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L-Asparaginase of *Saccharomyces cerevisiae*: an Extracellular Enzyme

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During recent studies conducted with suspensions of three strains of *Saccharomyces cerevisiae*, it was observed that ammonia was rapidly liberated when L-asparagine was added to the medium. Subsequent investigation has revealed that these strains of *S. cerevisiae* have an externally active asparaginase as well as an internally active one. The appearance of the external asparaginase is stimulated by nitrogen starvation, requires an available energy source, and is prevented by cycloheximide. The internal enzyme appears to be constitutive. The external activity is relatively insensitive to para-hydroxymercuribenzoate inhibition, whereas the internal activity is highly inhibited by this compound.

L-Asparaginase (L-asparagine amido hydrolase EC 3.5.1.1.) catalyzes the cleavage of L-asparagine to aspartic acid and ammonia. The occurrence of this enzyme in yeast, as well as other microbial organisms, is well documented (1, 3, 14, 16); however, since its substrate, L-asparagine, is a required component in several important metabolic processes, it seems unlikely that high levels of active asparaginase would be freely accessible within the cell. The hydrolytic asparaginase activity must, of necessity, be regulated through either metabolic alteration or cellular compartmentation.

Arima et al. (2) showed that a number of bacteria and fungi produced true extracellular asparaginase activity, i.e., the active enzyme could be isolated from culture filtrates. However, in most of the strains of *Saccharomyces* tested no extracellular activity was detected. Cedar and Schwartz (4) have reported that two asparaginases exist in *Escherichia coli* strain K-12, and that the enzyme designated asparaginase II is located at or near the cell surface. Intact cell preparations had asparaginase activity; however, no free enzyme could be detected in culture filtrates. The enzyme was released into the supernatant fluid only after the cells were subjected to osmotic shock or spheroplast formation. Their conclusion was that this enzyme was located in the periplasmic region external to the bacterial cytoplasmic membrane and thus could be classified as extracellular.

Jones and Mortimer (9), in an effort to compare asparaginase data derived from bacterial studies with that observed for yeast, investigated several wild-type and mutant strains of *S. cerevisiae*. They suggested that a single yeast L-asparaginase existed which was synthesized constitutively, was entirely intracellular in nature, and was functionally unaffected by the products of its activity. Concurrently, they investigated the genetic control of L-asparaginase synthesis and through the aid of asparaginaseless mutants were able to show a single structural gene (called *asp*1) was responsible for asparaginase synthesis (7, 8).

Recently we have found that native intact cells of three strains of *S. cerevisiae* quantitatively release ammonia from L-asparagine. This could be explained if certain yeast strains have two forms of asparaginase similar to that seen for *E. coli* strain K-12. Because of the substantial genetic information and technology available for *S. cerevisiae*, the existence of these two enzyme forms would present an ideal system for the study of enzyme multiplicity and the mechanisms responsible for enzyme compartmentation. Therefore, an investigation of the nature of the strain-specific asparaginase activities of *S. cerevisiae* was undertaken. The results of this investigation are reported in this paper.

MATERIALS AND METHODS

Organism. The haploid yeast strain used in the majority of these studies was *S. cerevisiae* X-2180-A2 (*a SUC2 mal gal2* CUP1) obtained from the Yeast Genetics Stock Center, Donner Laboratory, University of California, Berkeley. Other wild-type yeast strains used were obtained from the following sources: D273-10B (β haploid) from F. Sherman, 3962c (a haploid) and ϵ 1278b (α haploid) from M. Grenson, 9896 (mating type unknown) and 10275 (α haploid) from the American Type Culture Collection, XT 1172 (diploid) from T. Cooper and Harden and Young strain from M. Utter.

Growth conditions. The standard medium contained (per liter): 20 g of D-glucose, 2 g of yeast

nitrogen base (without amino acids and ammonium sulfate), and 1.32 g of ammonium sulfate. After inoculation to an initial optical density (660 nm) of 0.10, the yeast cultures were grown at 25 C on a New Brunswick gyrotory shaker at 150 rpm for 16 h. Cells were harvested by centrifugation and washed with 20 mM potassium phosphate buffer, pH 7.0, just before use.

Cells not used immediately were stored as a pellet, after centrifugation, at 4 C. The time of storage never exceeded 3 h. No difference in cell response was observed between stored and fresh cell preparations. All cell populations used in this study were grown under the above conditions.

Cell preparation methods. For the investigation of yeast asparaginase activity three methods of cell preparation were routinely used. (i) Unstarved cells: cell populations designated in this manner were assayed for asparaginase activity directly after harvest by suspension in 20 mM potassium phosphate buffer, pH 7.0, to a final volume of 100 ml. (ii) Nitrogen-starved cells: cell populations designated in this manner were suspended after harvest in a nitrogen-free medium containing 20 mM potassium phosphate buffer, pH 7.0, and 3% glucose and allowed to incubate for 3 h at 25 C. After the incubation cell samples were centrifuged, washed and resuspended in 20 mM potassium phosphate buffer, pH 7.0, to a volume of 100 ml. (iii) Benzene-treated cells: in those studies where the internal level of asparaginase activity was to be assayed, the cell pellet either before or after nitrogen starvation was resuspended to 4 ml with 20 mM potassium phosphate buffer, pH 7.0, and 3% glucose. Reagent grade benzene (1 ml) was added to the cell suspension and the reaction mixture was placed in the cold (4 C) for 2 h. After 2 h the cells were allowed to equilibrate to room temperature and diluted to a final volume of 100 ml with 20 mM potassium phosphate buffer, pH 7.0. (Benzene had no effect on the enzymatic activity or the assay system.)

Measurement of asparaginase activity in whole cells. The cell suspensions described above were then used in either of two assays. For the extended time course assay, L-asparagine was added to the cell suspension (optical density [660 nm] of 0.10) to a final concentration of 500 μM. At 5-min intervals, 5.0-ml samples were removed and centrifuged at 3,000 rpm for 1 min, and the supernatant fluid was reserved for later development. For the rapid interval assay, 50 ml of the cell suspension (optical density [660 nm] of 0.7) was rapidly mixed with L-asparagine to a final concentration of 2 mM and drawn into a syringe. The syringe was then fitted with a 25-mm Gelman filter holder containing a 0.45-μm filter. At 15-s intervals, 1.5 ml of the fluid was passed through the filter and the cell-free filtrate was collected for later development.

Methods of assay. (i) Ammonia assay. The ammonia concentration in the filtrate or supernatant fluid was determined spectrophotometrically through coupling with L-glutamic dehydrogenase (EC 1.4.1.3). The assay mixture contained in a final volume of 1.0 ml: 200 mM phosphate buffer, pH 7.4; 25 mM α-ketoglutarate; 5.65 U of L-glutamic dehydrogenase; 200 μM of β-nicotinamide adenine dinucleotide, reduced form (NADH); and 0.4 ml of an ammonia sample. The mixture was incubated at 21 C for 1 h and the absorbance at 340 nm was recorded. This assay gave linear results for ammonia concentrations between 0 and 200 μM. Asparaginase activity was expressed as nanomoles of ammonia produced per minute per milligram of cells (dry weight) at 21 C (room temperature).

(ii) Aspartate assay. The asparate concentration in the filtrate or supernatant fluid was measured spectrophotometrically through coupling with L-glutamic-oxaloacetic transaminase (EC 2.6.1.1) and L-malic dehydrogenase (EC 1.1.1.37). The assay mixture contained in a final volume of 1.0 ml: 20 mM phosphate buffer, pH 7.4, 2 mM α-ketoglutarate, 5.4 U of L-glutamic-oxaloacetic transaminase, 6.9 U of L-malic dehydrogenase, 250 μM NADH and 0.4 ml of aspartate sample. After incubation for 15 min at 21 C, the absorbance at 340 nm was recorded. This assay gave linear results for aspartate concentrations between 0 and 200 μM. Asparaginase activity was expressed as nanomoles of aspartate produced per minute per milligram of cells (dry weight) at 21 C (room temperature).

Measurement of asparaginase activity in cell extracts. Extracts were prepared using an Aminco French press with an internal pressure of 20,000 lb/in^2. Ruptured cell samples were centrifuged at 18,000 rpm for 30 min and the supernatant fluid was used as the crude extract. The release of aspartate owing to asparaginase activity was monitored spectrophotometrically at 21 C through coupling with L-glutamic-oxaloacetic and L-malic dehydrogenase. The assay system contained in 0.95 ml: 25 mM phosphate buffer (pH 7.4), 125 μM L-asparagine, 1 mM α-ketoglutarate, 16 U of L-glutamic-oxaloacetic, 7.0 U of L-malic dehydrogenase, and 250 μM of NADH. At zero time, 50-μl crude extract was added and the change in absorbance at 340 nm per unit time was recorded. The rate of aspartate production was corrected through subtraction of the rate of NADH oxidation observed in the absence of L-asparagine. This never resulted in a correction of greater than 10%. Protein concentration was determined by the biuret method. Activity was reported as nanomoles of aspartate released per minute per milligram of protein.

Determination of the pH profile for L-asparagine transport. Yeast cells were suspended in medium containing 20 mM potassium phosphate, pH 6.5, and 2% glucose. After incubation for 3 h at 25 C, 5-ml samples were collected by filtration and quantitatively resuspended in the same medium at the pH indicated. After 15 min, 4.5 ml of the cell suspension was mixed with 0.5 ml of 1 mM L-asparagine (G-^{3}H) (general label). Samples (1.0 ml) of this mixture were removed at 1-min intervals for 4 min. Each sample was pipetted onto a Gelman membrane filter (25 mm diameter, 0.45-μm pore size) and then immediately washed with 10 ml of ice cold water. The washed filters were placed into vials containing 5 ml of liquid scintillation counting fluid and the level of radioactivity was measured on a Beckman LS 100 liquid scintillation counter. The scintillation fluid contained 9.9 g of 2,5-diphenyloxazole, 609 mg of 1,4-bis-[2]-(5-phenyloxazole) benzene, 3,000 ml of toluene, and

390 ml of Bio-Solve BBS-3 (obtained from Beckman Instruments, Inc.).

RESULTS

Hydrolysis of L-asparagine by whole cell suspensions of S. cerevisiae. (i) Enzyme activity. The time course for the production and subsequent uptake of ammonia from L-asparagine by cell suspensions of nitrogen-starved *S. cerevisiae* X-2180-A2 is shown in Fig. 1. The initial rate of ammonia liberation was 55 nmol/min per mg of cells. The maximum observed rate of ammonia uptake was 10 nmol/min per mg of cells. The release of ammonia was a linear function of cell concentration (Fig. 2) and exhibited pseudo first order kinetics at saturating levels of substrate (12). Other nitrogen-containing compounds did not serve as a source of ammonia. The stoichiometry of the reaction (Table 1) was observed to be 1:1:1 for the disappearance of reactant (L-asparagine) and the formation of products (L-aspartate and ammonia). No other ninhydrin-detectable amino derivatives were observed. Similar results were obtained for *S. cerevisiae* strains D 273-10B and XT 1172, whereas suspensions of strains 3962C, 10275, ϵ 1278b, 9896, and Harden and Young did not exhibit this activity.

(ii) Enzyme appearance. *S. cerevisiae* X-2180-A2 cells suspended in a nitrogen-free medium with and without a metabolizable source of energy (3% glucose) were assayed for asparaginase activity over a 5-h period. The results, as shown in Fig. 3, reveal: (i) in the absence of an energy source, little activity is detectable; (ii) in the presence of an energy source, there is a low initial activity of 9 nmol/min per mg of cells (dry weight) which increases in a hyperbolic fashion for 4 h appraoching a maximal rate >63 nmol/min per mg of cells. Similar results were obtained if fructose of mannose are substituted for glucose as alternative sources of energy. However, when galactose, which is not metabolized by this strain, was used the results were identical to a system containing no energy source.

To determine whether protein synthesis is involved in asparaginase appearance, induction experiments were run as follows. Cycloheximide (1 mg/100 ml of cells) was added to cell suspensions at 0.5-h intervals during the incubation

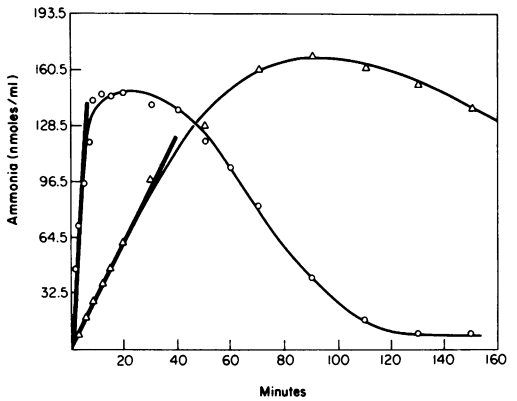

FIG. 1. *Release of ammonia from L-asparagine by whole cell suspensions of S. cerevisiae strain X-2180-A2. Cells were incubated (25 C) for 2.5 h in nitrogen-free medium (see Materials and Methods) before assay. The extended time course assay coupled to ammonia determination was used to measure the level of asparaginase activity. Cell densities were 0.6 mg/ml (O) and 0.12 mg/ml (Δ). L-Asparagine concentration was 500 μM.*

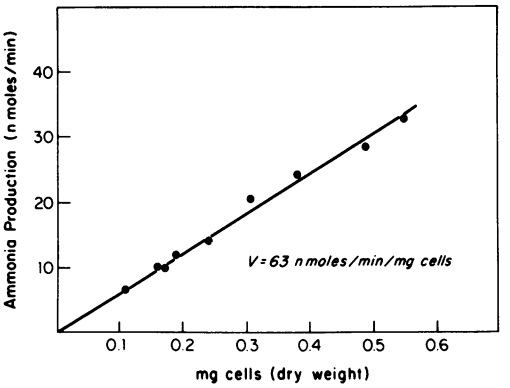

FIG. 2. *Ammonia production as a function of cell concentration. Cells were incubated (25 C) for 3 h in nitrogen-free medium (see Materials and Methods) before assay. The extended time course assay was used for cell concentrations between 0.1 mg/ml and 0.3 mg/ml. Above 0.3 mg/ml the rapid internal assay was used. L-Asparagine concentration was 1 mM.*

TABLE 1. *Stoichiometric relationship between ammonia L-aspartate and L-asparagine during asparaginase reaction time course with whole cells*[a]

Reaction time (min)	L-Asparagine[b] consumed (nmol/ml)	L-Aspartate[b] produced (nmol/ml)	Ammonia[c] produced (nmol/ml)
10	163	121	139
20	246	249	231
30	352	325	323
40	436	455	432

[a] Results obtained with the extended time course assay using 500 μM asparagine and 0.2 mg of cell concentration per ml.

[b] Results obtained by amino acid analysis of individual time point samples by using a single column Beckman model no. 119 analyzer.

[c] Results obtained with glutamic dehydrogenase coupling assay.

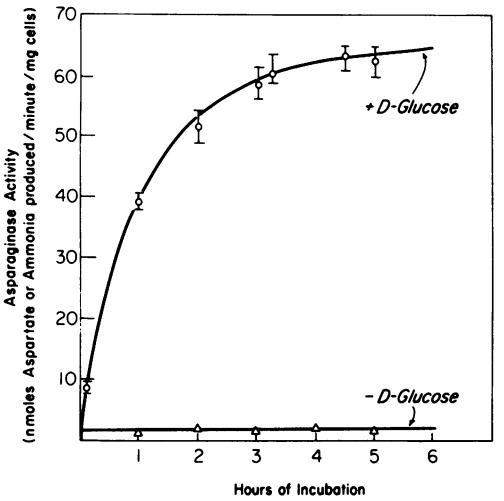

FIG. 3. *Response of asparaginase activity to time of cell incubation in a nitrogen-free medium. Fresh cells were suspended at zero time in 20 mM potassium phosphate buffer (pH 7.0), and 3% D-glucose at a cell density of 0.1 mg/ml. At the time points indicated a 50-ml sample of cells was removed and used in an extended time course assay. L-Asparagine concentration was 1 mM; aspartic acid concentration was monitored to determine asparaginase activity. The data points represent an average value obtained from five separate determinations and the bar lines (I) indicate the limits of the variation in activity.*

period, and after 3 h all the samples were assayed for asparaginase activity. As shown in Fig. 4, a hyperbolic curve was obtained which is similar to that seen in Fig. 3. This suggests that the level of observable asparaginase is directly dependent upon protein synthesis.

We then investigated the nature of the stimulus for enzyme appearance. All previous work had been done in nitrogen-free medium at pH 7.0 because of the original finding of this enzyme in such a system. Since the initial enzyme level in cells grown on ammonium sulfate at pH 4.5 was low, it seemed possible that the introduction of a nitrogen source and/or a low pH could affect the level of enzyme appearance. To test the former possibility, nitrogen compounds at 2 mM concentrations were introduced into the incubation medium at pH 7.0. The data (Table 2) show a decrease in the amount of detectable activity for all the amino acids tested and a variable effect with ammonium chloride. No effect was observed with urea and allantoin. Furthermore, the extent to which the individual amino acids reduced the activity level can be directly correlated with their rate of uptake into the cell at a specific pH (15). These results indicate that the nitrogen starvation state of the cell [i.e., the internal concentrations of ammonia (NH_4^+) and amino acids] determines the level of externally accessible asparaginase.

The possible effects of several amino acids (glutamine, glutamate, aspartate, alanine, serine, and arginine) as inhibitors of asparaginase activity was also studied. Whole cell asparaginase activity, present after nitrogen starvation, was neither enhanced nor inhibited in the presence of these compounds. Similarly, the presence or absence of an energy source after nitrogen starvation had no effect on asparaginase activity.

The effect of pH on both the appearance and activity of whole cell asparaginase was examined (Fig. 5). The pH curve of enzyme appearance, obtained through incubating the cells at various pH values for 3 h and then assaying activity at pH 7.0, is a bell-shaped profile exhibiting a maximum at pH 6.5. The profile for activity, obtained by incubating cells at pH 7.0 for 3 h and then assaying activity at specific pH values, is also bell-shaped; however, the maximum is shifted to pH 7.0.

Cellular organization of L-asparaginase in S. cerevisiae. (i) Strain-specific L-asparaginase activity. To establish a distinction between two asparaginase enzyme forms, the

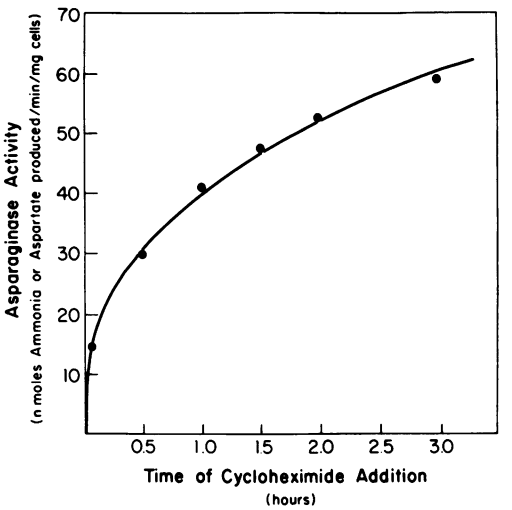

FIG. 4. *Effect of cycloheximide on the appearance of asparaginase in S. cerevisiae strain X-2180-A2. At zero time the cells were suspended in 20 mM potassium phosphate buffer (pH 7.0) and 3% D-glucose to a cell density of 0.1 mg/ml, divided into seven separate samples, and cycloheximide (1 μg/ml) was added to the first sample. Thereafter at 0.5-h intervals cycloheximide was added to consecutive samples. After 3 h the cells of the individual samples were washed, resuspended, and used in the extended time course assay. L-Asparagine concentration was 1 mM; aspartic acid concentration was monitored to determine the level of asparaginase activity.*

TABLE 2. *Effect of nitrogen compounds on the appearance of asparaginase activity*

Nitrogen compound[a] (2 mM)	Asparaginase activity detected (%)
None	100
Arginine	15–30[b]
Serine	45–55
Asparagine	15–30
Alanine	30–40
Glycine	25
Histidine	25
Aspartate	
pH 7.0	84
pH 6.0[c]	40
Glutamate	
pH 7.0	66
pH 6.0[c]	22
Glutamine	15
Lysine	10
γ-Amino butyric acid	40
Ammonium chloride	<50
Urea	100
Allantoin	97

[a] Cells were incubated in 20 mM potassium phosphate buffer (pH 7.0) and 3% D-glucose for 3 h in the presence of the indicated nitrogen compound and then used in the extended time course assay described under methods.

[b] Results obtained using both ammonia and aspartate development systems. Slightly lower values are always observed due to the uptake of ammonia by whole cells.

[c] Compared to control cells starved at same pH.

levels of asparaginase activity in cells exhibiting solely intracellular activity as well as those with extracellular activity had to be determined. The L-asparaginase activity levels of those strains used in this study were measured by using cell extracts and benzene-treated cells (11, 13). The results are shown in Tables 3 and 4, respectively. The extract studies show that for all strains examined before incubation in nitrogen-free medium a constitutive asparaginase level of 15 ± 5 nmol/min per mg of protein was observed. Under conditions of 3 h of nitrogen starvation, the five strains exhibiting no external asparaginase activity show a level identical to that observed before starvation. However, the three strains exhibiting whole cell asparaginase show an increase in extractable activity. Hydrolysis rates of 80 to 125 nmol/min per mg of protein were observed representing a five- to eightfold increase in activity. Studies using benzene-treated cells show a constitutive asparaginase activity level of 7 ± 2 nmol/min per mg of cells (dry weight) (1 mg [dry weight] ~ 0.5 mg of protein). These results are in excellent agreement with those observed with the cell extracts. Moreover, upon nitrogen starvation, the same five- to eightfold increase in activity was observed for the three strains normally exhibiting whole cell asparaginase, whereas no increase was observed for the other strains.

Concurrent with the above determinations, the strain-specific asparaginase activities of various yeast strains were observed in the presence of para-hydroxymercuribenzoate (*p*-OHMB). It had been reported previously that *p*-OHMB caused a partial inhibition (56%) of yeast asparaginase (6). The data (Table 5) show a distinct difference between individual strain responses. Those strains exhibiting only intracellular asparaginase appear to be highly inhibited after incubation with *p*-OHMB. The strains showing internal as well as whole cell asparaginase activity show a varied response to *p*-OHMB, depending on their state of nitrogen starvation. Unstarved cells with a constitutive enzyme level showed a 70 to 80% inhibition by *p*-OHMB, whereas after nitrogen starvation

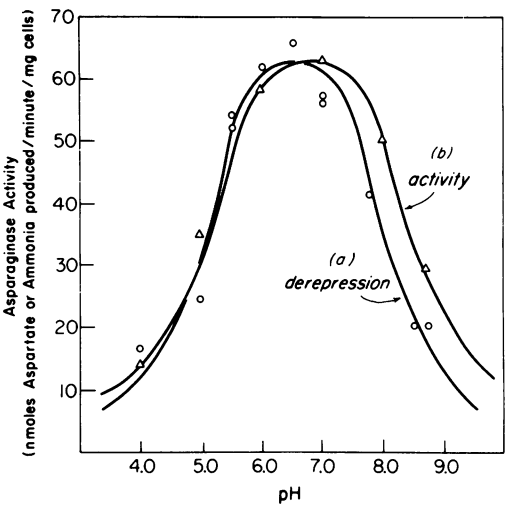

FIG. 5. *pH profiles for derepression and activity of L-asparaginase from whole cells of S. cerevisiae strain X-2180-A2. (a) Fresh cells were suspended in 20 mM potassium phosphate buffer and 3% D-glucose at appropriate pH values for 3 h prior to assay. After incubation, the cells were removed by centrifugation, resuspended in 20 mM potassium phosphate buffer, pH 7.0, and used in the rapid interval assay. Cell density was 0.3 mg/ml (dry weight). L-Asparagine concentration was 1 mM. (b) Cells were incubated in 20 mM potassium phosphate buffer (pH 7.0) and 3% D-glucose for 3 h prior to assay. For activity determinations, the cells were resuspended in 20 mM phosphate buffer at appropriate pH levels and used in an extended time course assay. Cell density was 0.1 mg/ml (dry weight). L-Asparagine concentration was 500 μM.*

TABLE 3. *L-Asparaginase activity in cell extracts of S. cerevisiae*

Strain (*S. cerevisiae*)	Asparaginase activity (nmol/min per mg of protein)	
	Starved cells[a]	Unstarved cells[b]
X-2180-A2	125 ± 7	17.2 ± 1.4
D 273-10B	80 ± 5	10.6 ± 1.2
XT 1172	97 ± 6	13.6 ± 1.6
10275	16.1 ± 1.4	19.8 ± 1.5
3962C	12.9 ± 1.0	
ϵ 1278b	13.0 ± 1.5	
9896	19.8 ± 1.8	12.9 ± 1.3
Harden and Young	17.3 ± 1.6	

[a] Cells were incubated in a nitrogen-free media (20 mM potassium phosphate buffer [pH 7.0] and 3% glucose) for 3 h prior to extraction.

[b] Ammonia grown cells were used directly.

TABLE 4. *L-Asparaginase activity of benzene-treated cells*[a]

Strain (*S. cerevisiae*)	Asparaginase activity (nmol/min per mg of cells [dry weight])	
	Starved cells	Unstarved cells
X-2180-A2	64.5 ± 5.0	7.6 ± 1.1
D 273-10B	41.5 ± 7.0	5.0 ± 1.3
XT 1172		13.3 ± 1.5
10275	4.6 ± 0.8	7.1 ± 0.9
3962C	2.0 ± 0.8	5.6 ± 0.9
ϵ 1278b	3.3 ± 0.9	
9896	3.6 ± 0.7	8.1 ± 1.1
Harden and Young	8.4 ± 1.5	7.6 ± 1.0

[a] Cells were exposed to benzene at 4 C in a volume ratio of 4:1 (cell suspension to benzene) for a period of 2 h before whole cell extended time course asparaginase assays were conducted.

only a 10 to 20% inhibition by *p*-OHMB was observed.

(ii) L-Asparaginase activity in L-asparagine transport-deficient S. cerevisiae. The cellular transport of L-asparagine in wild-type X-2180-A2 cells was examined to determine if uptake into the cell was a prerequisite to hydrolysis. The uptake of labeled L-[H^3]asparagine by nitrogen-starved *S. cerevisiae* at various pH levels was measured. The results indicate a maximal rate of uptake of 25 ± 5 nmol/min per mg of cells at pH 4.0 with a rate of only 6.5 nmol/min per mg of cells at pH 7.0 (Fig. 6). This represents only one-tenth of the observed rate of hydrolysis of L-asparagine at pH 7.0

After these experiments, wild-type X-2180-A2 cells were exposed to a mutagenic agent (ethane methylsulfonic acid) and cell colonies were selected by their ability to grow on 2 mM D-asparagine as the sole source of nitrogen (methodology to be described in a subsequent communication). These yeast strains, designated D-ASN^R, were then examined for their ability to transport L-asparagine (Fig. 6), as well as their ability to produce whole cell asparaginase activity (Table 6). The data show that although the level of exo-L-asparaginase is somewhat variable in the mutant cells, it is generally in excess of asparagine transport by approximately 100-fold.

DISCUSSION

In our investigation of L-asparaginase, we have found three strains of *S. cerevisiae* which show a hydrolytic cleavage of L-asparagine with intact whole cells. The expression of this activity is increased by nitrogen starvation, requires an available energy source, and is prevented by cycloheximide. These phenomena suggest that the synthesis of an externally active form of asparaginase is derepressed during nitrogen starvation. After enzyme synthesis, no cofactor requirements for enzymic activity have been observed.

The localization of this asparaginase activity external to the cell membrane is confirmed by the 100-fold difference in rates between aspara-

TABLE 5. *Effect of 2 mM p-OHMB on the asparaginase of S. cerevisiae strains*

Strain (*S. cerevisiae*)	Asparaginase remaining benzene-treated cells (%)[a]	Asparaginase remaining cell extracts (%)[b]
X-2180-A2		
(starved)	88 ± 5	76 ± 4
(unstarved)	35 ± 4	24 ± 5
D 273-10B		
(starved)	84 ± 4	69 ± 3
(unstarved)	14 ± 5	28 ± 6
XT 1172		
(unstarved)	65 ± 5	42 ± 5
10275	<15	<10
3962C	<18	<10
ϵ 1278b	<18	<9
9896	<12	<5
Harden and Young	<10	<7

[a] Benzene-treated cells were incubated with p-OHMB for 60 min prior to assay. Asparaginase activity determined using the extended time course assay with a cell density of 0.1 mg/ml (dry weight).

[b] Cell-free extracts were exposed to p-OHMB 30 min before assay. Asparaginase activity was measured using direct spectrophotometric assay described in Materials and Methods.

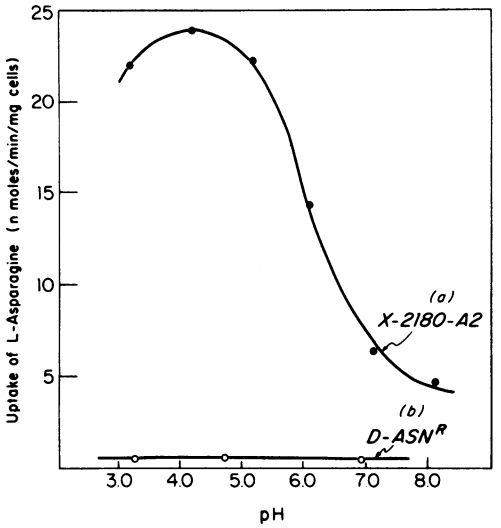

FIG. 6. *pH profile of L-asparagine (G-^{3}H) uptake in wild-type and asparagine transport-deficient (D-ASN^R) S. cerevisiae strain X-2180-A2. (a) The uptake of L-asparagine (G-^{3}H) (specific activity = 870 counts/min per nmol) was measured in 20 mM potassium phosphate buffer and 2% D-glucose at a final cell density of 0.2 mg/ml (dry weight). (b) The uptake of L-asparagine (G-^{3}H) (specific activity = 757 counts/min per nmol) was measured in 20 mM potassium phosphate buffer and 2% D-glucose at a final cell density of 0.2 mg/ml (dry weight).*

gine transport and asparaginase activity observed with cell suspension of asparagine transport-deficient mutants. Generally, the liberation of enzymic activity from intact protoplast cells is considered the single conclusive proof for the existence of an extracellular enzyme. Our data on the accessibility of whole cell asparaginase activity to effects of pH and inhibitors, coupled with the transport studies, represent strong indirect evidence for such an assignment. We are presently working on the isolation of asparaginase from intact protoplast cells to show conclusively the external location of this enzyme.

Although these results appear to be in contrast to the reports of Jones and Mortimer (9) who found a single, constitutive, intracellular asparaginase in wild-type strains of yeast, we believe the variance can be explained. Their studies were conducted with fresh non-nitrogen-starved cells at low pH (about 4.0) using Nessler reagent to detect ammonia. Under similar conditions, we observe an external asparaginase activity of less than 1 nmol/min per mg of cells. It would be very difficult to detect this level of activity above the normal background of ammonia using the Nessler method.

The properties of yeast asparaginase(s) are presently being studied in more detail; differences in inhibitor response (*p*-OHMB), pH optima, gel filtration patterns, and heat and alcohol stability between the constitutive internal enzyme of unstarved cells and the external enzyme found in starved cells suggest that two distinct forms do exist (data to be presented in a subsequent publication). Asparaginase synthesis may thus be quite analogous to that of the other known extracellular enzymes of yeast such as invertase and alpha-glucosidase (5, 10). These inducible, hydrolytic glycoproteins have no cofactor requirements and are composed of a protein core which is similar to that of their respective inner enzyme forms. We are attempting to purify the internal and external enzymes to determine whether they are indeed derivative species of a single protein core which differ in carbohydrate content and/or polymeric form or if they are distinct protein moieties. In addition, we are investigating the effect of 2-deoxy-D-glucose which has been shown to interfere with the glycosylation steps of glycoprotein formation for other yeast enzymes (5). We hope this latter approach will clarify whether a mannan-protein complex is involved in external asparaginase formation and will aid in the isolation of exo-asparaginase precursor by causing it to accumulate in excess of normal levels.

TABLE 6. *Rates of L-asparagine uptake versus asparagine hydrolysis for wild-type and D-ASN^R mutants of S. cerevisiae X-2180-A2*

Strain (*S. cerevisiae*)	Uptake of L-Asparagine (nmol/min per mg of cells)[a]	Asparaginase activity[b] (nmol/min per mg of cells)
X-2180-A2	25 ± 5 nmol	65 ± 5 nmol
D-ASN$^R_2$	<0.35 nmol	35 ± 7 nmol
D-ASN$^R_5$	<0.35 nmol	86 ± 5 nmol
D-ASN$^R_8$	<0.35 nmol	27 ± 4 nmol
D-ASN$^R_9$	<0.35 nmol	91 ± 6 nmol

[a] Uptake measured in 20 mM potassium phosphate buffer (pH 4.8), 2% D-glucose at a final cell density of 0.2 mg/ml (dry weight) using L-asparagine (G-^{3}H), specific activity = 757 counts/min per nmol.

[b] Asparaginase activity measured via extended time course assay at a final cell density of 0.15 mg/ml.

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