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Induction of the Allantoin Degradative Enzymes in *Saccharomyces cerevisiae* by the Last Intermediate of the Pathway

(allophanic acid/urea carboxylase/allantoin degradation defective mutants)

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ABSTRACT ***Saccharomyces cerevisiae*** **can degrade allantoin in five steps to glyoxylate, ammonia, and "CO_2." We previously demonstrated that synthesis of the urea carboxylase–allophanate hydrolase multienzyme complex is contingent upon the presence of allophanic acid, the product of the urea carboxylase reaction. Since these enzymes catalyze the last two reactions of allantoin degradation, experiments were performed to establish whether or not the presence of allophanic acid was required for synthesis of any other enzymes participating in this degradative pathway. The data presented here indicate that allophanic acid is required for synthesis of all enzymes participating in allantoin degradation. This conclusion is based upon the observation that: (*i*) wild-type strains produced a large amount of allantoinase upon addition of allantoin, allantoate, ureidoglycolate, or urea to the medium, (*ii*) no increase in activity was observed unless the added compound could be metabolized to allophanate, (*iii*) strains lacking allophanate hydrolase contained large amounts of allantoinase even in the absence of added urea, and (*iv*) the urea analogue, formamide, was capable of inducing allantoinase synthesis in wild-type strains but would not serve this function in a strain lacking urea carboxylase.**

Saccharomyces cerevisiae can grow using allantoin as sole nitrogen source by virtue of its ability to degrade the compound in five steps, to ammonia, glyoxylate, and "CO_2." We have obtained data (Lawther and Cooper, unpublished observations) showing that mutant strains devoid of allantoinase, allantoicase, urea carboxylase, or allophanate hydrolase are incapable of using allantoin as sole nitrogen source. This finding suggests that the reaction sequence for allantoin degradation is similar to that shown in Fig. 1. This reaction sequence is also operative in several other yeasts (1, 2). We have shown that allophanate is quite likely the inducer of the urea carboxylase–allophanate hydrolase multienzyme complex (3). This was concluded from experiments demonstrating that: (*i*) strains lacking allophanate hydrolase possessed a large constitutive amount of urea carboxylase whether or not the culture was grown in the presence of urea, whereas strains lacking urea carboxylase produced a basal amount of allophanate hydrolase that did not increase upon addition of urea; (*ii*) formamide and hydantoic acid induced these activities in wild-type strains, but would not serve as nitrogen sources; and (*iii*) formamide, a compound structurally related to and selected as a putative physiological analogue of urea, was not capable of inducing the hydrolase in a carboxylase defective strain, whereas hydantoate, an allophanate analogue, was able to induce the hydrolase in such a strain. Since the urea degradative complex may be considered as the final two steps in the degradation of arginine and purines, it was pertinent to identify the inducer of other enzymes participating in this area of nitrogen metabolism. The data reported here suggest that induction of all five enzymes participating in allantoin degradation is contingent upon the presence of allophanic acid, the last intermediate in the pathway.

MATERIALS AND METHODS

Strains. Genotypes of the strains used in this work are listed in Table 1. All strains were prototrophic diploids, homozygous for the significant alleles. Biochemical characteristics of the mutant strains have been determined (Lawther, R. P., Riemer, E. T. & Cooper, T. G., manuscript in preparation).

Media. The minimal medium used in all experiments, except those conducted in the absence of biotin, contained per liter: 0.875 mg of KH_2PO_4, 125 mg of K_2HPO_4, 500 mg of $MgSO_4 \cdot 7H_2O$, 100 mg of NaCl, 10 μg of H_3BO_3, 10 μg of $CuSO_4 5H_2O$, 10 μg of KI, 50 μg of $FeCl_3 \cdot 6H_2O$, 70 μg of $ZnSO_4 \cdot 7H_2O$, 400 μg of thiamine hydrochloride, 400 μg of D-calcium pantothenate, 400 μg of pyridoxine hydrochloride, 2 mg of inositol, 5 μg of D-biotin, 6 g of glucose, and 1 g of $(NH_4)_2 SO_4$.

Induction Experiments. Before initiation of an experiment,

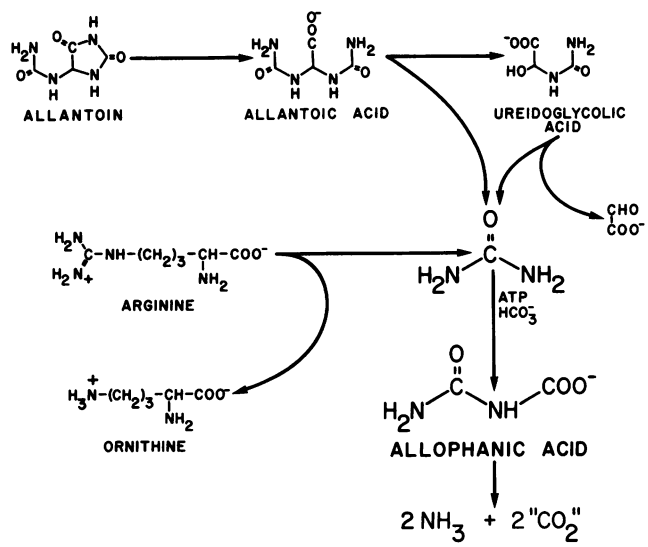

FIG. 1. Reactions involved in degradation of arginine and allantoin.

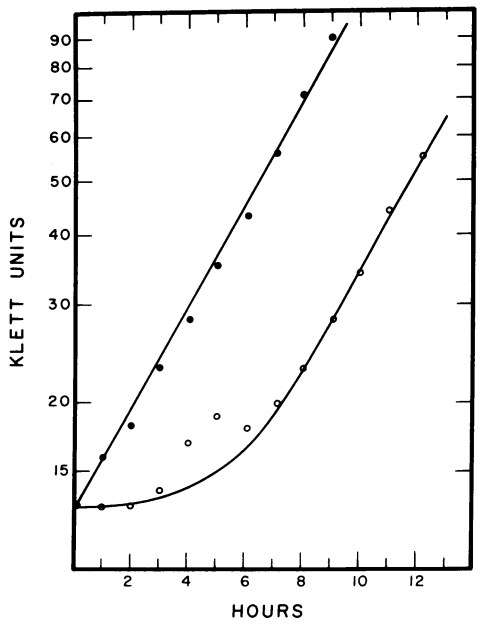

FIG. 2. Growth of *Saccharomyces* on minimal-allantoin medium from an inoculum that was previously grown to saturation on minimal-ammonia (O) or minimal-allantoin (●) medium. Cultures of M-25 were grown on the standard minimal medium with 0.1% of either ammonium sulfate (O) or allantoin (●) as sole nitrogen source. An appropriate sized sample of each culture was then inoculated into fresh minimal medium containing allantoin as sole nitrogen source.

the desired strain was grown to saturation on minimal medium. A sample of the saturated culture was diluted to a final concentration of 0.5–1.0 klett units and permitted to grow to a density of 20–30 klett units (100 klett units is approximately equivalent to 2×10^7 cells per ml). At the indicated time or cell density, the various compounds being tested were added to a final concentration of 10 mM. At appropriate times thereafter, 10-ml samples were removed from the culture, chilled on ice, and brought to 10 μg/ml of cycloheximide. After all samples were taken, the cells were collected by centrifugation and suspended in 2 ml of a wash solution containing 100 mM Tris-acetate buffer adjusted to pH 7.0, 0.5 mM EDTA, and 1% glycerol. To this suspension, nystatin and cycloheximide were added to final concentrations of 15 and 10 μg/ml, respectively. The cells were incubated in this solution for 2–6 hr at 0° and then collected by centrifugation. The permeabilized cells were washed with 2 ml of the above buffer to remove any remaining nystatin (its presence results in increased background values), and suspended in either 0.5 or 1.0 ml of buffer solution for assay. Allantoinase was assayed by the method of Van de Poll (4). All activities are reported as the concentration of glyoxylate produced per min per ml of culture.

RESULTS

The growth patterns observed when *Saccharomyces* is cultured on a medium containing allantoin as sole nitrogen source are determined by the previous culture conditions of the inoculum. As shown in Fig. 2, cells previously grown on minimal medium containing ammonia as sole nitrogen source exhibited a 6- to 8-hr lag when transferred to a medium containing allantoin as sole nitrogen source. This lag, however,

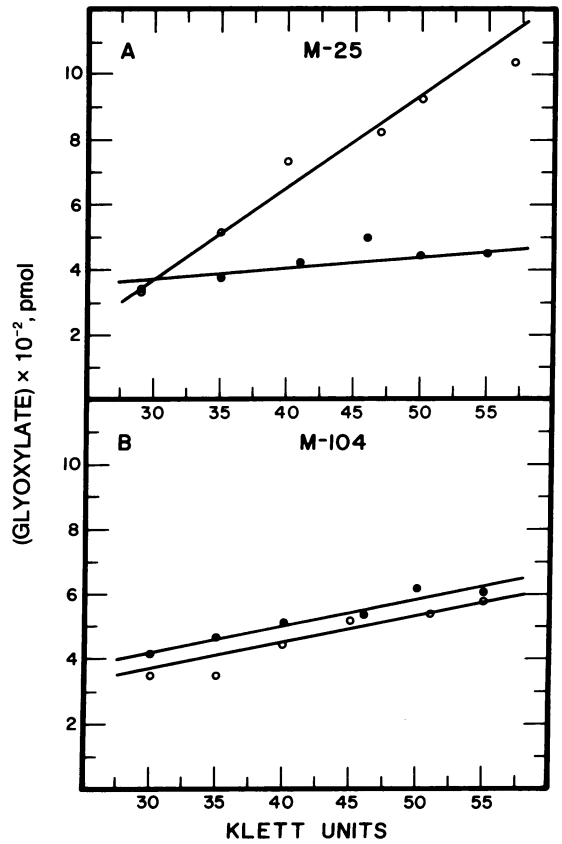

FIG. 3. Differential rate of allantoinase synthesis in wild-type and allantoicase-defective strains of *Saccharomyces*. Cultures of M-25 and M-104 were grown on standard minimal medium containing 0.1% ammonium sulfate as sole nitrogen source. At a cell density of 30 klett units, allantoin was added to a final concentration of 10 mM (O). The control cultures (●) received no additions. Samples were removed at the cell densities indicated and assayed.

was not observed if the cells were grown initially on minimal-allantoin medium. This behavior suggested that at least one inducible protein was functioning in the degradation of allantoin. Since allantoinase is an inducible enzyme in some strains of yeast (5), its activity was monitored after addition of

TABLE 1. *Strains*

Number	Genotype	Defective enzyme
M-25	$\frac{a \; + \; + \; his6 \; lys1 \; ura1}{\alpha \; ade6 \; leu1 \; + \; + \; +}$	None
M-58	$\frac{a \; + \; + \; his6 \; ura1 \; aga2}{\alpha \; ade6 \; leu1 \; + \; + \; aga2}$	Arginase
M-62	$\frac{a \; + \; + \; his6 \; ura1 \; dur1\text{-}145}{\alpha \; ade6 \; leu1 \; + \; + \; dur1\text{-}145}$	Urea carboxylase
M-64	$\frac{a \; + \; + \; his6 \; ura1 \; dur2\text{-}142}{\alpha \; ade6 \; leu1 \; + \; + \; dur2\text{-}142}$	Allophanate hydrolase
M-104	$\frac{a \; + \; + \; his6 \; all2\text{-}18 \; ura1}{\alpha \; ade6 \; leu1 \; + \; all2\text{-}18 \; +}$	Allantoicase
M-130	$\frac{a \; + \; + \; his6 \; all2\text{-}18 \; aga2}{\alpha \; ade6 \; leu1 \; + \; all2\text{-}18 \; aga2}$	Arginase & allantoicase

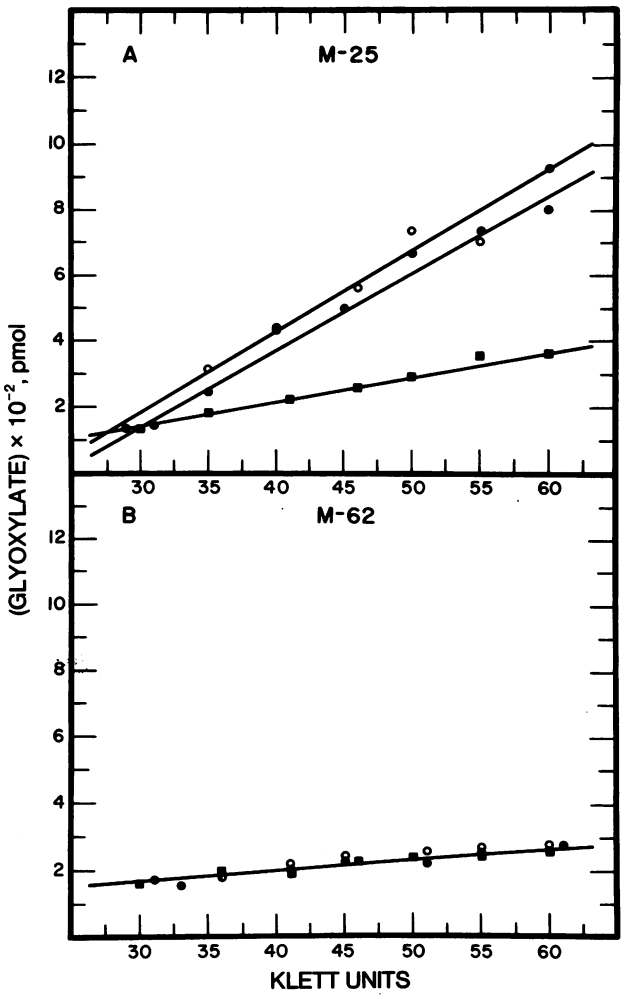

FIG. 4. Differential rate of allantoinase synthesis in wild-type and urea carboxylase-defective strains of *Saccharomyces*. The procedures used were identical to those in Fig. 3. Strains M-25 and M-62 were grown in the absence of inducer (■) or in the presence of 10 mM urea (●) or formamide (○).

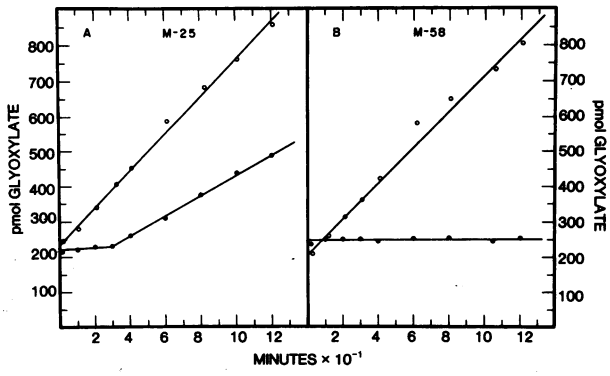

FIG. 5. Induction of allantoinase activity in wild-type and arginase-defective strains of *Saccharomyces*. Strains M-25 and M-58 were grown on minimal-ammonia medium in the presence of 10 mM arginine (●) or 10 mM urea (○). The procedures used in this experiment were identical to those in Fig. 3 except that samples were removed at very short time intervals instead of at given cell densities. The cell density at the beginning of the experiment was 30 klett units.

allantoin to cells growing on ammonia. As shown in Fig. 3*A*, there is an 8- to 9-fold increase in the differential rate of allantoinase synthesis if allantoin is added to the medium. However, (Fig. 3*B*), this increase in activity is not observed in a strain that is devoid of allantoicase activity. Since this mutant would be expected to accumulate allantoin and allantoic acid (it has a measurable uninduced level of allantoinase activity), it is reasonable to conclude that neither of these compounds serves as the inducer of allantoinase. The immediate products of the allantoinase reaction are ureidoglycolic acid and urea. Therefore, this experiment was repeated with urea or the physiological analogue of urea, formamide, in place of allantoin. Fig. 4*A* depicts the 4-fold increase in allantoinase activity that was observed with these two compounds. It is known from our previous work that urea is being continually produced as a result of arginine turnover (3, 6). This fact prompted us to determine the kinetics of allantoinase induction resulting from addition of arginine to a wild-type culture. As shown in Fig. 5*A*, addition of arginine brought about an increase in allantoinase activity after a 30- to 40-min lag, but arginine was not as good an inducer as urea, which did not exhibit such a lag. This finding suggests that induction of allantoinase by arginine is the result of catabolism of arginine to urea. To test this hypothesis, we performed a similar experiment using a mutant strain that lacks arginase activity; as shown in Fig. 5*B*, allantoinase is induced by urea, but not by arginine in this strain. These data confirm that induction of allantoinase by arginine is the result of cleavage of this compound to urea. It is now pertinent to ask whether urea or its carboxylation product, allophanate, is the inducer of allantoinase activity. We approached this question by determining the ability of urea to bring about induction of allantoinase in a strain that is unable to synthesize allophanate from urea (urea carboxylase-defective strain). As shown in Fig. 4*B*, urea and formamide were both ineffective in this strain as, indeed, were all of the allantoin degradative intermediates (Table 2).

These experiments lead to the conclusion that allophanate is required for the increase in allantoinase activity. If this is true, then a strain defective in allophanate hydrolase should contain a large amount of allantoinase even in the absence of added urea. This result is predicted, because an allophanate

TABLE 2. *Differential rate of allantoinase synthesis in wild-type and urea carboxylase-defective strains of Saccharomyces cerevisiae*

Compound added to growth medium	M-25 wild type (Increase in enzyme activity* per 10 klett units of growth)	M-62 urea carboxylase-defective (Increase in enzyme activity* per 10 klett units of growth)
None	75	66
Allantoin	575	66
Allantoate	575†	58†
Ureidoglycolate	575†	66†
Urea	575	66

* Activities are expressed in pmol of glyoxylate produced per min per ml of culture. The values are the slopes of induction curves obtained with log-phase cultures.

† Data from two separate experiments were used and normalized to the urea value.

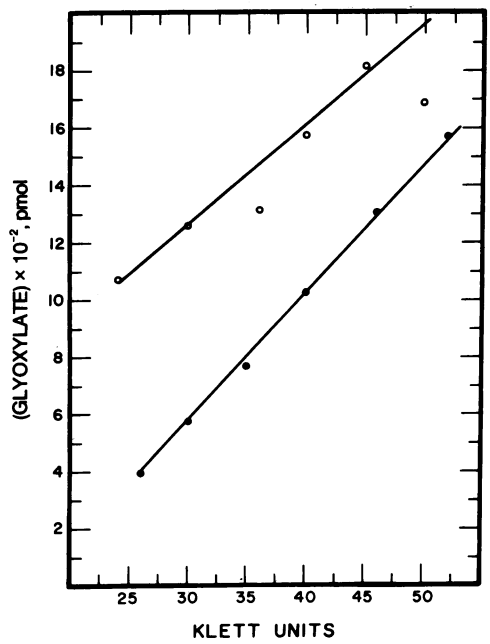

FIG. 6. Differential rate of allantoinase synthesis in a wild-type strain (M-25) grown in the presence of 10 mM urea (●) and an allophanate hydrolase-defective strain (M-64) grown in the absence (○) of added urea. Culture and assay conditions are identical to those in Fig. 3.

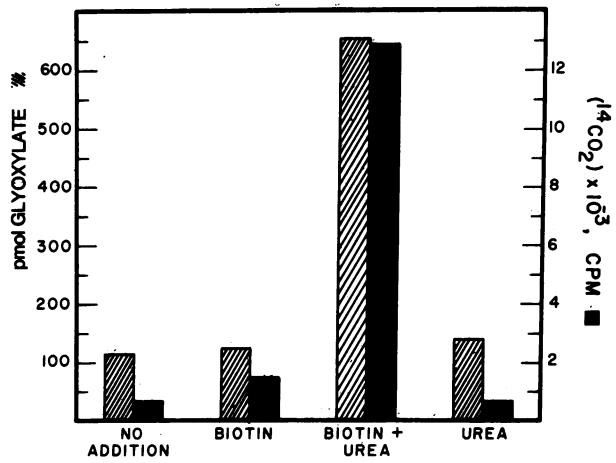

FIG. 7. Effect of biotin and urea on the levels of allantoinase and allophanate hydrolase activity found in wild-type strains of *Saccharomyces* grown on a biotin-free medium. Strain M-25 was grown to saturation five times on the biotin-free medium of Sumper and Riepertinger (14) with aspartate as sole nitrogen source. Samples of this culture were then inoculated into fresh medium containing (in final concentration): (*i*) no additions, (*ii*) 4 μg/ml of biotin, (*iii*) 4 μg/ml of biotin and 10 mM urea, or (*iv*) 10 mM urea. After 1½ generations of growth, samples were taken from each subculture and assayed for allantoinase activity. Allophanate hydrolase activity was assayed by the described procedures (10).

hydrolase-defective strain would be expected to accumulate urea and allophanate through normal turnover of the large arginine pools that are present in these strains (6). As shown in Fig. 6, the initial amount of allantoinase in a hydrolase-defective strain (M-64) is much larger than that found in the wild type and increases with a differential rate that is just slightly less than that observed when urea is added to a wild-type culture. The ratio of the differential rate of activity observed in a hydrolase-defective strain (M-64) to that observed in the wild type is 0.79 and 0.69 for allantoinase and urea carboxylase, respectively (compare ratios obtained from Fig. 6 to those obtained from Fig. 2 of ref. 3).

In the above discussion, identification of allophanate as the inducer of allantoinase depends heavily upon the physiological behavior of a urea carboxylase-defective strain. Although the biochemical lesion in this strain has been documented and we can demonstrate complementation among urea carboxylase, allantoinase-, and allantoicase-defective mutants, it could be argued that M-62 carried an additional defect with regard to allantoinase synthesis. To guard against this possibility we performed the following experiment. A wild-type culture was grown for many generations on a biotin-free medium and then divided into four subcultures. Removal of biotin from the medium will result in production of an inactive urea carboxylase; we have shown that this enzyme contains biotin as an active prosthetic group (7). After 1½ generations of growth on biotin-free medium in the presence of urea, biotin, or biotin plus urea, the cells were collected and assayed for allophanate hydrolase and allantoinase activity. Fig. 7 shows that the two activities are found only if both urea and biotin were present in the medium. It should be pointed out that although allantoinase activity increased dramatically in this experiment, it did not reach the full levels expected.

The two principal sources of allophanate in the cell are allantoin and arginine (Fig. 1). If these two pathways are appropriately blocked, then the intracellular level of allophanate would decrease and a greater-fold increase in allan-

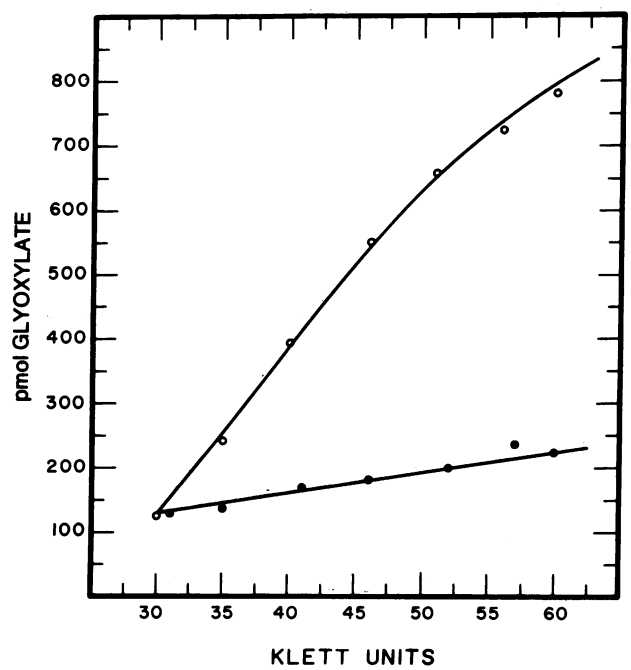

FIG. 8. Differential rate of synthesis of allantoinase in an arginase- and allantoincase-defective strain of *Saccharomyces*. Strain M-130 was grown on minimal-ammonia medium in the presence (○) and absence (●) of added urea. Culture and assay conditions were identical to those described in *Methods*.

TABLE 3. *Amounts of allantoin degradative enzymes in Saccharomyces cerevisiae grown in the presence or absence of urea*

Strain	Allantoinase	Allantoicase	Ureidoglycolate hydrolase
	(nmol/min per mg of soluble protein)		
M-25			
+ urea	0.144	3.53	57.2
− urea	0.044	0.68	12.2
M-62			
+ urea	0.029	0.62	11.2
− urea	0.026	0.68	12.2

Cultures of M-25 and M-62 were grown on minimal-ammonia medium in the presence or absence of 10 mM urea. At a cell density of 60 klett units, the cells were harvested and assayed for allantoicase and ureidoglycolate hydrolase (5, 13). Enzyme activities are all expressed as nmol of glyoxylate produced per min per mg of soluble protein. Allantoate and ureidoglycolate were synthesized by established procedures (11, 12).

toinase synthesis would be expected upon addition of urea. This result was observed experimentally when allantoinase activity, of a strain lacking both arginase and allantoicase, was determined in the presence and absence of urea; a 9- to 10-fold increase in activity was observed (Fig. 8). The curvature observed at high cell densities is due to quenching of the colored complex by the high concentrations of protein present in the assay reaction mixture (Lawther, R. P., Riemer, E. T. & Cooper, T. G., manuscript in preparation).

In order to ascertain whether or not synthesis of allantoicase and ureidoglycolic acid hydrolase was influenced by allophanate in a manner similar to that observed with allantoinase, these activities were assayed in wild-type and urea carboxylase-defective strains that had been cultured for many generations in the presence or absence of urea. As shown in Table 3, both activities increased upon addition of urea to wild-type cells, but did not increase upon addition of urea to the mutant strain. It should be emphasized that the values reported here are the amounts observed at the conclusion of the growth period and not differential rates of synthesis. If these enzymes exhibit high uninduced levels of activity, as does allantoinase, then the fold induction observed would greatly increase when the differential rates of synthesis are determined.

DISCUSSION

In the above experiments, we have shown that growth of wild-type and some mutant strains of *Saccharomyces* in the presence of compounds derived from allantoin results in an 8- to 10-fold increase in allantoinase activity. This increase, however, is observed only if the compounds can be metabolized to allophanic acid, the last intermediate in the allantoin degradative pathway. A similar type of behavior was also observed for allantoicase and ureidoglycolate hydrolase. These results and those we reported earlier lead to the conclusion that synthesis of all enzymes participating in allantoin degradation is contingent upon the presence of allophanic acid. Induction of enzyme synthesis by other than the first intermediate of a pathway is well documented in bacterial systems (8, 9). However, this is likely the first case in either bacterial or eukaryotic systems of the last intermediate in a pathway being required for induction of the enzymes leading to its synthesis.

The central position of allophanate as the last intermediate in the degradation of both arginine and allantoin endows it with a unique capability in coordination of arginine and purine degradation. In the presence of excess nitrogen there is little need to catabolize arginine or any purine-related metabolites that are in the cell. The remoteness of the inducer, allophanate, from these compounds and its high rate of degradation by allophanate hydrolase (10), insure that its concentration will remain at a minimal level. Hence, the urea and allantoin degradative enzymes will be induced only if the concentrations of the pertinent precursors increase above a threshold value. Such a response would be expected during periods of severe nitrogen limitation. Under these conditions, the large pools of arginine contained in *Saccharomyces* are mobilized with the resulting increase in the cellular amounts of allophanate. It is also during these starvation conditions that it would be most beneficial to degrade any purine-related metabolites that might be available. In the scheme we have outlined, control of induction of the enzymes in this area of nitrogen metabolism is exerted in a two-step process. Arginine must become metabolically available in quantities sufficient to induce arginase. The action of arginase on arginine will then produce the threshold concentrations of urea and, hence, allophanate necessary to bring the entire system to full degradative capacity. The delicate balance between the concentration of available metabolites and enzymes used for their degradation is further refined by imposing the antagonistic effects of nitrogen repression upon the induction events.

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