JOURNAL OF BACTERIOLOGY, Aug. 1978, p. 498-510
0021-9193/78/0135-0498$02.00/0

Vol. 135, No. 2
Printed in U.S.A.

Induction and Inhibition of the Allantoin Permease in *Saccharomyces cerevisiae*

ROBERTA SUMRADA, CAROL A. ZACHARSKI, VANESSA TUROSCY, AND TERRANCE G. COOPER*

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Received for publication 6 February 1978

Allantoin uptake in *Saccharomyces cerevisiae* is mediated by an energy-dependent, low-K_m, active transport system. However, there is at present little information concerning its regulation. In view of this, we investigated the control of allantoin transport and found that it was regulated quite differently from the other pathway components. Preincubation of appropriate mutant cultures with purified allantoate (commercial preparations contain 17% allantoin), urea, or oxalurate did not significantly increase allantoin uptake. Preincubation with allantoin, however, resulted in a 10- to 15-fold increase in the rate of allantoin accumulation. Two allantoin analogs were also found to elicit dramatic increases in allantoin uptake. Hydantoin and hydantoin acetic acid were able to induce allantoin transport to 63 and 95% of the levels observed with allantoin. Neither of these compounds was able to serve as a sole nitrogen source for *S. cerevisiae*, and they may be non-metabolizable inducers of the allantoin permease. The *rna1* gene product appeared to be required for allantoin permease induction, suggesting that control was exerted at the level of gene expression. In addition, we have shown that allantoin uptake is not unidirectional; efflux merely occurs at a very low rate. Allantoin uptake is also transinhibited by addition of certain amino acids to the culture medium, and several models concerning the operation of such inhibition were discussed.

Allantoin uptake in both growing and resting cultures of *Saccharomyces cerevisiae* has been shown to involve a low-K_m (ca. 15 μM), active transport system. This conclusion was based on the observations that: (i) intracellular allantoin was concentrated at least 7,700 times over external levels present in the medium and (ii) accumulation did not occur in the absence of glucose or the presence of dinitrophenol, carbonyl cyanide-*m*-chlorophenyl hydrazone, potassium fluoride, or potassium arsenate (16, 22). However, two characteristics often observed in bacterial transport systems were apparently missing. First, allantoin accumulation did not exhibit time-dependent saturation. This was somewhat disturbing because a variety of other *Saccharomyces* transport systems have been reported to reach influx-efflux equilibrium within 3 to 90 min (6, 9, 15, 19). Second, accumulation appeared to be unidirectional. Preloaded, radioactive allantoin was not lost from cells suspended in allantoin-free buffer and did not exchange with exogenously added, non-radioactive allantoin. Treatment of preloaded cells with nystatin, however, released the accumulated radioactive material which was shown to be chemically unaltered allantoin (22). Similar characteristics have been observed for a variety of other yeast transport systems (10-12).

There is presently little information concerning control of the allantoin transport system. All of the enzymes associated with allantoin degradation are inducible and repressible (5, 8, 27). Their production is contingent on the presence of allophanate, the last intermediate in the allantoin-degradative pathway (8, 27) or the gratuitous inducer, oxaluric acid (21). Urea, a major intermediate in allantoin degradation, may enter cells by two routes whose regulatory characteristics have been determined previously (9, 23). A facilitated diffusion system operating at urea concentrations above 0.5 mM is apparently constitutive. The second means of entry is via an energy-dependent, low-K_m, active transport system (9). Production of this system is dependent on allophanate or oxalurate in a manner similar to that of the other pathway enzymes (9).

Several yeast and fungal systems have been reported to exhibit the phenomenon of transinhibition. This term has been used to denote an inhibition of uptake of one compound by accumulation of that compound or another one in the cell. Some systems such as histidine transport are highly specific (10). Histidine uptake

was reported to be inhibited by intracellular histidine, but not by lysine, arginine, methionine, glycine, serine, or glutamate. Each of these amino acids in turn inhibited its own uptake. These results were obtained from experiments in which cells were preloaded with a non-radioactive amino acid for increasing amounts of time, washed, and permitted to accumulate the same radioactive amino acid for a short time (sufficient to obtain an initial rate of uptake). The time observed for "transinhibition" to become complete was the same as that required for uptake of histidine to reach a plateau of time-dependent accumulation. These results may not represent the same phenomenon discussed below even though they have been so classified by several investigators (17, 18, 30). In other systems, such as methylamine and α-aminoisobutyric acid transport, there is less specificity. Uptake of methylamine is non-competitively inhibited by addition of aspartate or alanine (20), whereas α-aminoisobutyric acid uptake is non-competitively inhibited by glycine (12). Roon et al. have suggested that inhibition of methylamine transport by amino acids resulted from a reduction of the plasma membrane adenosine triphosphatase-generated proton-motive force when both methylamine and an amino acid were being transported simultaneously compared with the transport of either compound alone (20). It is not known whether allantoin transport is subject to inhibition of the types described above.

The purpose of the present report is to fill some of the above-mentioned gaps in our understanding of allantoin transport. The data obtained also permit us to separate phenomena, previously all thought to be transinhibition, into two classes. At the molecular level, these two classes of phenomenona likely operate differently.

MATERIALS AND METHODS

Strains. All strains used in this work were prototrophic diploid organisms that have been described previously (2, 14, 26). Their genotypes and biochemical defects are summarized in Table 1.

Culture conditions. The medium used throughout these experiments was that of Wickerham (28). Glucose (0.6%) and ammonia (0.1%) were added as sole sources of carbon and nitrogen, respectively. Cell density measurements were made with a Klett-Summerson colorimeter (500- to 570-nm band-pass filter). One hundred Klett units is approximately equivalent to 3 × 10^7 cells per ml of culture.

Resting cell cultures were prepared as follows. Cells were grown to a cell density of about 40 Klett units, harvested by filtration, washed with several volumes of nitrogen-free medium, and suspended in one-half the original volume of prewarmed, preaerated buffer.

TABLE 1. *Strains Used*

Strain designation	Genotype	Phenotype
M25	α *ade6 leu1* / a *his6 ura1 lys1*	Wild type
M85	α *ade6 leu1 dal1* / a *his6 ura1 dal1*	Allantoinase minus
M104	α *ade6 leu1 dal2* / a *his6 ura1 dal2*	Allantoicase minus
M62	α *ade6 leu1 dur1* / a *his6 ura1 dur1*	Urea carboxylase minus
M664	α *his6 ura1 dal1 dur1* / a *ade6 leu1 dal1 dur1*	Allantoinase and urea carboxylase minus
M674	α *ade1 tyr1 dal1 rna1* / a *lys1 dal1 rna1*	Allantoinase and RNA metabolism minus

Buffer consisted of 0.1 M citrate (pH 5.0) containing 0.6% glucose. After incubation at 30°C for 18 to 24 h, the culture was ready for use (22). The cell density at this time was approximately 150 to 210 Klett units. These cells were used directly for the transport assays.

Allantoin uptake assay. At zero time, an 8.5-ml portion of the culture to be assayed was transferred to a prewarmed flask containing concentrations of [^{14}C]allantoin (specific activity, 0.2 μCi/μmol) that ranged from 0.3 to 0.9 mM. These concentrations of allantoin are 25- to 75-fold greater than the apparent Michaelis constant of the transport system. [^{14}C]allantoin was synthesized as described earlier (22). Incubation was carried out at 30°C in a shaking water bath under conditions identical to those used for growth. At the times indicated, 1.0-ml samples were removed and transferred to nitrocellulose membrane filters. These filters were then washed five times with 4 ml of cold minimal medium containing 0.1% allantoin. The temperature of the wash solution did not appear to be significant, because the same values of accumulation were observed when cells were washed with medium at either 4 or 27°C. Washed filters were placed in 5 ml of aqueous scintillation fluid (Aquasol, New England Nuclear Corp.), and their radioactivity content was determined 16 to 24 h later. The incubation time in Aquasol was needed to allow the filters to become transparent. Failure to do this resulted in unevenly quenched samples and loss of assay precision. All data are expressed as amounts of radioactive allantoin accumulated per milliliter of culture unless otherwise indicated. It should be noted that resting cell cultures accumulated more allantoin than growing cells. This difference results in part from the greater cell densities of resting cell cultures.

Whenever a potential inducing compound was present in the growth medium, the cells were harvested by centrifugation, washed, and resuspended in fresh prewarmed, preaerated, glucose-ammonia medium just before being assayed.

Purification of allantoate. Allantoate was purified by ion-exchange chromatography. Dowex-1-acetate ion-exchange resin was prepared as described by Cooper and Beevers (7). Potassium allantoate (1 g) was dissolved in 200 ml of water and

percolated through a column (12.5 by 2.5 cm) of the above resin. The resin was washed with 200 ml of water and eluted with a linear 0 to 0.33 M NaCl gradient (800-ml total volume). Sodium allantoate recovered from the resin was concentrated 10- to 15-fold with a Brinkmann rotary evaporator and crystallized with cold (−20°C) ethanol. The crystals were collected by filtration, washed with ether, and dried. This material on rechromatography contained neither allantoin nor urea.

RESULTS

Time-dependent saturation of allantoin accumulation. An important characteristic of active transport systems is their saturability with increasing time and substrate concentration. As expected, the rate of allantoin accumulation plateaus at high substrate concentrations (see Fig. 5 of reference 22). Time-dependent saturability, on the other hand, was not observed even when accumulation was followed for 120 min. To ascertain whether or not allantoin accumulation ever reached a plateau, we performed an experiment which made use of the fact that intra- and extracellular allantoin do not measurably exchange over short time periods (22). A culture of an allantoinase-minus strain of *S. cerevisiae* was grown in minimal glucose-ammonia medium containing 6 mM non-radioactive allantoin. At various times after addition of allantoin, cell samples were harvested by filtration, resuspended in fresh medium containing [^{14}C]allantoin, and incubated for a short time (35 min). If, during the initial incubation period, the uptake system became saturated with non-radioactive allantoin, it would not be able to accumulate the radioactive metabolite. The rate of [^{14}C]allantoin accumulation remained more or less constant for nearly six generations, indicating that allantoin uptake did not undergo time-dependent saturation in growing cells. However, as shown in Fig. 1, resting cells that were not undergoing division slowly lost their ability for continued allantoin accumulation. This was not observed earlier (22), because 14 to 22 h elapsed before allantoin accumulation reached a plateau.

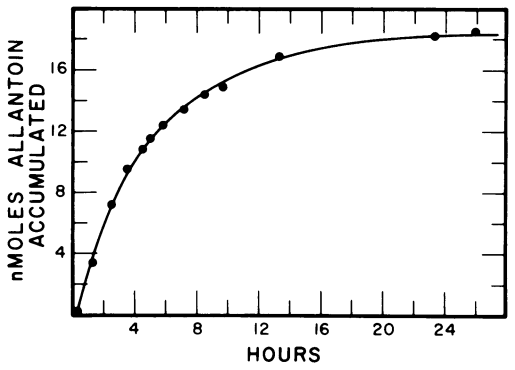

FIG. 1. *Time-dependent accumulation of [^{14}C]allantoin by resting cultures of S. cerevisiae. Resting cell cultures of strain M85 were prepared as described in the text. The cell density of the culture used in the experiment was 147 Klett units. A sample of this culture was incubated in the presence of [^{14}C]allantoin (6 mM final concentration; specific activity, 0.2 μCi/μmol). At the times indicated, a 1-ml sample was removed from the incubation mixture and processed as described in the text. The data are expressed as nanomoles of radioactive allantoin accumulated per milliliter of culture.*

Reversibility of the allantoin transport system. All of our previous experiments attempting to demonstrate efflux or exchange of intracellular and extracellular allantoin yielded negative results (22) This may be interpreted as unidirectional transport, a characteristic previously reported for many amino acid uptake systems (10–12). Alternatively, such behavior might reflect a very slow rate of efflux or exchange that would likely be missed in standard experimental formats. To evaluate the latter possibility, we preloaded growing and resting cells with radioactive allantoin, transferred them to fresh medium devoid of radioactive allantoin, and then monitored both cells and medium for extended periods of time. As shown in Fig. 2A, a small amount of efflux (appearance of [^{14}C]allantoin in medium devoid of non-radioactive allantoin and its simultaneous loss from cells) was observed with logarithmically growing cells. At 1 to 2 h, the normal time used in the past for such experiments, the amount of radioactive material lost from cells and appearing in the medium was barely above background. However, at 6 h the degree of allantoin efflux was clearly measurable. Exchange of intra and extracellular allantoin (appearance of [^{14}C]allantoin in medium containing excess non-radioactive allantoin and its simultaneous disappearance from cells) was somewhat greater. Here also, the degree of exchange observed after 1 to 2 h would likely have been dismissed as experimental error. Somewhat different results were observed when this experiment was repeated with resting cells. As shown in Fig. 2B, no efflux was observed over the 8-h duration of the experiment. In addition to the data shown here, this experiment was carried out for a total of 34 h, and no efflux whatsoever was observed. Exchange proceeded in a manner similar to that seen previously with growing cells; approximately 40% of the preloaded, radioactive material had been liberated after 8 h.

Induction of allantoin accumulation. Initial characterization of the allantoin accumulation system was performed with nitrogen-

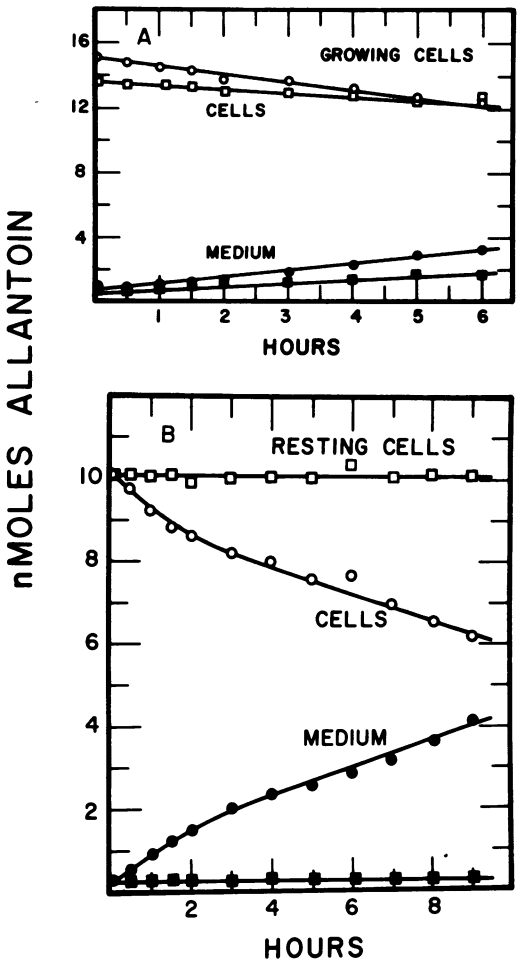

FIG. 2. *Efflux and exchange of [^{14}C]allantoin from growing (A) and resting (B) cultures of S. cerevisiae. (A) A culture of strain M 85 was grown in minimal ammonia medium containing 0.2 mM [^{14}C]allantoin (specific activity, 0.2 μCi/μmol) to a cell density of 53 Klett units. At that time, the cells were harvested by filtration and washed with excess medium devoid of radioactive allantoin. The washed cells were then resuspended either in minimal ammonia medium containing an excess (0.1% final concentration) of non-radioactive allantoin (○ and ●) or in medium devoid of non-radioactive allantoin (□ and ■). The cell density after resuspension was 13 Klett units. At the times indicated, 5-ml samples were removed from this culture and separated by filtration into cell and medium fractions. The radioactivity content of both fractions was then determined. The data are expressed as nanomoles of allantoin observed in 5 ml of cells (□ and ○) or medium (■ and ●). (B) Data shown in (B) were obtained in exactly the same manner as used for (A). Here, however, resting cells were used in place of growing cells. Here, as in (A), the cell density at the beginning of the experiment was 13 Klett units. Data are expressed as the nanomoles of [^{14}C]allantoin observed in cells (○) and medium (●) which contained excess non-radioactive allantoin and in cells (□) and medium (■) which did not contain allantoin. As in (A), the data are given for 5-ml samples.*

starved, resting cells and cells growing logarithmically in minimal glucose-ammonia medium. Comparison of data obtained from these two experimental conditions provided the first hint that allantoin uptake might be inducible. The rate of allantoin accumulation was 25- to 30-fold greater in starved cells than it was in growing cells (see Fig. 4E, 4F, 7A, and 7B of reference 22). This difference was explained in part by the greater cell density of the starved cultures. However, a significant fraction of the increased accumulation remained unaccounted for. To determine whether or not allantoin uptake was an inducible process, appropriate cultures were grown overnight in glucose-ammonia medium with and without addition of possible inducers. Strains for these experiments were selected such that neither allantoin nor the test compound could be metabolized. Because different strains exhibited some variation in inducibility, positive and negative controls were included along with each test compound. Before measuring allantoin uptake, the test compound was removed by harvesting the cells and resuspending them in prewarmed, preaerated, glucose-ammonia medium. As shown in Table 2, the rate of allantoin accumulation was 10- to 15-fold higher in cells grown in the presence of allantoin than it was in cells grown in only glucose-ammonia medium (experiments I and VI). Adenine, commercially prepared allantoic acid, and oxalurate all elicited somewhat increased rates of allantoin accumulation (experiments I, III, and IV), whereas urea was totally ineffective (experiment II).

The ability of oxalurate, adenine, and allantoate to moderately increase allantoin accumulation raised the possibilities of their being inducers or inducer analogs or being contaminated with allantoin. To evaluate these possibilities, we first assayed each preparation for material with the characteristics of allantoin (ureido group-positive material that would not bind to either Dowex-50 cation-exchange resin under acidic conditions or Dowex-1-acetate anion-exchange resin under slightly alkaline conditions). For adenine and oxalurate, no such material was found. However, commercially prepared allantoic acid contained about 17% material that behaved like allantoin. This was not suprising because allantoate is routinely prepared by heating allantoin in the presence of KOH (22). We belatedly discovered that contamination of allantoate preparations with allantoin had been reported earlier (1). In view of this finding, we

TABLE 2. *Induction of allantoin permease activity*[a]

Expt	Strain genotype	Test compound	Allantoin accumulated (nmol/40 min per ml of culture)
I	*dal1*	None	0.22
		Allantoin	2.22
		Adenine	1.04
II	*dal1 dur1*	None	0.34
		Allantoin	1.76
		Urea	0.38
III	*dal1*	None	0.27
		Allantoin	3.94
		Oxalurate	1.08
IV	*dal1 dal2*	None	0.24
		Allantoin	2.78
		Allantoate (commercial)	1.92
V	*dal1 dal2*	None	0.30
		Allantoin	2.43
		Allantoate (chromatographically purified)	1.07
VI	*dal1*	None	0.29
		Allantoin	3.75
		Hydantoin	2.36
		Hydantoic acid	0.68
		2-Thiodhydantoin	0.69
VII	*dal1*	None	0.32
		Allantoin	3.55
		Methyl hydantoin	0.95
		Hydantoin acetic acid	3.37

[a] Diploid strains with the genotypes indicated in the table were grown in minimal ammonia medium containing the test compounds at the following final concentrations: allantoin and allantoate, 0.1%; urea, 10 mM; oxalurate, 0.5 mM; adenine, 0.15%; and all others, 1 mM. On reaching a cell density of 50 Klett units, a sample of each culture was harvested by filtration, washed, and resuspended in an identical amount of prewarmed, preaerated minimal medium devoid of the test compound. After a brief equilibration period at 30°C, [^{14}C]-allantoin (0.1 mM final concentraton; specific activity, 0.2 μCi/μmol) was added. From this point forward, the samples were processed as described for the standard assay in the text. Data are expressed as nanomoles of allantoin accumulated per 40 min per milliliter of the original culture (that at 50 Klett units).

purified allantoate, by the methods described above. As shown in experiment V of Table 2, use of chromatographically purified allantoate resulted in a markedly diminished response.

Because non-metabolizable inducers can often be used to great advantage, a search was mounted for a compound that would effectively perform this function for the allantoin permease. A number of allantoin analogs were tested, and, as shown in experiments VI and VII of Table 2, hydantoin and hydantoin acetic acid induced allantoin accumulation to 63 and 95%, respectively, of the rates observed by preincubating cells overnight with allantoin. These analogs did not contain material with the characteristics of allantoin and were totally unable to serve as sole nitrogen sources.

To test the effects of hydantoin and hydantoin acetic acid on production of the other pathway components, allophanate hydrolase activity was measured after incubation of the cells in the presence and absence of both analogs overnight. Neither of these compounds had any effect on the amounts of hydrolase synthesized.

Induction of several enzymes associated with arginine or allantoin degradation has been shown to require a functional *rna1* gene product (2–4). Strains carrying mutations within this locus have been suggested to be defective in transport of RNA from the nucleus to the cytoplasm. However, identification of the precise biochemical function that has been lost in these strains is lacking, and the above suggestion remains somewhat open to question (2, 3). To ascertain whether or not the *rna1* gene product was required for production of the allantoin transport system, cultures of *rna1* mutant (M674) and wild-type (M85) strains of *cerevisiae* were grown to a cell density of 30 Klett units at 22°C. The cultures were then transferred to medium maintained at 36°C and incubated for 10 min. Finally, [^{14}C]allantoin was added to each culture, and its accumulation was assayed. As shown in Fig. 3,

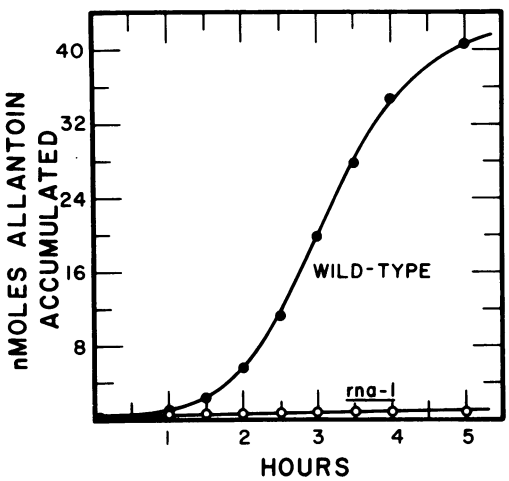

FIG. 3. *Production of the allantoin transport system in wild-type and rna1 mutant strains of S. cerevisiae incubated at 36°C. Cultures of strains M85 and M674 were grown at 22°C in minimal ammonia medium to cell densities of 30 Klett units each. At this time, they were harvested by filtration and resuspended in the same medium maintained at 36°C. After 10 min of equilibration at this temperature, [^{14}C]allantoin (0.2 mM final concentration) was added to each culture. At this concentration, radioactive allantoin used for the accumulation assay will bring about induction of the system. At the times indicated in the figure, 5-ml samples were removed from each culture and processed as described in the text. The data are expressed as the nanomoles of [^{14}C]allantoin accumulated per 5 ml of culture for the times indicated.*

loss of the *rna1* gene product resulted in drastically reduced ability of the cells to accumulate allantoin.

Inhibition of allantoin uptake. To test the sensitivity of allantoin accumulation to nitrogen repression, cultures of *S. cerevisiae* were grown in minimal glucose medium provided with various nitrogen sources. As shown in Table 3, accumulation was greatest with proline, followed closely by ammonia. When serine, aspartate, or asparagine was provided, negligible accumulation was observed. Repression of this magnitude by aspartate had not been previously observed for the allantoin pathway components and suggested that two levels of inhibition might be involved: repression at the level of gene expression and transinhibition at the level of transport protein operation. To test this hypothesis, a culture of strain M85 was permitted to accumulate [^{14}C]allantoin for 30 min. At that time it was divided into two portions; one received no further additions, whereas asparagine (0.1% final concentration) was added to the second. As shown in Fig. 4, uptake ceased immediately on addition of the amino acid. Because transport activity already present before addition of asparagine no longer functioned, transinhibition of allantoin uptake was clearly evident.

Another means of eliciting the transinhibition phenomenon is by inhibiting protein synthesis (11). Therefore, we measured allantoin accumulation in the presence of protein synthesis inhibitors. As shown in Fig. 5A, allantoin accumulation proceeded for only a short time after addition of either cycloheximide or trichodermin. Inhibition of protein synthesis would be expected to result in an accumulation of intracellular amino acids. They in turn might be hypothesized to prevent allantoin uptake. To evaluate this possibility, the above experiment was repeated with cells that had been starved before addition of inhibitor. Starvation would be expected to deplete any intracellular amino acid reserves, and, hence, subsequent inhibition of protein synthesis would not be expected to result in accumulation of amino acids. As shown in Fig. 5B, allantoin accumulation was the same whether or not trichodermin was added to a culture that had been starved for nitrogen overnight before addition of inhibitor.

TABLE 3. *Uninduced levels of allantoin uptake in cells using various nitrogen sources*[a]

Nitrogen source	Allantoin accumulated (nmol/40 min per ml of culture)
Proline	2.98
Ammonia	2.46
Aspartate	0.28
Serine	0.28
Asparagine	0.27

[a] Strain M85 was grown in minimal medium containing one of the indicated nitrogen sources (0.1% final concentraton) to a cell density of 50 Klett units. At that time, 8.5 ml was removed and directly assayed as described in the text. Incubation time of the assay was 40 min. Data are expressed as nanomoles of allantoin accumulated per 40 min per milliliter of culture at 50 Klett units.

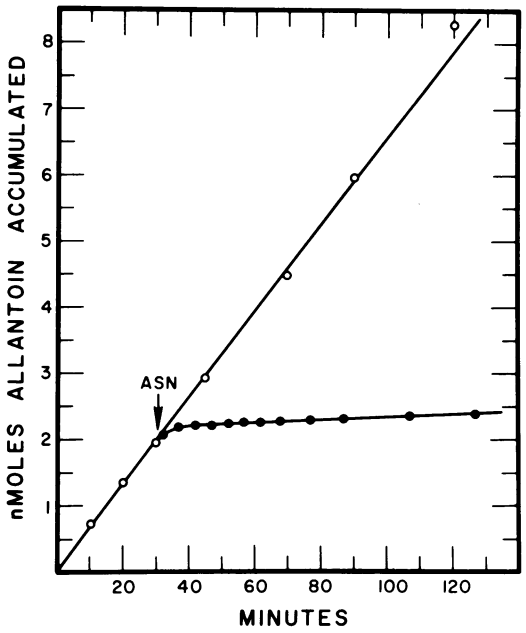

FIG. 4. *Inhibition of allantoin accumulation by asparagine (ASN). A culture of strain M85 was grown to a cell density of 30 Klett units in minimal ammonia medium. At that time [^{14}C]allantoin was added to a final concentration of 0.9 mM. Thereafter, samples were removed for 31 min as indicated in the figure. At this time, the culture was divided into two portions.* L-*Asparagine (0.1% final concentration) was added to one portion, and no further additions were made to the other. Sampling was continued as before at the times indicated in the figure. All samples were processed as described in the text. Data are expressed as nanomoles of radioactive allantoin accumulated per milliliter of culture for the times indicated in the figure.*

Failure of cells to accumulate allantoin after addition of asparagine or cycloheximide to the medium could result from either loss of ability to transport allantoin into the cell or acquisition of an increased rate of allantoin efflux from the cell. To distinguish between these alternatives, a culture was preloaded with radioactive allantoin and transferred to fresh, prewarmed, preaerated medium devoid of allantoin. After addition of asparagine to this culture, the radioactivity content of both cells and medium was measured as a function of time. As shown in Fig. 6, the rate

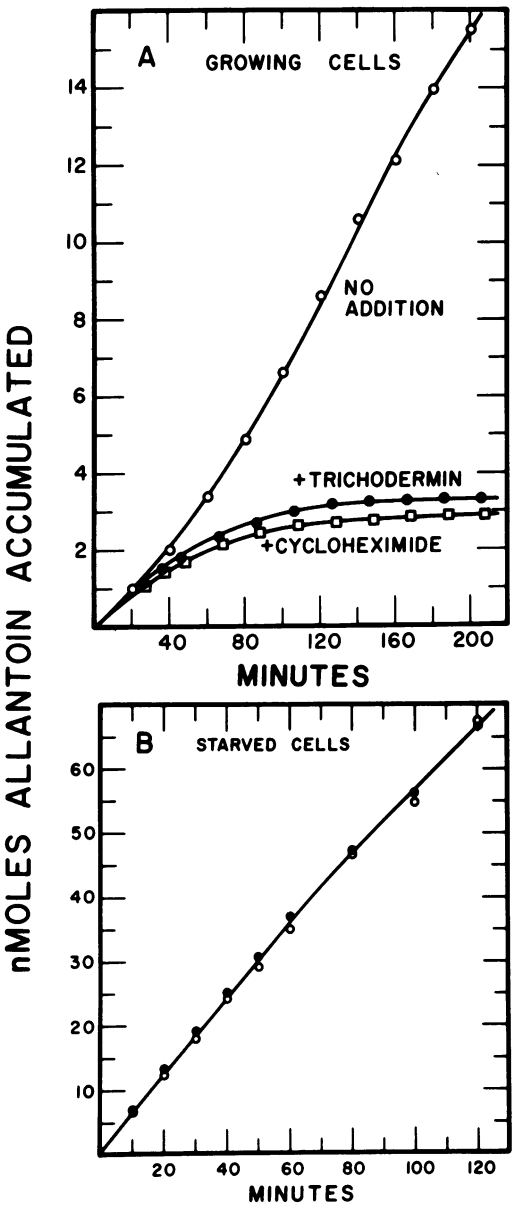

FIG. 5. *Effect of protein synthesis inhibitors on accumulation of allantoin in growing and starved cultures of S. cerevisiae. (A) A culture of strain M85 was grown overnight in minimal ammonia medium containing 1 mM allantoin. At a cell density of 45 Klett units, the cells were harvested, washed with prewarmed, preaerated minimal ammonia medium devoid of allantoin, and resuspended in this medium at the original 45-Klett unit cell density. At this time [^{14}C]allantoin (0.1 mM final concentration; specific activity, 0.2 μCi/μmol) was added. This is zero time in the figure. The culture was then divided into three portions. Trichodermin (30 μg/ml final concentration), cycloheximide (100 μg/ml final concentration) and no additions were made to the three portions respectively. At the times indicated 1-ml samples were removed from each culture and processed for accumulation of allantoin as described in the text. Data are expressed as nanomoles of radioactive allantoin accumulated per milliliter of culture. (B) Data were obtained in a manner identical to that described for (A). Here, however, a resting cell culture was used; the cell density at the beginning of the experiment (zero time) was 155 Klett units. One portion of the culture received trichodermin at a final concentration of 20 μg/ml (○), whereas the remaining portion received no further additions (●).*

of allantoin loss from the cells was significantly higher in cultures treated with asparagine. Although this experimental result seems to suggest that asparagine stimulated allantoin efflux, it is equally consistent with suggesting that efflux was the same in the presence or absence of asparagine, but recapture of [^{14}C]allantoin lost via efflux was not possible in cells treated with asparagine. To distinguish between these two explanations, we repeated the above experiment

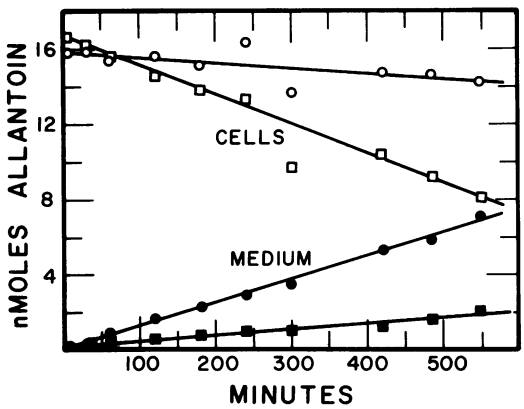

FIG. 6. *Effect of asparagine on the release of preloaded [^{14}C]allantoin from S. cerevisiae. A culture of strain M85 was grown overnight to a cell density of 53 Klett units in minimal ammonia medium containing 0.2 mM [^{14}C]allantoin. At that time, the cells were harvested by filtration, washed thoroughly with prewarmed, preaerated medium devoid of radioactive allantoin and resuspended in the same medium to a cell density of 13 Klett units. The culture was then divided into two portions and asparagine (0.1% final concentration) was added to one portion (□ and ●). At the times indicated, samples were removed from each culture and divided into the cell (□ and ○) and medium (● and ■) fractions. The amount of radioactivity contained in each fraction was then determined. Cells and medium that were incubated in the absence of asparagine are represented as open circles (○) and closed squares (■), respectively. Data are expressed as nanomoles of radioactive allantoin observed in 5 ml of cells or medium. The data may be compared with those shown in Table 4.*

with two additional cultures being added. Asparagine and excess non-radioactive allantoin were added to one of these cultures, whereas only excess non-radioactive allantoin was added to the other. In cultures containing non-radioactive allantoin, the specific activity of any radioactive allantoin leaving the cells would be enormously diminished. In formal terms, this is equivalent to eliminating recapture of allantoin lost from cells via efflux. As shown in Table 4, [^{14}C]allantoin was lost from cells about 50% faster when asparagine was present than when it was absent.

Some insight into possible mechanisms of transport function and inhibition can be obtained at times from the kinetic characteristics of the inhibitors and substrate analogs. We, therefore, measured the effects of several allantoin analogues and asparagine on the K_m and V_{max} of the allantoin uptake system. As shown in Table 5, ureidoglycolate, oxalurate, urea, and allophanate had only a marginal effect on the apparent Michaelis constant for allantoin or the V_{max} of allantoin uptake. Hydantoin and hydantoin acetic acid modestly increased the apparent K_m of the transport system for allantoin, whereas the presence of commercially prepared allantoate resulted in a drastic 60-fold increase. When contaminating allantoin was removed from the allantoate preparation, the apparent K_m increased by only threefold. None of the above metabolites or analogs caused a significant alteration of the system's V_{max}, i.e., inhibition was competitive in each case. Asparagine, on the other hand, did not alter the K_m of the system for allantoin, but strikingly altered the V_{max} of the uptake system. The degree of change in V_{max} was a function of asparagine concentration. At 1 mM asparagine, the transport system was totally inactive, whereas at 0.05 mM its activity decreased by 50% (Table 5).

TABLE 4. *Loss of preloaded [^{14}C]allantoin from S. cerevisiae*[a]

Medium supplements	[^{14}C]allantoin released (nmol)
None	2.0
Allantoin	5.2
Allantoin and asparagine	7.4
Asparagine	7.4

[a] A culture of strain M85 was permitted to accumulate [^{14}C]allantoin (0.2 mM final concentration; specific activity, 0.2 μCi/μmol) overnight. When the culture reached a density of 50 Klett units, a 50-ml sample of the culture was harvested and washed with three volumes of prewarmed, preaerated medium devoid of radioactive allantoin. Washed cells were resuspended in minimal medium containing the supplements indicated; all supplements were provided at final concentrations of 0.1%. The cell density at this time was 13 Klett units. Thereafter, 2-ml samples were removed from these cultures and transferred to a filter apparatus (Millipore Corp.). The cells and medium were separated by filtration, and the radioactivity content in the medium was determined. The data are expressed as nanomoles of allantoin released in 9 h per 5 ml of culture that was provided with the supplements. The data were derived by taking the best-fit line through the nine data points collected for each experimental condition and may be directly compared with the data in Fig. 6.

TABLE 5. *Apparent K_m values of allantoin permease for allantoin in the presence of various allantoin analogs and metabolites*[a]

Inhibitor added	Apparent K_m (μM)	V_{max} (nmol accumulated per 20 min)
None	13.2	17.0
Ureidoglycolate	10.0	17.0
Oxalurate	15.9	14.7
Urea	11.3	16.4
Allophanate	10.0	19.0
Allantoate (commercial)	795	17.0
Allantoate (chromatographically purified)	42.6	12.5
Hydantoin	21.5	17.0
Hydantoin acetic acid	20.0	18.6
Asparagine (0.05 mM)	13.2	9.3

[a] Data presented in this table were obtained from experiments that were performed as described in the legend to Fig. 5 of reference 23. Assays were performed with resting cells that were incubated in the presence or absence of the test compound (final concentration of the test compounds were 1 mM in all cases except asparagine, which was 0.05 mM).

As shown in Fig. 7, inhibition of allantoin accumulation resulting from addition of asparagine was freely reversible. However, 30 min elapsed between removal of asparagine from the culture medium and the onset of allantoin accumulation. Transfer of cells previously incubated with asparagine to fresh medium devoid of the amino acid but containing cycloheximide resulted in their failure to recover allantoin-accumulating ability.

DISCUSSION

The work reported here has provided information concerning production, operation, and inhibition of the allantoin active transport system in *S. cerevisiae*. It appears that production of this system is inducible and is regulated quite differently from other allantoin-degradative pathway components. Production of all components studied so far is dependent on allophanic acid, the last intermediate of the pathway. Allantoin uptake, on the other hand, increased significantly only when allantoin was provided

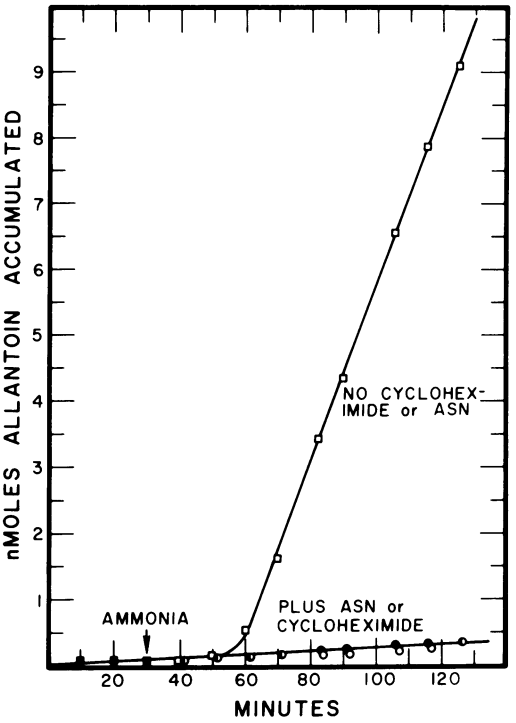

FIG. 7. *Effect of cycloheximide and asparagine on allantoin uptake activity after transfer of cells from a medium containing asparagine as a nitrogen source to one containing ammonia as sole nitrogen source. A culture of strain M85 was grown in minimal asparagine medium to a cell density of 30 Klett units. At that time (zero time in the figure) [^{14}C]allantoin (0.9 mM, final concentration) was added to the culture, and it was sampled for assay of allantoin accumulation. After removing three samples, the remaining portion of the culture was harvested by filtration, washed with prewarmed, preaerated medium devoid of asparagine, and resuspended in an identical volume of fresh minimal ammonia medium containing 0.9 mM [^{14}C]allantoin. This is indicated with an arrow in the figure. Thereafter, the culture was divided into three portions: one received no additions (□), a second received asparagine at a final concentration of 0.1% (●), and the remaining portion received cycloheximide at a concentration of 10 μgm/ml (○). After these additions were made, the culture was sampled at the times indicated and the samples were processed as described in the text. Data are expressed as nanomoles of radioactive allantoin accumulated per milliliter of culture.*

in the culture medium. Previous studies provided rigorous evidence that allantoin accumulated by cells was not altered chemically (22). Preincubation with allantoate also elicited increased allantoin uptake, but subsequent experiments demonstrated this effect, and allantoate's effectiveness as a competitive inhibitor of allantoin transport resulted from its being contaminated with substantial amounts of allantoin. The ability of the allantoin analogs, hydantoin and hydantoin acetic acid, to serve as inducers of allantoin transport activity is also consistent with the suggestion that allantoin is the native inducer. If the degree of induction is correlated with the chemical structures of inducing compounds (see Table 2 and Fig. 8), it appears that both the ring and ureido group side chain are needed for full activity.

Although it would be desirable to designate hydantoin and hydantoin acetic acid as nonmetabolizable or gratuitous inducers, this is not possible at present because only two of the three gratuitous inducer characteristics have been rigorously demonstrated. Both compounds brought about significant increases in the rate of allantoin transport and neither was at all capable of supporting growth of wild-type strains. However, we do not have radioactive preparations of these compounds and, hence, cannot certify that they were not modified in some way once they entered the cell. The latter possibility is felt to be remote because almost any alteration, other than addition of a neutral group to the ring, would have either modified the compound in a way that we could detect or converted it to a form where the ureido nitrogen atoms were available to serve as a nitrogen source. Another way these two compounds might have conceivably served as inducers was by displacing the large amounts of allantoin sequestered in the cell vacuole (31). This, too, is considered unlikely, because if induction occurred by this mechanism, allantoin displaced from the vacuole would be degraded to allophanate, which in turn would have brought about induction of the other pathway enzymes. This, however, is incompatible with our experimental observations.

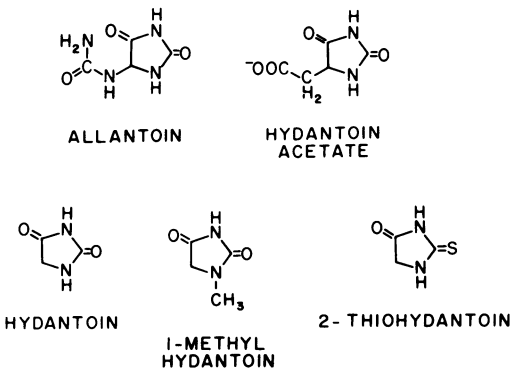

FIG. 8. *Chemical structures of allantoin analogs that were tested for their ability to bring about induction of the allantoin transport system.*

Identification of allantoin as inducer of its own transport and finding that cells normally contain significant levels of sequestered allantoin explains a peculiarity in experiments that we reported earlier (22). In those studies we observed that starved cells possessed much greater abilities to accumulate allantoin than growing cells. In part, this discrepancy could be explained by a greater cell density and possible release from nitrogen repression of the starved cells. Unfortunately, a significant portion of the increase in allantoin uptake remained unexplained. If we assume that sequestered allantoin was released on starvation, it is reasonable to suggest that the unaccounted increase in transport activity resulted from induction. We will report experiments elsewhere (Sumrada and Copper, manuscript in preparation) demonstrating that sequestered allantoin becomes metabolically available when cells are starved for nitrogen and a variety of other metabolites.

Although an increased rate of allantoin accumulation might result from induction, it might alternatively result from activation of the transport protein. One means of distinguishing these possibilities was to perform an induction experiment in the presence of protein or RNA synthesis inhibitors. Use of protein synthesis inhibitors was precluded because they caused transinhibition, as discussed later. Our past work has shown that the *rna1* gene product functions temporally midway between the conclusion of transcription and initiation of protein synthesis (2, 3). We have also shown that using strains with a conditionally defective *rna1* gene product is the most reliable means of interrupting mRNA metabolism (2, 3; T. G. Cooper, G. Marcelli, and R. Sumrada, Biochim. Biophys. Acta, in press). In view of these considerations, the requirement of a functional *rna1* gene product for allantoin transport induction (Fig. 3) is suggestive that induction likely involves gene expression rather than protein activation. A potential criticism can be made of this argument; interruption of mRNA metabolism will eventually result in loss of protein synthesis. Simultaneously, amino acids would accumulate, causing transinhibition of allantoin transport. Although such an argument might be valid for times in excess of 2 or 3 h, it is difficult to seriously entertain it before that time because proteins are still being synthesized with pre-existing mRNA as template (13; Cooper et al., Biochim. Biophys. Acta, in press).

Inducers of allantoin transport have minimal or no effect on induction of other allantoin pathway components. Conversely, allophanate induces these components and has no effect on allantoin transport induction. This, coupled with our past studies, permits a more complete picture of allantoin metabolism regulation. Allantoin must be considered as derived from two sources: internal and external. During periods of metabolite limitation, allantoin is made available presumably from the vacuole (31). Here, we have shown that it does not readily leave the cell. The high basal levels of allantoin-degrading enzymes degrade it to urea. Allantoin provided in the medium can enter the cell by way of basal allantoin transport activity normally present. Once inside, this allantoin can bring the allantoin transport system to full capacity via induction. Superimposed on this level of regulation is nitrogen repression and transinhibition, which likely override signals to increase allantoin transport if a more readily used nitrogen source is available.

Modest induction of allantoin transport was observed with adenine, allantoate, and oxalurate. The physiological significance of these effects is not at all clear. Adenine might be considered a remote analog of allantoin, as might oxalurate and allantoate. Alternatively, some fraction of the adenine provided exogenously might be degraded to allantoin. Purine degradation is the only known source of allantoin, and adenine is an effective inducer only when provided at high concentration. A similar argument cannot be made for oxalurate and allantoate.

This report clarifies two apparent paradoxical characteristics of allantoin transport. In previous work we concluded that accumulation of allantoin never reached a time-dependent plateau, a situation also seen in at least one other system (12). As shown here, accumulation slowly plateaued in 14 to 24 h if cell division was inhibited, but did not plateau during logarithmic growth. These observations suggest that cells did not accumulate sufficient cytoplasmic allantoin during the course of one generation to impede further uptake. If, however, cell division was inhibited, cytoplasmic allantoin had a chance to attain levels sufficient to prevent further accumulation. There are at least two potential reasons for such behavior: (i) the rate of allantoin uptake is intrinsically slow or (ii) allantoin initially reaching the cytoplasm was efficiently sequestered in the vacuole or other cellular organelle, preventing its cytoplasmic buildup. This conclusion is consistent with our observation that most [^{14}C]allantoin provided exogenously is sequestered in compartments other than the cytoplasm (31). These considerations raise a serious problem concerning a precise understanding of allantoin transport. If, for the sake of argument, allantoin transport into the cell was considered to be fast and freely reversible as found for urea uptake (9), then many of the

characteristics attributed to allantoin transport would in reality be characteristics of allantoin transport into the sequestering organelle. Although we do not feel that this is likely, it does point to the fact that allantoin participates in two sequential transport reactions: allantoin (external) $\rightleftarrows$ Allantoin (cytoplasmic) $\rightleftarrows$ Allantoin (sequestered), and the need for future mutational separation and study of these systems. These problems are not specific to allantoin transport because transport and compartmentation characteristics of several amino acids appear to be similar (24, 25, 29).

A second paradox explained here is the apparent unidirectional transport of allantoin. Allantoin uptake in *S. cerevisiae* is not rigorously unidirectional; efflux is merely much slower than influx, which accounts for its being missed earlier (22). It is probable that this is also the case for reported examples of unidirectional amino acid transport in yeast.

An interesting relationship seems to exist between efflux and apparent exchange in growing and resting cells. We have operationally used the words "efflux" and "exchange" to denote loss of radioactive allantoin from preloaded cells into medium either devoid of or containing excess non-radioactive allantoin, respectively. Used in this way, both growing and resting cells were able to carry out exchange, whereas only growing cells were capable of efflux. The reason for this difference is not clear, but further study may require clearer definitions of efflux and exchange. Concern for our operational definition of exchange derives from the fact that it may cover at least two very different molecular processes. For example, it is reasonable to suggest that the allantoin "carrier" can be moved from an external to an internal position only when allantoin is bound to it. Because efflux would move the carrier from an internal to an external position, exogenously provided allantoin would be required for efflux of internal allantoin. Here, the process is exchange as designated. Alternatively, restrictions in carrier movement may not be operative. Allantoin lost from the cell into medium devoid of non-radioactive allantoin would be subject to recapture. If, however, non-radioactive allantoin were present in the medium, chances of recapturing a radioactive allantoin molecule would be very small. Here, what might be termed exchange would in fact be prevention of radioactive metabolite recapture. In our experiments there is no easy way of distinguishing these possibilities, so an ambiguity in the interpretation of these results must remain.

It is likely that production of the allantoin transport system is subject to nitrogen repression as are all other components of the allantoin-degradative system (5), but the data presented here are only suggestive. The increased rate of allantoin uptake observed when proline was provided as sole nitrogen source compared with that seen with ammonia is characteristic of nitrogen repression. This argument, however, cannot be extended to the amino acids aspartate, serine, and asparagine, because they also clearly cause transinhibition of allantoin transport.

Little is known about the mechanism of transinhibition; operationally, the term has been used to denote both inhibition of metabolite transport by that same metabolite (10) or a different one (12, 20). For example, it has been claimed that accumulation of histidine prevented its continued accumulation. This was not seen when amino acids other than histidine were preloaded in its place. Allantoin accumulation, on the other hand, was prevented by asparagine, aspartic acid (Fig. 4 and Table 3) and lysine (Zacharski and Cooper, unpublished data). In the past, both of the above situations have been designated as transinhibition and felt to be of a regulatory nature. It is reasonable to ask whether or not similar events produced these two observations. High intracellular concentrations of histidine can be easily visualized to prevent further uptake either by increasing histidine efflux or perhaps by occupying the histidine carrier, a molecule that might be envisioned to return to the external surface of the plasma membrane only when it is unoccupied. However, it is conceptually more difficult to see how amino acids that are structurally unrelated to allantoin would be capable of inhibiting its uptake by a similar mechanism. It is also reasonable to question whether or not transinhibition represents a useful and specific regulation of allantoin metabolism. Lysine and asparagine were both able to equally transinhibit allantoin transport, whereas proline was ineffective. It is useful to keep in mind that lysine is totally ineffective as a nitrogen source, whereas proline and asparagine are poor and good nitrogen sources, respectively. It might well be advisable to term the phenomenon reported for histidine-dependent loss of histidine uptake, "feedback inhibition" and the amino acid-dependent inhibition of allantoin uptake, transinhibition, because it is likely that they operate by two different mechanisms. Both types of inhibition are observed with allantoin uptake, but feedback inhibition was only seen when cell division was prevented.

Roon and his collaborators have suggested that transinhibition results from a reduction of the plasma membrane adenosine triphosphatase-generated proton-motive force when both methylamine and an amino acid are being trans-

ported simultaneously compared with the transport of either compound alone. This suggestion, in its present form, may be somewhat inconsistent with data shown in Fig. 5A which demonstrate that protein synthesis inhibition resulted in severe inhibition of allantoin uptake in normal unstarved cultures but not in starved cultures. Here, materials already in the cell appear to have caused inhibition of transport. This effect has also been reported by others (11). Roon's conclusion also does not easily explain our observation that treatment of cells with asparagine, an agent shown to cause transinhibition, increases efflux of allantoin from preloaded cells whether or not excess non-radioactive allantoin was present in the medium along with the amino acid. Although the degree of efflux increase was certainly insufficient to totally prevent net accumulation of allantoin after addition of asparagine, it is an observation that must be conceptually accounted for in explanations of this phenomenon. It could be argued that asparagine-mediated transinhibition resulted in displacement of allantoin from its sequestered location (presumably the cell vacuole) by asparagine. This argument, however, is not supported by the data shown in Fig. 5. Allantoin uptake was inhibited only by addition of cycloheximide to unstarved cells. We will present evidence elsewhere (submitted for publication) demonstrating that allantoin is released from sequestration both by starvation and cycloheximide treatment. Because the present experiments were carried out in allantoinase-minus strains, it is reasonable to assume that allantoin was present in the cytoplasm of starved and unstarved cells. Yet allantoin uptake was inhibited only under conditions in which intracellular amino acids have been shown to accumulate. Therefore, simple displacement of allantoin by amino acids is not likely to account for its inhibition of uptake.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM-19386 and GM-20693 from the National Institute of General Medical Sciences. T.G.C. was supported by Public Health Service Research Career Development Award K04-GM-00091, also from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Ammann, E. C. B., and V. H. Lynch.** 1964. Purine metabolism of unicellular algae. I. Chromatographic detection of some purines, pyrimidines, and imidazoles by their mercuric complexes. Anal. Biochem. **7**:387–392.
2. **Bossinger, J., and T. G. Cooper.** 1976. Sequence of molecular events involved in induction of allophanate hydrolase. J. Bacteriol. **126**:198–204.
3. **Bossinger, J., and T. G. Cooper.** 1976. Execution times of macromolecular synthetic processes involved in the induction of allophanate hydrolase at 15°C. J. Bacteriol. **128**:498–501.
4. **Bossinger, J., and T. G. Cooper.** 1977. Molecular events associated with induction of arginase in *Saccharomyces cerevisiae*. J. Bacteriol. **131**:163–173.
5. **Bossinger, J., R. P. Lawther, and T. G. Cooper.** 1974. Nitrogen repression of the allantoin degradative enzymes in *Saccharomyces cerevisiae*. J. Bacteriol. **118**:821–829.
6. **Chevallier, M. R., R. Jund, and F. Lacroute.** 1975. Characterization of cytosine permeation in *Saccharomyces cerevisiae*. J. Bacteriol. **122**:629–641.
7. **Cooper, T. G., and H. Beevers.** 1969. Mitochondria and glyoxysomes from castor bean endosperm. Enzyme constituents and catalytic capacity. J. Biol. Chem. **244**:3507–3513.
8. **Cooper, T. G., and R. P. Lawther.** 1973. Induction of the allantoin degradative enzymes in *Saccharomyces cerevisiae* by the last intermediate of the pathway. Proc. Natl. Acad. Sci. U.S.A. **70**:2340–2344.
9. **Cooper, T. G., and R. Sumrada.** 1975. Urea transport in Saccharomyces cerevisiae. J. Bacteriol. **121**:571–576.
10. **Crabeel, M., and M. Grenson.** 1970. Regulation of histidine uptake by specific feedback inhibition of two histidine permeases in *Saccharomyces cerevisiae*. Eur. J. Biochem. **14**:197–204.
11. **Greth, M. L., M. R. Chevallier, and F. Lacroute.** 1977. Ureidosuccinic acid permeation in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **465**:138–151.
12. **Kotyk, A., and L. Rihova.** 1972. Transport of α-aminoisobutyric acid in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. **288**:380–389.
13. **Lawther, R. P., S. L. Phillips, and T. G. Cooper.** 1975. Lomofungin inhibition of allophanate hydrolase synthesis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **137**:89–99.
14. **Lawther, R. P., E. Riemer, B. Chojnacki, and T. G. Cooper.** 1974. Clustering of the genes for allantoin degradation in *Saccharomyces cerevisiae*. J. Bacteriol. **119**:461–468.
15. **Macris, B. J.** 1975. Mechanism of benzoic acid uptake by *Saccharomyces cerevisiae*. Appl. Microbiol. **30**:503–506.
16. **Morlion, M., and A. Domnas.** 1962. Uptake and use of allantoin and allantoic acids by yeasts. Natuurwet Tijdschr. (Ghent) **44**:100–121.
17. **Pall, M. L., and K. A. Kelly.** 1971. Specificity of transinhibition of amino acid transport in *Neurospora*. Biochem. Biophys. Res. Commun. **42**:940–947.
18. **Pateman, J. A., J. R. Kinghorn, and E. Dunn.** 1974. Regulatory aspects of L-glutamate transport in *Aspergillus nidulans*. J. Bacteriol. **119**:534–542.
19. **Roon, R. J., H. L. Even, P. Dulop, and F. L. Larimore.** 1975. Methylamine and ammonia transport in *Saccharomyces cerevisiae*. J. Bacteriol. **122**:502–509.
20. **Roon, R. J., J. S. Levy, and F. Larimore.** 1977. Negative interactions between amino acid and methylamine/ammonia transport systems of *Saccharomyces cerevisiae*. J. Biol. Chem. **252**:3599–3604.
21. **Sumrada, R., and T. G. Cooper.** 1974. Oxaluric acid: a non-metabolizable inducer of the allantoin degradative enzymes in *Saccharomyces cerevisiae*. J. Bacteriol. **117**:1240–1247.
22. **Sumrada, R., and T. G. Cooper.** 1977. Allantoin transport in *Saccharomyces cerevisiae*. J. Bacteriol. **131**:839–847.
23. **Sumrada, R., M. Gorski, and T. G. Cooper.** 1976. Urea transport-defective strains of *Saccharomyces cerevisiae*. J. Bacteriol **125**:1048–1056.
24. **Svihla, G., J. L. Dainko, and F. Schlenk.** 1963. Ultraviolet microscopy of purine compounds in the yeast vacuole. J. Bacteriol. **85**:399–402.
25. **Svihla, G., and F. Schlenk.** 1960. *S*-adenosylmethionine in the vacuole of *Candida utilis*. J. Bacteriol. **79**:841–848.
26. **Whitney, P. A., and T. G. Cooper.** 1970. Requirement

for HCO_3^- by ATP:urea amido-lyase in yeast. Biochem. Biophys. Res. Commun. **40**:814–819.

27. **Whitney, P. A., T. G. Cooper, and B. Magasanik.** 1973. The induction of urea carboxylase and allophanate hydrolase in *Saccharomyces cerevisiae*. J. Biol. Chem. **248**:6203–6209.
28. **Wickerham, L. J.** 1946. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. J. Bacteriol. **52**:293–301.
29. **Wiemken, A., and P. Nurse.** 1973. Isolation and characterization of the amino-acid pools located within the cytoplasm and vacuoles of *Candida utilis*. Planta **109**:293–306.
30. **Woodward, J. R., and V. P. Cirillo.** 1977. Amino acid transport and metabolism in nitrogen-starved cells of *Saccharomyces cerevisiae*. J. Bacteriol. **130**:714–723.
31. **Zacharski, C. A., and T. G. Cooper.** 1978. Metabolite compartmentation in *Saccharomyces cerevisiae*. J. Bacteriol. **135**:490–497.