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Induction of *N*-Acetylglucosamine-Catabolic Pathway in Spheroplasts of *Candida albicans*

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Synthesis of *N*-acetylglucosamine-catabolic enzymes, namely permease (high-affinity uptake system), kinase and deaminase was studied in the spheroplasts of the yeast *Candida albicans*. The presence of *N*-acetylglucosamine as inducer is essential for the induced synthesis of these enzymes in the spheroplasts, which were active for at least 8–9h. However, some of the newly synthesized kinase and deaminase leaked out from the spheroplasts into the medium during induction. Experiments with inhibitors of RNA and protein synthesis indicate that the appearance of new enzyme activities is dependent on concomitant new protein synthesis and the inducer operates at a transcriptional level. However, inhibitors of DNA synthesis, e.g. mitomycin-C and hydroxyurea, had no effect on the synthesis of these enzymes.

The metabolism of *N*-acetylglucosamine, which is shown here, has been established in animal tissues and bacteria, but has not been previously demonstrated in yeasts:

N-Acetylglucosamine $\xrightarrow{\text{Kinase}}$ *N*-acetylglucosamine 6-phosphate $\xrightarrow{\text{Deacetylase}}$ glucosamine 6-phosphate $\xrightarrow{\text{Deaminase}}$ fructose 6-phosphate

In *Escherichia coli*, it has been shown that *N*-acetylglucosamine kinase (ATP–2-acetamido-2-deoxy-D-glucose 6-phosphotransferase; EC 2.7.1.59) is synthesized constitutively (Asensio & Ruiz-Amil, 1966), but *N*-acetylglucosamine 6-phosphate deacetylase (2-acetamido-2-deoxy-D-glucose 6-phosphate aminohydrolase; EC 3.5.1.–) and glucosamine 6-phosphate deaminase [2-amino-2-deoxy-D-glucose 6-phosphate ketol-isomerase (deaminating); EC 5.3.1.10] are induced in the presence of *N*-acetylglucosamine (White & Pasternak, 1967; White, 1968). Furthermore, it has been established that deacetylase and deaminase loci are very close to each other on the genetic map of *E. coli* K12 (Holmes & Russell, 1972). In our laboratory, Bhattacharya *et al.* (1974) reported for the first time the induction of *N*-acetylglucosamine kinase, the first enzyme in the metabolism of *N*-acetylglucosamine, in the pathogenic yeast *Candida albicans*. Moreover, osmotically fragile spheroplasts, which are more or less synchronous and permeable to small molecules, offer a useful system to study RNA and protein synthesis. With this in view, we have characterized the induction of enzymes of *N*-acetylglucosamine metabolism in spheroplasts of *Candida albicans*. In the present paper, we also present evidence that the synthesis of RNA and protein is necessary for the induction of these enzymes, whereas the synthesis of DNA is not.

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Materials and Methods

Organism and growth conditions

Candida albicans 3100, a wild-type pathogenic yeast strain, was obtained from the National Chemical Laboratory, Pune, India. In each instance, cells were transferred from a slant (2–3 days old) into a medium containing 0.5% peptone, 0.3% KH_2PO_4 and 1% glucose. Cells were grown at 30°C for about 15–17h, harvested by centrifugation (1000*g*, 5min), and an inoculum was then transferred to a medium in which the glucose concentration was lowered to 0.5%. Cell growth was monitored turbidimetrically by reading the A_{595} in a Bausch and Lomb Spectronic 20 spectrophotometer.

Assay of N-acetylglucosamine 'permease' (high-affinity uptake system) in spheroplasts

Metabolically active spheroplasts were prepared by the method of Bhattacharya & Datta (1977). Spheroplasts were suspended in 100ml of induction medium containing 1% *N*-acetylglucosamine and 0.3% KH_2PO_4 supplemented with 1M-D-sorbitol to

provide osmotic support (A_{595} 10–14). At indicated times samples (10ml) were taken, centrifuged (1000*g*, 5min), washed with 1M-D-sorbitol and then resuspended in 10ml of 1M-D-sorbitol. For a typical uptake experiment, spheroplast suspensions in 5ml of 1M-D-sorbitol (A_{595} 0.2) were incubated at 30°C for 5min in a shaking water bath and then *N*-[^{3}H]-acetyl-D-glucosamine (1μCi/ml) was added to give a final concentration of 0.05mM. At various times, the cells from 0.5ml samples of the incubation mixtures were collected on Millipore filters and washed twice with 5ml of ice-cold 1M-D-sorbitol. The filters were dried, and their radioactivity was determined in 10ml of a toluene-based scintillation mixture in a Packard Tri-Carb liquid-scintillation spectrometer.

Preparation of crude extract and enzyme assay

Frozen spheroplasts resuspended in 2ml of potassium phosphate buffer (50mM, pH7.6), containing 1mM-EDTA and 1mM-2-mercaptoethanol, were ground for 5min in a mortar and pestle with fine glass powder. After centrifugation at 14000*g* for 30min, the supernatant fraction (crude extract) was assayed for *N*-acetylglucosamine kinase (Datta, 1970) and glucosamine 6-phosphate deaminase (White & Pasternak, 1975). Kinase and deaminase activities in the medium were assayed after dialysis for 12h with two changes against potassium phosphate buffer (50mM, pH7.6) containing 1mM-EDTA and 1mM-2-mercaptoethanol, followed by concentration with saturated sucrose solution. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Syntheses of protein, RNA and DNA were followed by incorporation of [^{3}H]lysine, [^{3}H]uridine and [^{3}H]thymidine respectively into the trichloroacetic acid-precipitable fraction (Bhattacharya & Datta, 1977).

N-[^{3}H]Acetyl-D-glucosamine (686mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. *N*-Acetylglucosamine, glucosamine 6-phosphate, ATP, sodium dodecyl sulphate, NADP$^+$, glucose phosphate isomerase (D-glucose 6-phosphate ketol-isomerase; EC 5.3.1.9; 500–800 units/mg of protein), glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–NADP$^+$ 1-oxidoreductase; EC 1.1.1.49; 300–400units/mg of protein), cycloheximide, actinomycin D, ethidium bromide, cordycepin and mitomycin-C were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and hydroxyurea was a product of Calbiochem, Los Angeles, CA, U.S.A. Glusulase, the intestinal juice of the snail *Helix pomatia*, which contains a mixture of enzymes including β-glucuronidase and sulphatase, was purchased from Endo Laboratories, Garden City, NY, U.S.A. All other reagents were of analytical grade.

Results and Discussion

The utilization of *N*-acetyl-D-glucosamine as a carbon source by *C. albicans* cells is as effective as that of glucose. Like glucose, *N*-acetylglucosamine is taken up readily as the cells start to multiply after a 2–3h lag and rate of multiplication is comparable on both the media (Bhattacharya *et al.*, 1974).

Induction of N-acetylglucosamine-catabolic enzymes

N-Acetylglucosamine permease (high-affinity uptake system) and *N*-acetylglucosamine kinase activities could not be detected in the spheroplasts prepared from glucose-grown cells of *C. albicans* (Figs. 1 and 2). However, basal glucosamine 6-phosphate deaminase activity is present in these spheroplasts (Fig. 3). Addition of *N*-acetylglucosamine to the medium elicits an almost immediate synthesis of all these three enzymes without any detectable lag period. The values at zero time indicate the enzyme activity in uninduced spheroplasts. Enzyme activities increased steadily up to 9h as long as *N*-acetylglucosamine was present in the medium (Figs. 1–3); however, some of the newly synthesized kinase and deaminase leaked out into the medium. Further, removal of *N*-acetylglucosamine by replacement with a medium devoid of

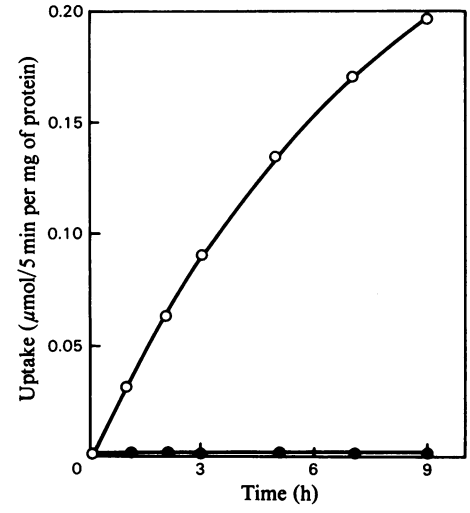

Fig. 1. *Induction of high-affinity uptake system for N-acetylglucosamine in Candida albicans spheroplasts in the presence of N-acetylglucosamine* (10*g*/*litre*)
At various times during induction uptake of *N*-acetylglucosamine was studied, as described in the Materials and Methods section. Protein concentration of spheroplasts was 80μg/ml in the final reaction mixture. In a duplicate culture, cycloheximide was added at zero time together with *N*-acetylglucosamine. ○, Control; ●, with cycloheximide (20μg/ml).

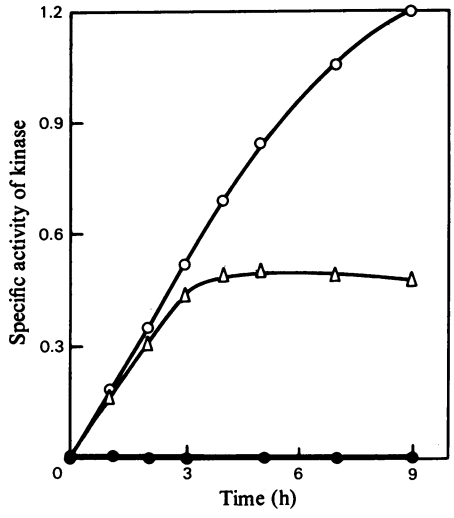

Fig. 2. *Induction of N-acetylglucosamine kinase in spheroplasts of Candida albicans in the presence of N-acetylglucosamine* (10*g*/*litre*)
Cycloheximide (20μg/ml) was added at zero time together with *N*-acetylglucosamine (●). A comparable suspension with no cycloheximide serves as control. ○, Kinase in spheroplasts, control; △, kinase in medium, control. Specific activity is expressed as μmol of *N*-acetylglucosamine esterified/45 min per mg of protein at 37°C under the assay conditions.

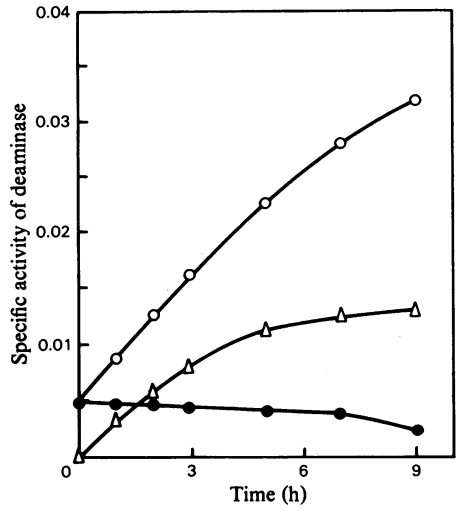

Fig. 3. *Induction of glucosamine 6-phosphate deaminase in Candida albicans spheroplasts in the presence of N-acetylglucosamine* (10*g*/*litre*)
Cycloheximide (20μg/ml) was added at zero time together with *N*-acetylglucosamine (●). A comparable suspension with no cycloheximide served as control. ○, Deaminase in spheroplasts, control; △, deaminase in medium, control. Specific activity is expressed as μmol of glucosamine 6-phosphate deaminated/min per mg of protein at 25°C under the assay conditions.

N-acetylglucosamine causes a gradual decrease in inducible enzyme activities (results not shown). This result suggests that the presence of inducer is necessary for the synthesis of the enzymes, as in other inducible systems. However, the possibility that *N*-acetylglucosamine may stabilize the newly synthesized enzymes has not been entirely ruled out.

An investigation was carried out to detect these inducible enzyme activities in spheroplasts suspended in other carbon sources, namely glucose, glutamic acid, succinic acid and glycerol. However, *N*-acetylglucosamine permease and *N*-acetylglucosamine kinase activities could not be detected in any of these conditions, suggesting that these enzymes are not formed constitutively in *C. albicans*. Furthermore, the basal activity of glucosamine 6-phosphate deaminase also remained unaltered in the presence of substrates other than *N*-acetylglucosamine.

Effect of inhibition of macromolecule synthesis on induction

To find out the mechanism of induction several inhibitors of macromolecule (protein and nucleic acids) synthesis were tested. The induction of permease, kinase and deaminase requires continuous protein synthesis and is prevented completely if cycloheximide (at a concentration of 20μg/ml, i.e. enough to stop protein synthesis in these cells) is added together with the inducer (Figs. 1–3). Further, addition of cycloheximide at any time after the beginning of induction prevents any further increase in enzyme activities and causes these to reach a plateau (results not shown). This result suggests that the induction involves new protein synthesis and the inducer does not function by simply converting enzyme precursors into an active form.

Actinomycin D, a potent inhibitor of RNA synthesis, and ethidium bromide, a known inhibitor of RNA synthesis in yeast (Tonnesen & Friesen, 1973), have been used to analyse the mechanism of induction in the present study. Actinomycin D at 4μg/ml and ethidium bromide at 25μg/ml completely stop the inducible synthesis of all these three enzymes (Table 1). These amounts of inhibitors inhibit uridine incorporation into RNA by more than 95%. Tomkins *et al.* (1966) reported a paradoxical increase in the synthesis of the inducible enzyme tyrosine aminotransferase in the presence of actinomycin D, in cultured hepatoma cells. The phenomenon, sometimes termed superinduction, is rather frequently observed in eukaryotic systems (Tomkins *et al.*, 1972). However,

Table 1. *Effect of inhibitors of macromolecule synthesis on the synthesis of high-affinity uptake system, N-acetylglucosamine kinase and glucosamine 6-phosphate deaminase in Candida albicans spheroplasts*

Spheroplasts were prepared from mid-exponential phase cells of *Candida albicans* and resuspended in a medium containing 1 M-D-sorbitol, 0.3% KH_2PO_4, 1% *N*-acetylglucosamine and inhibitor (where indicated). At 5 h after the beginning of induction, spheroplasts were harvested by centrifugation and enzyme activities were determined as described in the Materials and Methods section.

	N-Acetylglucosamine uptake		*N*-Acetylglucosamine kinase		Glucosamine 6-phosphate deaminase	
Treatment	Specific activity (μmol/min per mg of protein)	Inhibition (%)	Specific activity (μmol of *N*-acetylglucosamine esterified/min per mg of protein)	Inhibition (%)	Specific activity (μmol of glucosamine 6-phosphate deaminated/min per mg of protein)	Inhibition (%)
Non-induced spheroplasts	0.001	—	0	—	0.005	—
Induced spheroplasts	0.028	—	0.019	—	0.022	—
+cycloheximide (20 μg/ml)	0.001	100	0	100	0.004	100
+actinomycin D (4 μg/ml)	0.002	96	0	100	0.005	100
+ethidium bromide (25 μg/ml)	0.002	96	0.001	95	0.005	100
+cordycepin (20 μg/ml)	0.004	89	0.003	84	0.007	88
+mitomycin-C (100 μg/ml)	0.029	0	0.020	0	0.022	0
+hydroxyurea (200 μg/ml)	0.028	0	0.019	0	0.023	0

in the present study no such superinduction was observed for *N*-acetylglucosamine-induced enzymes in *C. albicans*.

Poly(A) sequences, 100–200 nucleotides long, have been detected at the 3′-end of the mRNA of various eukaryotes, including yeast (Darnell *et al.*, 1971*a*; McLaughlin *et al.*, 1973). Although their precise function has not yet been established it has been suggested that poly(A) may be involved in the processing or transport of mRNA from nucleus to cytoplasm (Darnell *et al.*, 1971*b*), or in the stability and activity of templates (Mendecki *et al.*, 1972; Johnston & Bose, 1972). As a first step in investigating the role of poly(A) in this induction system, we have studied the effect of cordycepin, a reported inhibitor of poly(A) synthesis (Penman *et al.*, 1970; Darnell *et al.*, 1971*b*). Like actinomycin D and ethidium bromide, cordycepin at 20 μg/ml strongly inhibits the synthesis of inducible permease, kinase and deaminase (Table 1). Further, this concentration of the drug specifically inhibits the incorporation of adenosine into an mRNA fraction containing poly(A) (Bhattacharya, 1976). Our result supports the view that poly(A) addition is necessary for maturation of mRNA and its subsequent transport across the nuclear membrane. However, the other possibility, that the drug may inhibit the transcription of the RNA essential for the induction of these enzymes, has not been entirely excluded. All these findings suggest that the induction involves the new synthesis of specific mRNA(s) and possibly selective promotion of gene transcription by the inducer.

The question of whether DNA synthesis is absolutely required for hormone-regulated induced enzyme synthesis has been dealt with by several groups in the past years (Moscona *et al.*, 1970; Jailkhani & Talwar, 1972). To answer this question, we have also studied the effect of hydroxyurea and mitomycin-C on induction. Mitomycin-C at 100 μg/ml completely inhibits DNA synthesis, whereas only 60% inhibition (maximum) is obtained with hydroxyurea at 200 μg/ml. Moreover, these doses do not have any effect on cellular RNA synthesis (as determined by [^{3}H]-uridine incorporation into trichloroacetic acid-precipitable fraction). Inhibitors of DNA synthesis (mitomycin-C or hydroxyurea) when added simultaneously with the inducer have no effect on inducible synthesis of the enzymes (Table 1). Further, when the inhibitors are added before inducer, there is still no perceptible effect on the inducible synthesis of the enzymes (results not shown). Thus continuous DNA synthesis is not necessary for enzyme induction, and DNA synthesis before induction is not a necessary prerequisite for the formation of *N*-acetylglucosamine-induced enzymes in *C. albicans*.

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