JOURNAL OF BACTERIOLOGY, Sept. 1979, p. 917-923
0021-9193/79/09-0917/07$02.00/0

Vol. 139, No. 3

Oxalurate Transport in *Saccharomyces cerevisiae*

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Received for publication 19 April 1979

Oxalurate, the gratuitous inducer of the allantoin degradative enzymes, was taken into the cell by an energy-dependent active transport system with an apparent K_m of 1.2 mM. Efflux of previously accumulated oxalurate was rapid, with a half-life of about 2 min. The oxalurate uptake system appears to be both constitutively produced and insensitive to nitrogen catabolite repression. The latter observations suggest that failure of oxalurate to bring about induction of allophanate hydrolase in cultures growing under repressive conditions does not result from inducer exclusion, but rather from repression of *dur1,2* gene expression.

Saccharomyces cerevisiae can utilize allantoin as sole nitrogen source by degrading it in five steps to ammonia, carbon dioxide, and glyoxylate (9, 14-16; T. G. Cooper, M. Gorski, and V. Turoscy, Genetics, in press). The enzymes catalyzing these degradative reactions are all inducible, and allophanic acid, the last pathway intermediate, has been shown to be the native inducer (7, 8, 16). Sumrada and Cooper reported that the allophanate analog oxalurate (OXLU) could bring about high levels of induction and was not apparently metabolized in any way by the cell (11). Subsequently Bossinger et al. (4) used this compound in studies of nitrogen catabolite repression. They concluded that decreases of the allantoin degradative enzymes observed in cultures growing under repressive conditions probably resulted from a loss of synthetic capacity.

The latter two studies were hampered by the unavailability of radioactive OXLU. In the first case we could not unequivocally exclude the possibility that a small amount of OXLU was modified after entry into the cell, because we were using low-resolution colorimetric assays to detect any changes that might have occurred. In the second instance questions could be raised concerning our interpretation of the repression experiments. It was possible that the inability of cells growing in rich medium to induce the allantoin degradative enzymes was the result of inducer exclusion. Although we showed this interpretation of our work to be unlikely by using 10 mM urea as inducer of the allantoin system (1) ([^{14}C]urea uptake via the facilitated diffusion system was directly shown to be insensitive to nitrogen repression), we could not effectively answer the question when OXLU was used as an inducer. In addition, the future use of OXLU as a highly effective means of inducing the allantoin pathway enzymes required that we have some understanding of its mode of entry into the cell and the effects of various physiological conditions on the rate of entry.

In view of these considerations, we synthesized radioactive OXLU and used it to study the characteristics of OXLU transport into the cell. We also used these preparations to more rigorously verify that OXLU is not altered in any way after its uptake into the cell.

A preliminary account of this work has already appeared (Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, K12, p. 147).

MATERIALS AND METHODS

Strains. All strains used in this work were prototrophic diploid organisms. Strain M25 is our standard wild type and was prepared as described earlier (14). Strain M62 lacks urea carboxylase [urea:CO_2 ligase (ADP)(6.3.4.6)] activity (*dur1*) and is hence unable to degrade urea to ammonia and carbon dioxide.

Culture conditions. The medium used throughout these experiments was that of Wickerham (17). Glucose (0.6%) and ammonium sulfate (0.1%) were used as sole sources of carbon and nitrogen unless otherwise indicated. Cell density measurements were routinely made with a Klett-Summerson colorimeter (500- to 570-mm band-pass filter). One hundred Klett units is equivalent to about 3×10^7 cells per ml.

Transfer of cells from one medium to another. In several experiments we had to transfer cells from one medium to another. This was done by filtering the culture through membrane filters and suspending the harvested cells in fresh medium. All filtrations were performed with nitrocellulose filters (0.45-μm pore diameter; Millipore Corp.) and were completed in less than 15 to 20 s. The extent of cell loss during this procedure was determined with radioactively labeled cells and was found to be negligible (2, 3).

Assay of OXLU uptake. A culture of the strain to

be assayed was grown to a cell density of 60 Klett units at 30°C. At that time (designated zero time in the figures), a portion of the culture was added to a prewarmed (30°C) flask containing sufficient [^{14}C]OXLU to yield a final concentration of 2 mM. [^{14}C]OXLU was dissolved in dimethyl sulfoxide before being placed in the flask such that the final concentration of dimethyl sulfoxide in the culture never exceeded 1%. Control experiments demonstrated that this amount of solvent neither perturbed OXLU uptake nor adversely affected cell growth. Samples (2.0 ml) were taken from the test culture at 2-min intervals and transferred to a Millipore filter apparatus where the cells were collected. The harvested cells were quickly washed three times with 4 ml of cold wash solution. This solution contained all of the components of our standard minimal medium except ammonia and glucose; non-radioactive OXLU (1 mM) was also present; the same values were observed whether or not non-radioactive OXLU was present in the wash medium. The temperature of the wash solution did not appear to be significant since the same values of accumulation were observed when cells were washed with solutions maintained at 4 and 30°C. Washed filters were placed in 5 ml of aqueous scintillation fluid, and their radioactivity content was measured after 18 to 24 h of incubation at room temperature. Failure to do this resulted in unevenly quenched samples and loss of assay precision. In all cases where uptake rates were determined, assays were conducted for less than 10 min. Since this represents less than 7% of the cell doubling time (140 to 160 min), no corrections were made for increases in cell number.

Synthesis of radioactive OXLU. Oxaluric acid was synthesized by condensation of radioactive urea and ethyl malonate followed by stepwise oxidation of the product with chromium trioxide and hydrogen peroxide (Cooper and Sumrada, manuscript in preparation). The final product was obtained at an overall yield of 24% and a specific activity of 0.1 to 0.5 mCi/mol. The specific activity of the radioactive OXLU was determined by weighing a sample, dissolving it in a precisely known volume of water, and measuring the radioactivity content of the solution. The scintillation spectrometer used for these measurements was operating at an efficiency of 89%.

Paper chromatography. Compounds to be analyzed were spotted on Whatman no. 1 medium-flow-grade filter paper. Forced air was not used for drying here because it caused partial degradation of the OXLU. The chromatogram was developed for approximately 16 h in a descending orientation with 1-butanol–acetic acid–water (100:22:50) as the solvent system. After development, the paper was air dried in a fume hood and then either cut into 0.5-cm strips for radioactivity determination or sprayed with Ehrlich reagent (10) for visual observation of the ureido-containing compounds. Allantoin, allantoate, urea, and OXLU can all be resolved with this chromatographic system.

Ion-exchange chromatography. Ion-exchange resins were prepared as described by Cooper and Beevers (5) and equilibrated with the indicated counterions. The conditions of chromatography are described in appropriate figure legends.

RESULTS

Purity and authenticity of radioactive OXLU. Our characterization of the OXLU transport system in *S. cerevisiae* was based on the observed intracellular accumulation of [^{14}C]OXLU. It was, therefore, important to demonstrate the authenticity and purity of our [^{14}C]OXLU preparations. First, the radioactive material that we made possessed the same melting point as authentic OXLU (208 to 211°C with some discoloration). A sample of our preparation was dissolved in water along with several milligrams of authentic OXLU and passed over a Dowex-1-X8–acetate ion-exchange resin. The chromatogram was then developed with a 400-ml gradient of 0 to 0.5 M NaCl. All of the radioactive material was eluted as a single sharp peak which coincided with elution of authentic OXLU followed colorimetrically. No other radioactive material was observed in the column eluate. The elution profile was similar to that shown in Fig. 1. The radioactive preparation also comigrated with authentic OXLU as a single species on a paper chromatogram (data not shown). The solvent system used to develop the chromatogram was capable of resolving OXLU from all of the allantoin pathway intermediates and the known by-products of the synthetic scheme used for its synthesis.

Two additional tests of identity and purity were performed with strains of *S. cerevisiae* in a biological assay. In the first test, a sample of our radioactive material was incubated with permeabilized cell suspensions of *dur1* (urea carboxylase minus) and wild-type strains; the urea degradative enzymes were fully induced in both strains. After prolonged incubation, all carbon dioxide produced was collected, and its radioactivity content was determined (15). Any urea or allantoin degradative intermediates would be degraded to CO_2 by wild-type cells, but not by the mutant. We observed only 0.44 and 0.18% of the input radioactivity as CO_2 after incubation of wild-type and mutant cells, respectively. Therefore, our maximum contamination by urea or compounds that could be degraded to it was 0.26%.

The only known function of oxalurate in *S. cerevisiae* is to serve as gratuitous inducer of the allantoin degradative enzymes. Therefore, our second test was to compare the abilities of authentic OXLU and our radioactive material to induce allophanate hydrolase [allophanate amidohydrolase (EC 3.5.1.13)], the last enzyme of the pathway. As shown in the inset of Fig. 1, our radioactive preparation and standard OXLU yielded identical dose-response curves over a 100-fold concentration range.

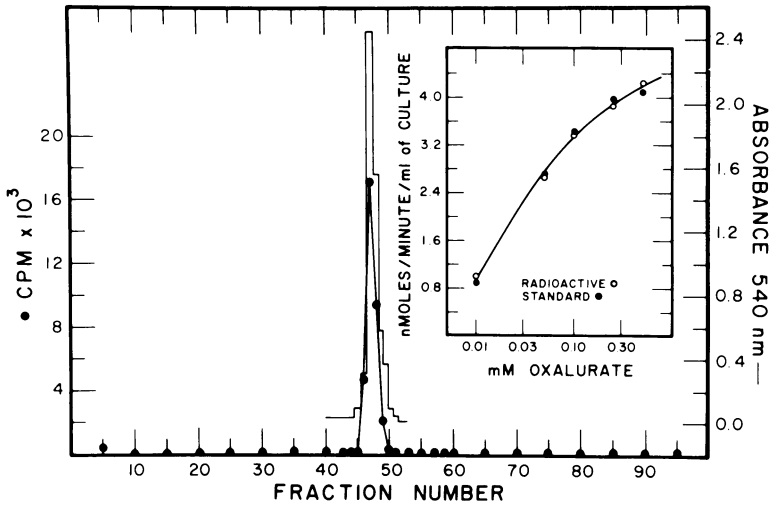

FIG. 1. *Ion-exchange chromatography of a cell extract derived from a wild-type strain after its accumulation of [¹⁴C]OXLU. A culture of strain M25 was grown to a cell density of 30 Klett units. At that time [¹⁴C]OXLU was added to a final concentration of 0.5 mM. After permitting the culture to grow for one generation in the presence of the OXLU, the cells were harvested by filtration, washed with medium devoid of OXLU, and suspended in a solution of chloroform, methanol, and water (1:7:2). Precipitated material was removed by centrifugation, and the supernatant solution was concentrated by evaporation under a stream of dry, high-purity nitrogen. Authentic non-radioactive OXLU was added to the extract, and it was passed over a column of Dowex-1-acetate resin. The fractions collected were assayed for their radioactivity content and also for ureido group-containing material (the authentic OXLU added) with a colorimetric assay (12). Inset, Comparison of the ability of authentic OXLU and the radioactive material that we prepared to serve as inducers of allophanate hydrolase. A culture of strain M25 was grown to a cell density of 30 Klett units. At that time the culture was divided into 10 portions, and the indicated concentrations of authentic oxalurate (●) or the material that we prepared (○) was added to each portion. One generation later, the cultures were sampled for assay of allophanate hydrolase by our standard procedures (4).*

Accumulation of allantoin against a concentration gradient. Three possible modes of OXLU uptake are possible: simple and facilitated diffusion and active transport. The latter is unique in that solute is accumulated inside the cell against a concentration gradient. As shown in Fig. 2, OXLU was accumulated in cells at a linear rate for about 6 min, after which the accumulation rate declined; a plateau was reached at 20 to 24 min. To ascertain whether or not intracellular concentration of OXLU occurred, we incubated a 2.0-ml culture of cells (density, 60 Klett units or 3.6×10^7 cells total) in the presence of approximately 2 mM [^{14}C]-OXLU for 33 min. The cells and medium were then separated by filtration, and the amount of radioactive OXLU in each fraction was determined; 12 and 3,549 nmol were observed in the cells and medium, respectively. If one assumes that the volume of a yeast cell is four times its dry weight, as we have on several past occasions (6, 12, 13), the above values are consistent with intra- and extracellular concentrations of 3.9 and 1.7 mM, respectively, or a two- to threefold concentration of OXLU. If, on the other hand, one uses the average volume of yeast cells (43 μm^3) determined with a Coulter Counter Channelyzer (10-μm latex beads were used as the standard), a four- to fivefold concentration was found. In toto, these data suggest that OXLU was accumulated against a concentration gradient, albeit a modest one.

To determine whether or not oxalurate was modified after entry into the cell, a large culture was permitted to accumulate OXLU, and the soluble cellular components were extracted with aqueous chloroform–methanol. Authentic carrier was added to this material, and it was passed over a Dowex-acetate column and eluted with a linear salt gradient. As shown in Fig. 1, all of the radioactive material was eluted from the resin as a single species coincident with authentic OXLU. Coincidence of migration was also observed on paper for authentic OXLU and radioactive material in the extract.

Requirement of energy for uptake of OXLU. Another unique characteristic of active transport is its requirement for energy. To test the energy dependence of OXLU uptake, we monitored the initial rates of uptake in the pres-

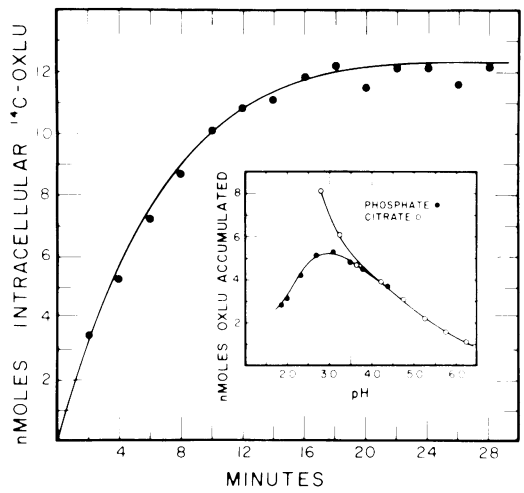

FIG. 2. *Time-dependent accumulation of [^{14}C]-OXLU by growing cultures of strain M25. A wild-type culture was grown to a cell density of 60 Klett units in minimal glucose-ammonia medium at 30°C. At that time, [^{14}C]OXLU was added to a final concentration of 2 mM. At the times indicated, a 2-ml sample was removed from the culture and processed as described in the text. The data are expressed as total nanomoles of OXLU accumulated per 2-ml sample. Inset, Effect of pH on the rate of OXLU uptake in S. cerevisiae. A culture of strain M25 was grown to a cell density of 60 to 70 Klett units. At that time, trichodermin was added to a final concentration of 30 μg/ml to stop cell division. Citrate buffer of the desired pH was then added to portions of the culture (100 mM, final concentration), and 1 min later 2.0 mM [^{14}C]OXLU was added. After 7 min of incubation, a sample of each portion was removed and processed as described in the text. The precise pH of each portion was also determined after removing the sample used for radioactivity measurements. This was necessary because minor changes in pH occurred when cells and buffer were mixed. Phosphate buffer was used in the same manner just described. However, in this case cell division was prevented with 50 μg of cycloheximide per ml.*

ence of several compounds known to inhibit various steps in energy metabolism. As shown in Table 1, OXLU uptake was severely depressed by inhibitors of glucose metabolism such as arsenate and fluoride and by ionophores such as dinitrophenol and carbonylcyanide-*m*-chlorophenyl hydrazone. However, uptake was insensitive to cyanide, a classic inhibitor of mitochondrial respiration.

pH optimum of OXLU uptake. Uptake of OXLU occurred over a broad range of pH, with an optimum at pH 3.0 (inset to Fig. 2). Uptake decreased by 50% at pH 2.0 and 5.0. This optimum is similar to that observed for urea (pH 3.0 to 3.5), but is much lower than that observed for allantoin (pH 5.0 to 5.5).

Efflux and exchange of preloaded OXLU. The most important role of OXLU in *S. cerevisiae* is its ability to serve as gratuitous inducer of the allantoin degradative system. In view of this, we were interested to know whether or not OXLU could be easily removed from cells by resuspending them in fresh medium devoid of OXLU. To answer this question, we allowed a wild-type culture to accumulate OXLU for 28 min. At that time we collected the cells and resuspended them in fresh medium devoid of OXLU. Thereafter, we sampled the culture and measured the amount of radioactive OXLU that they contained. As shown in Fig. 3, efflux of preloaded OXLU was greater than 90% complete within 5 min after resuspending the cells in fresh medium. Efflux brought about in this manner followed closely the time course of OXLU loss from cells whose membranes had been damaged by treatment with nystatin (Fig. 3, inset).

In addition to simple efflux, preloaded intracellular OXLU was readily exchanged with extracellular material. This was shown by allowing a culture to accumulate [^{14}C]OXLU for 10 to 15 min and then adding excess non-radioactive OXLU to the test culture. As shown in Fig. 4, about 25 to 30 min was required to exchange 50% of the previously accumulated OXLU. Efflux and exchange rates of intracellular oxalurate were similar to those of urea but were much greater than those of allantoin.

OXLU uptake in cultures provided with various nitrogen sources. Bossinger et al. (4) previously reported that induction of the allantoin degradative system was not possible in cul-

TABLE 1. *Sensitivity of OXLU uptake to inhibitors of energy metabolism*[a]

Inhibitor	OXLU uptake (nmol/6 min)
None	3.45
1 mM KCN	3.83
1 mM DNP[b]	0.03
0.1 mM CCCP[c]	0.51
5 mM fluoride	0.23
5 mM arsenate	0.06

[a] A culture of strain M25 was grown to a cell density of 60 Klett units. Portions of the culture were then transferred to flasks containing the indicated inhibitor. After 2 min of incubation at 30°C, 2 mM radioactive OXLU was added to each flask; this was considered zero time. Thereafter, samples were removed at 2-min intervals and processed as described in the text.

[b] DNP, Dinitrophenol.

[c] CCCP, Carbonylcyanide-*m*-chlorophenyl hydrazone.

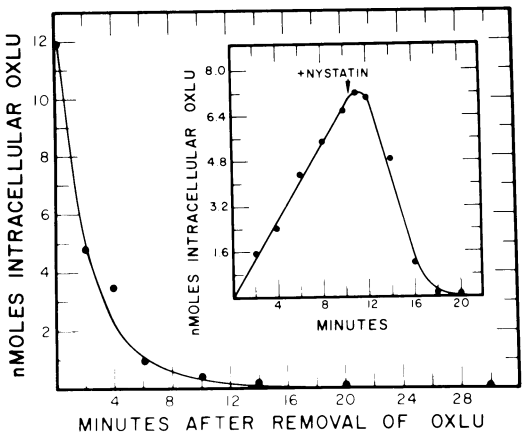

FIG. 3. *Efflux of [^{14}C]OXLU from growing cultures of S. cerevisiae. A culture of strain M25 was grown in glucose-ammonia medium to a cell density of 60 Klett units. Then [^{14}C]OXLU was added to a final concentration of 2.0 mM, and the culture was allowed to accumulate it for 20 min (20 min represents zero time in the figure). A 2.0-ml sample was removed at this time (the zero-time sample) for assay of its OXLU content, and the remaining cells were harvested by filtration, washed with fresh medium devoid of oxalurate, and resuspended in the same OXLU-free medium. Thereafter, 2.0-ml samples were removed and processed as described in the text. The data are expressed as nanomoles of intracellular [^{14}C]OXLU observed in 2.0-ml samples of culture. The times indicated in the figure refer to the time spent in OXLU-free medium before the sample was removed for assay. Inset, Effect of nystatin on oxalurate retention. A culture of strain M25 was grown to a cell density of 60 Klett units. At that time, [^{14}C]OXLU was added to a final concentration of 2.0 mM. Thereafter, samples were removed for 10 min as indicated in the figure. At this time, nystatin (9 μg/ml) was added to the culture, and sampling was continued as before. All samples were processed as described in the text. Data are expressed as nanomoles of intracellular OXLU observed in 2.0 ml of culture.*

tures provided with good nitrogen sources such as serine, glutamine, or asparagine. One explanation suggested by some investigators was that OXLU, the inducer, could not enter the cell (inducer exclusion) and hence induction did not occur. Although we did not agree with this view, we could not test the hypothesis directly at that time. However, we now have had an opportunity to make such tests. They were performed by measuring the initial rate of OXLU uptake in cultures growing on a wide variety of nitrogen sources. As shown in Table 2, the rate of uptake varied up to fourfold depending on the nitrogen source provided. However, there was no demonstrable relationship between uptake rate and the ability of a particular nitrogen source to repress synthesis of the allantoin degradative enzymes. Cells growing in minimal proline medium, one of our least repressive nitrogen sources, accumulated OXLU much more slowly than cells provided with either serine or glutamine. The latter compounds are the most repressive nitrogen sources available.

The rate of OXLU uptake was also measured after addition of several compounds to the medium just before assay (Table 2). Allantoate was the only compound in this group to significantly affect OXLU uptake. In the presence of this metabolite, OXLU uptake was depressed up to sevenfold. As shown in Fig. 5, allantoate appeared to be a competitive inhibitor of OXLU and thus increased the apparent Michaelis constant for OXLU. This result is not too suprising because allantoate is a reasonable analog of OXLU.

Effect of protein synthesis inhibitors on OXLU uptake. In past studies, we observed that starvation results in a dramatic increase in the rate of allantoin uptake (13). To ascertain whether a similar reponse occurred with respect to OXLU uptake, we starved a wild-type culture overnight for ammonia and then monitored its

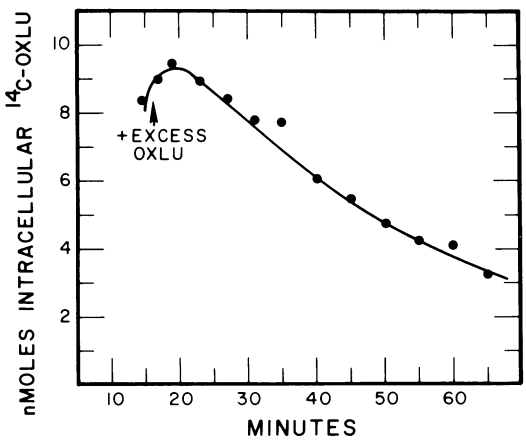

FIG. 4. *Uptake of [^{14}C]OXLU and its exchange with non-radioactive OXLU provided to a growing culture of S. cerevisiae. A culture of M25 was grown as described in Fig. 3. At zero time, 2 mM radioactive OXLU was added, and the culture was allowed to incubate for 14.5 min. At that time, a 2.0-ml sample was removed for assay as described in the text. At 15 min, non-radioactive OXLU was added to the remaining culture (3.0 mM final concentration of non-radioactive OXLU). Thereafter, 2.0-ml samples were removed and processed. The data are expressed as nanomoles of radioactive oxalurate contained in 2.0 ml of culture at the times indicated in the figure.*

TABLE 2. *OXLU uptake in cultures provided with various nitrogen sources*[a]

Nitrogen source or compound added	OXLU uptake (nmol/4 min)
Urea	8.11
Allantoin	7.12
Citrulline	5.82
Glutamine	4.29
Serine	4.13
Ammonia	3.66
Alanine	3.58
Ornithine	2.93
Glutamate	2.18
Asparagine	2.10
Proline	1.77
Ammonia + ureidoglycollate added	3.89
Ammonia + hydantoate added	4.08
Ammonia + ureidosuccinate added	3.53
Ammonia + allantoate added	0.55

[a] These experiments were done as described in Table 1. Nitrogen sources were present at 0.1% final concentration.

rate of uptake. As shown in Table 3, the initial rate of OXLU uptake was decreased seven- to eightfold. Similar decreases in OXLU uptake were observed after prolonged treatment of the test culture with trichodermin, a potent inhibitor of protein synthesis (3, 8).

DISCUSSION

The data presented above demonstrate that OXLU is accumulated within the cell against a modest concentration gradient. Uptake is mediated by an energy-dependent active transport system possessing an apparent Michaelis constant of 1.2 mM. Since OXLU is not a normal metabolite of *S. cerevisiae*, it is presumably transported by an uptake system associated with some other cellular constituent. Information presently available does not permit identification of this system or even certify that only one system performs this function. However, we have been able to delineate many of the physiological parameters associated with OXLU uptake, and this, after all, is the information needed most when the compound is employed as a nonmetabolizable inducer. The system(s) associated with OXLU transport appears to be constitutively produced, a conclusion based on our inability to increase the rate of uptake regardless of what compounds were added to the culture medium. The transport system(s) also appears to be insensitive to nitrogen repression. These characteristics raise the possibility that OXLU uptake may be mediated by a biosynthetic transport system, because most, if not all, of the catabolic uptake systems are inducible and/or repressible (4, 6, 12, 13). Another very useful characteristic of OXLU uptake is the fact that it can be quickly removed from cells by resuspending them in medium devoid of the compound. This is most advantageous for experiments involving pulse induction of the allantoin degradative enzymes by inducer addition and removal.

The direct demonstration that OXLU uptake is insensitive to nitrogen catabolite repression in strain M25 supports our earlier conclusions concerning repression of the allantoin degradative enzymes (1, 4). In those early experiments, we suggested that synthesis of allophanate hydrolase was repressed by growth on good nitrogen sources. The possibility that lack of enzyme induction by cultures growing in repressive minimal glutamine, asparagine, or serine media was the result of inducer exclusion, an alternative interpretation of our data, seems to be excluded by the present observations.

The present studies also offer further support for the conclusion that OXLU is a true nonmetabolizable inducer of the allantoin system

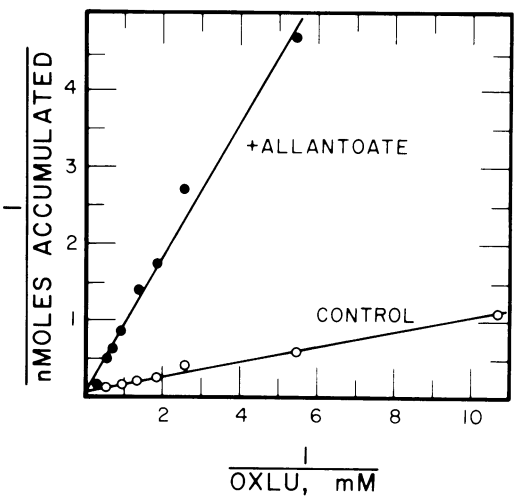

FIG. 5. *Effect of increasing concentrations of OXLU on its uptake and characterization of allantoate inhibition of that uptake. A culture of strain M25 was grown in glucose-ammonia medium to a cell density of 60 Klett units. At this time, cycloheximide was added to a final concentration of 20 μg/ml. This culture was then divided into two portions and 0.1% allantoate (final concentration) was added to one of them. The remaining portion received no additions. Samples of each of these two portions were allowed to accumulate oxalurate, at varying external concentrations, for 8 min. At that time the samples were processed as described in the text. The rates plotted in the figure are expressed as nanomoles of OXLU accumulated in 8 min by 2.0 ml of culture.*

TABLE 3. *Effect of protein synthesis inhibition on the rate of OXLU uptake*[a]

Inhibitory condition and time of pretreatment before assay of uptake	OXLU uptake (nmol/6 min)
None	7.43
Trichodermin (30 μg/ml) for:	
0 min	8.06
10 min	7.31
20 min	6.32
30 min	5.97
180 min	4.26
1,400 min	1.69
Starvation for 1,400 min	1.08

[a] Cultures of strain M25 were grown to a cell density of about 60 Klett units. At that time the inhibitory condition was established (addition of trichodermin or the resuspension of cells in medium devoid of a nitrogen source). After the time indicated, a sample of the treated culture was assayed for OXLU uptake by the procedures outlined in the text.

(11). Our original experiments identifying this function provided low-resolution evidence that OXLU was both taken into the cell and thereafter remained unmodified. These conclusions were based on colorimetric assays of ureido group-containing compounds, and hence a certain amount of doubt was justified. However, the present experiments with radioactive material provide far greater verification of these observations.

Loss of protein synthesis affects OXLU uptake quite differently than it does urea or allantoin transport. Cycloheximide-treated cultures transported urea at rates close to those observed in control cultures. Allantoin uptake was far greater in starved cultures than it was in growing cells. OXLU uptake, on the other hand, was depressed by both of these conditions, and it is reasonable to ask whether loss of transport resulted from transinhibition or the loss of the transport system due to protein turnover. These and other characteristics of the OXLU uptake system are presently under investigation.

ACKNOWLEDGMENTS

These studies were supported by Public Health Service grants GM-19386, GM-20693, and GM-24383 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.

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