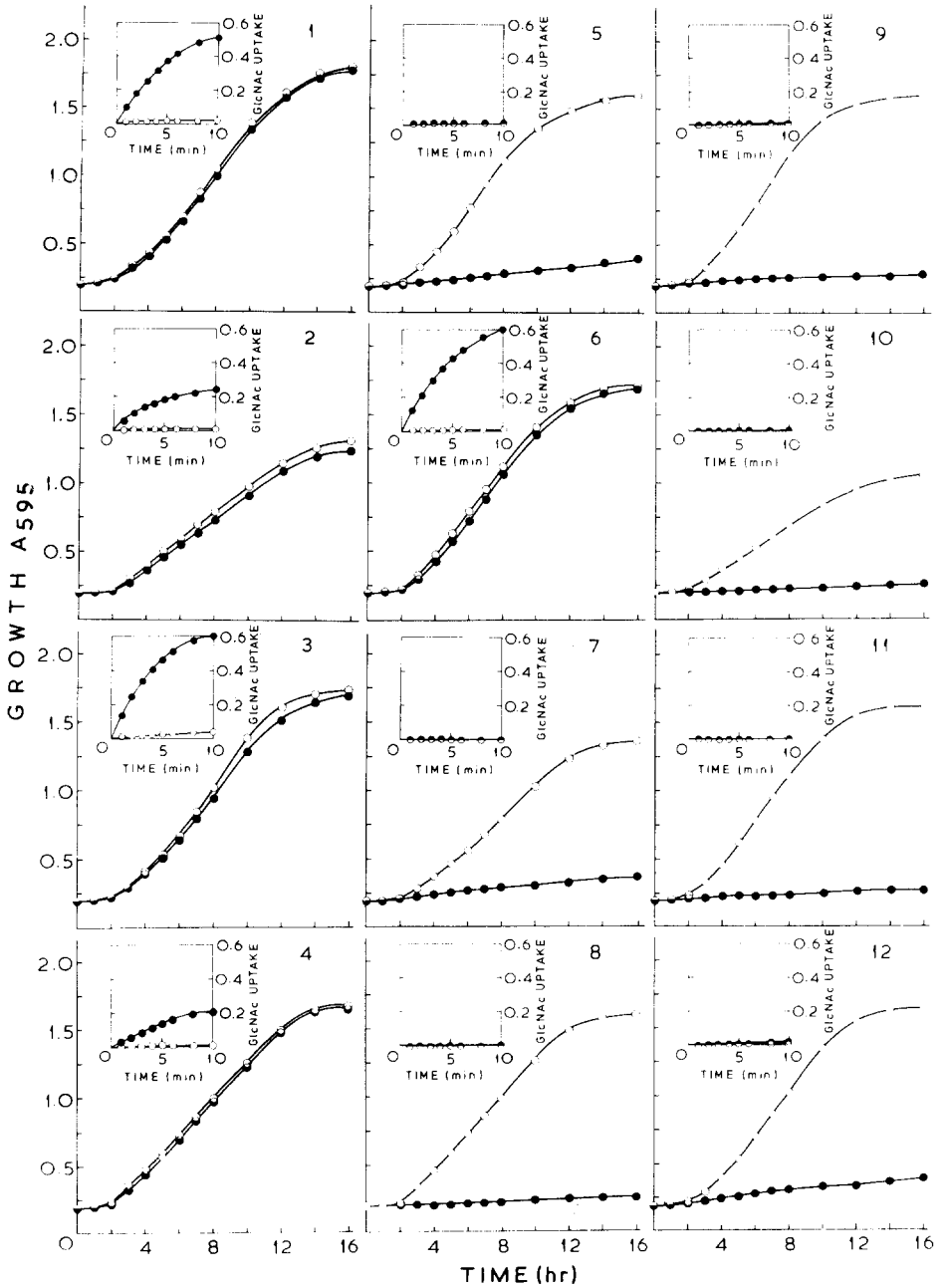


Fig. 1. Growth and uptake characteristics on glucose and *N*-acetylglucosamine media. Growth medium contained 0.5% peptone, 22 mM $KH_2PO_4$ and 28 mM glucose (○) or 22.5 mM *N*-acetylglucosamine (●). 1, *C. albicans*; 2, *C. krusie*; 3, *C. lipolytica*; 4, *T. candida*; 5, *C. utilis*; 6, *S. cerevisiae* 3059; 7, *S. cerevisiae* 211; 8, *S. cerevisiae ellipsoideus*; 9, *S. fragilis*; 10, *Schizosaccharomyces pombe*; 11, *H. angusta*; 12, *Rh. glutinis*. Uptake of *N*-[$^3$H]acetyl-D-glucosamine (0.05 mM) was studied in cells collected from mid-log phase (inset: ○, glucose-grown cells; ●, GlcNAc-grown cells). Uptake, μmol/mg of protein.

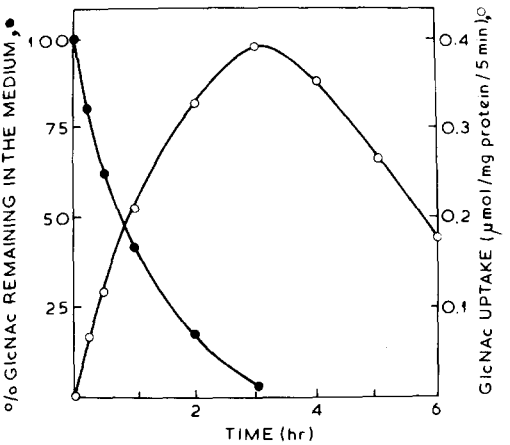


Fig. 2. Appearance of *N*-acetylglucosamine uptake system (System I) in washed cell suspension of *C. albicans* in the presence of 12.5 mM *N*-acetylglucosamine. *N*-Acetylglucosamine concentration of the induction medium during incubation was determined in the supernatant after centrifugation at 0°C to remove the cells. Uptake of *N*-acetylglucosamine (0.05 mM) in washed cells suspension at various times during induction was studied as described in Materials and Methods.

*Effect of inhibitors of macromolecule synthesis on induction*

As a first step towards the mechanism of induction, we have studied the effect of various inhibitors of macromolecule synthesis. Inhibitors of protein synthesis (cycloheximide), RNA synthesis (ethidium bromide) and poly(A) synthesis (cordycepin) had pronounced effect on the process whereas DNA synthesis inhibitors (mitomycin C and hydroxyurea) were without any perceptible effect (Table I). This result suggests that the induction involves new protein synthesis and that the inducer does not function by simply converting a precursor of the protein into an active form. Furthermore, continuous DNA synthesis is not necessary for enzyme induction and DNA synthesis before induction is not a necessary prerequisite for the induction.

TABLE I

EFFECT OF CYCLOHEXIMIDE, ETHIDIUM BROMIDE, CORDYCEPIN, MITOMYCIN C AND HYDROXYUREA ON THE INDUCIBILITY OF HIGH AFFINITY UPTAKE SYSTEM FOR *N*-ACETYLGLUCOSAMINE

*C. albicans* cells collected in their exponential phase of growth, were resuspended in 22 mM $KH_2PO_4$ containing 22.5 mM *N*-acetylglucosamine. At zero time, each inhibitor was added to one group of cultures and another group of cultures received no inhibitor. At 3 h after the addition of GlcNAc, cells were harvested by centrifugation and uptake of *N*-acetylglucosamine (0.05 mM) was studied in the washed cell suspensions as described in Materials and Methods.

| Treatment | *N*-Acetylglucosamine uptake (μmol/5 min per mg of protein) | % inhibition |
|---|---|---|
| Non-induced cells | 0.004 | — |
| Induced cells | 0.380 | — |
| + Cycloheximide (50 μg/ml) | 0.016 | 95 |
| + Ethidium bromide (50 μg/ml) | 0.007 | 98 |
| + Cordycepin (25 μg/ml) | 0.060 | 84 |
| + Mitomycin C (100 μg/ml) | 0.376 | 1 |
| + Hydroxyurea (200 μg/ml) | 0.360 | 5 |

*Existence of two uptake systems for N-acetylglucosamine*

While working on the mechanism of GlcNAc uptake (see below), we found that uranyl acetate (0.01 mM) completely inhibited the uptake of GlcNAc (0.05 mM) in induced cells. But when uranyl acetate at the same concentration was added to the cells of *C. albicans* or *S. cerevisiae* 3059 in presence of high concentration of GlcNAc (12.5 mM), the uptake of GlcNAc occurred normally (data not shown). This was an indication of the fact that in addition to inducible GlcNAc uptake system, there might exist a constitutive, low affinity uptake system for the aminosugar which is insensitive to uranyl acetate. This conclusion was confirmed by plotting external GlcNAc concentration ($S$) versus rate of uptake of GlcNAc inside the cells/30 s in glucose-grown and GlcNAc-induced cells of *C. albicans* (Fig. 3). At higher concentration of $S$ (GlcNAc) the uptake of GlcNAc occurs in glucose-grown cells, suggesting the presence of a low affinity uptake system. However, both constitutive (low affinity) and inducible (high affinity) systems were observed in induced cells. $K_m$ and $V$ for these two systems calculated from the Lineweaver-Burk plots (insets A and B of Fig. 3) are 0.01 and 1.0 mM for $K_m$ and 0.086 and 0.40 $\mu$mol · min$^{-1}$ · mg$^{-1}$ protein for $V$, for the high and low affinity uptake systems, respectively.

Fig. 4 shows the time course of total uptake of GlcNAc by both inducible and constitutive systems. Uranyl acetate which binds to polyphosphate present in the membrane [24,25], inhibited the inducible system by almost 100%, however, on the contrary, the constitutive uptake system was insensitive to uranyl acetate. In case of D-galactose uptake in *S. cerevisiae*, it was reported that inducible uptake was inhibited by low concentration of uranyl ions, however, a relatively higher concentration was required for the inhibition of

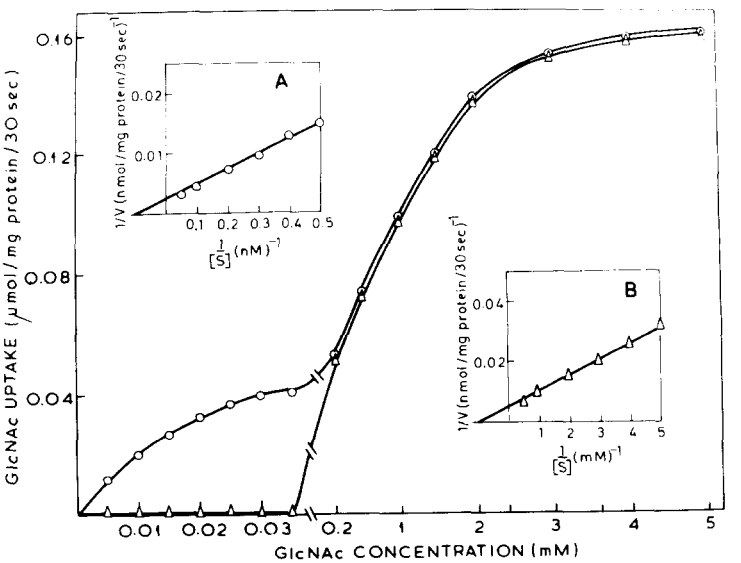


Fig. 3. Effect of *N*-acetylglucosamine concentration on the uptake velocity in *C. albicans*. *N*-Acetylglucosamine uptake was determined in glucose-grown cells (uninduced, △) and in the cells induced for 3 h in presence of 22.5 mM GlcNAc (○). Insets: double-reciprocal plots of high affinity uptake system (System I) and low affinity uptake system (System II) in A and B, respectively.

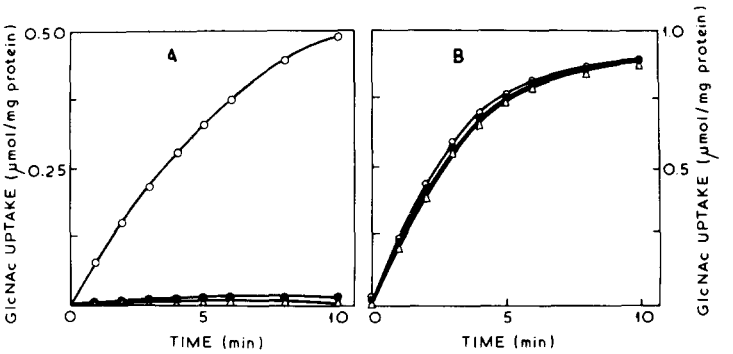


Fig. 4. Effect of uranyl acetate and cycloheximide on the inducible (System I) and constitutive (System II) uptake in *C. albicans*. Uptake of *N*-acetylglucosamine was studied in 3 h-induced cells (A) in glucose-grown cells (B) of *C. albicans*. Uranyl acetate (0.01 mM) was added 10 min before adding [$^3$H]GlcNAc. ○, control; ●, with uranyl acetate. Cycloheximide (50 μg/ml) was added to washed cell suspension, and after 10 min cells were divided into two flasks. In one flask, GlcNAc was added at a concentration of 12.5 mM (△, A) whereas the other flask remained without inducer (△, B). At 3 h, the cells were harvested, washed, resuspended in water, and uptake of GlcNAc was determined.

galactose uptake in glucose-grown cells [26,27]. Furthermore, cycloheximide also inhibits the new synthesis of this inducible system, suggesting the involvement of new protein synthesis in the process of induction.

*Mechanism of N-acetylglucosamine transport*

The crucial question in this study is whether GlcNAc is phosphorylated during its traversal of the cytoplasmic membrane, or whether it is phosphorylated after the sugar has entered into the cells in the free form. Van Steveninck's group [8,9,28] approached this question by measuring the rates of increase in specific activity of the non-phosphorylated and phosphorylated 2-deoxyglucose pools when 2-deoxy-D-[$^{14}$C]glucose was added to cells that were preloaded with non-radioactive 2-deoxy-D-glucose. They found that the specific activity of the phosphorylated sugar pool increased more rapidly than the non-phosphorylated sugar pool and concluded that the phosphorylation of 2-deoxy-D-glucose accompanied transport. However, the interpretation of these experiments was questioned by Kotyk and Michaljanikova [12] and their results indicated the facilitated diffusion of the free sugars, with subsequent intracellular phosphorylation. We have investigated this question using both wild-type cells of *C. albicans* (GlcNAc kinase plus) and *S. cerevisiae* (GlcNAc kinase minus), the latter offering the advantage of eliminating the ambiguities introduced by the kinase. In *N*-acetylglucosamine-grown cells, *N*-acetylglucosamine kinase is present only in *C. albicans* but totally absent in *S. cerevisiae* and other yeasts [14,15]. Moreover, hexokinase or other kinase cannot phosphorylate *N*-acetylglucosamine. We measured the rates of appearance of GlcNAc and GlcNAc-6-*P* during GlcNAc transport. Fig. 5 shows that in both yeasts, most of the label accumulated as sugar phosphate form. In these experiments, about 95% of the total radioactivity appeared as phosphorylated form. Also in other yeasts, i.e. *Candida krusie*, *Candida lipolytica* and *T. candida*, we have got the same type of result. As a further support, we have also found that in glucose-grown *C. albicans* (where kinase is absent) the sugar also enters into

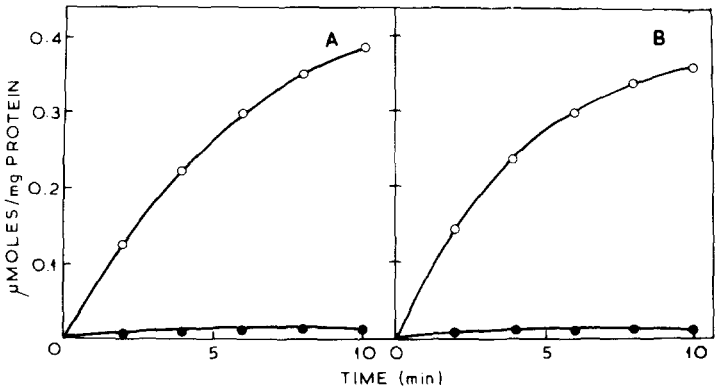


Fig. 5. Distribution of label in GlcNAc and GlcNAc-6-*P* during *N*-[$^3$H]acetyl-D-glucosamine uptake. Uptake of *N*-[$^3$H]acetyl-D-glucosamine (0.05 mM, 10 μCi/ml) was determined in 3 h-induced cells of *C. albicans* (A) and *S. cerevisiae* 3059 (B). At indicated times, 0.5 ml aliquots were taken out to determine free (●) and phosphorylated (○) derivative of GlcNAc according to the methods described in the text.

the cells as phosphorylated form (data not shown). Since GlcNAc kinase is absent in these yeasts, the plausible mechanism is the phosphorylation of aminosugar with membrane-associated polyphosphate as high energy phosphate donor as proposed by van Steveninck [10].

An investigation was carried out to detect the activities of *N*-acetylglucosamine deacetylase and oxidase. Since the radioactivity is present only in the acetyl group of GlcNAc, according to our present understanding of aminosugar metabolism radioactivity could not be lost before phosphorylation step without the participation of deacetylase. However, the failure to detect the activities of these enzymes also supports the view that GlcNAc is converted to its phosphorylated derivative during the uptake. Very little incorporation of GlcNAc into the cell wall or other glycoproteins is possible. This insignificant incorporation will not have any important bearing on our conclusions. Furthermore, the appearance of free GlcNAc (less than 2%) could be due to the action of endogenous phosphatase(s).

Uranyl acetate has been reported to bind to polyphosphate associated with the cell membrane [29] and does not inhibit cellular respiration [30]. As shown in Fig. 4, uranyl acetate strongly inhibited the inducible uptake of GlcNAc, supporting the involvement of membrane-associated polyphosphate as phosphate donor.

*Effect of inhibitors of energy metabolism on inducible uptake system*

In order to get further insight into the mechanism of GlcNAc uptake, effect of various inhibitors of energy metabolism (arsenate, azide and cyanide), proton conductor (*m*-CCCP), membrane permeable cations ($DDA^+$) and membrane permeable anion ($TPB^-$), on the inducible uptake of GlcNAc was studied. As shown in Table II, arsenate, azide and cyanide inhibited the uptake completely. Moreover, *m*-CCCP and $DDA^+$ also inhibited the uptake, emphasizing the importance of charge distribution across the cells membrane. However, as expected, $TPB^-$ which is negatively charged, was without any effect.

TABLE II

EFFECT OF VARIOUS INHIBITORS ON THE INDUCIBLE UPTAKE OF *N*-ACETYLGLUCOSAMINE IN *C. ALBICANS*

Uptake of *N*-[$^3$H]-acetylglucosamine (0.05 mM) was studied in 3 h-induced cells of *C. albicans*. Cells were preincubated for 10 min with various inhibitors and then uptake of *N*-[$^3$H]-acetylglucosamine was determined.

| Inhibitor | Uptake of GlcNAc (μmol/5 min per mg of protein) | % inhibition |
|---|---|---|
| Control | 0.380 | — |
| + Potassium cyanide ($1 \cdot 10^{-2}$ M) | 0.010 | 97 |
| + Sodium arsenate ($1 \cdot 10^{-2}$ M) | 0.005 | 99 |
| + Sodium azide ($4 \cdot 10^{-2}$ M) | 0.003 | 99 |
| + *m*-CCCP ($1.5 \cdot 10^{-3}$ M) | 0.040 | 89 |
| + $DDA^+$ ($1 \cdot 10^{-3}$ M) | 0.081 | 79 |
| + $TPB^-$ ($1 \cdot 10^{-4}$ M) | 0.380 | 0 |

Our results strongly support the transport-associated phosphorylation of *N*-acetylglucosamine in yeast. This conclusion is based on several observations. First, in labelling experiments GlcNAc was recovered mainly in phosphorylated form; second, GlcNAc uptake was inhibited by uranyl acetate (0.01 mM); third, yeasts which do not have *N*-acetylglucosamine kinase (kinase minus) also accumulated *N*-acetylglucosamine as phosphorylated form.

Supporting the view that increased uptake of GlcNAc in induced cells is because of induction of some component of uptake system we have isolated a specific GlcNAc binding protein. This binding protein is absent in glucose-grown cells, but its synthesis starts when inducer, i.e. GlcNAc is added to the medium (Singh, B., Biswas, M. and Datta, A., unpublished data). Further, the results suggest that the binding protein does not have any phosphorylating activity and shares many properties with the uptake system. Studies are in progress to isolate the kinase minus and other mutants to understand better the mechanism of GlcNAc uptake in particular and control of GlcNAc catabolic pathway in general.

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