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Urea Transport-Defective Strains of *Saccharomyces cerevisiae*

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Experiments characterizing the urea active transport system in *Saccharomyces cerevisiae* indicate that (i) formamide and acetamide are strong competitive inhibitors of urea accumulation, (ii) uptake is maximal at pH 3.3 and is 80% inhibited at pH 6.0, and (iii) adenosine 5'-triphosphate generated by glycolysis in conjunction with formation of an ion gradient is likely the driving force behind urea transport. Mutant strains were isolated that are unable to accumulate urea at external concentrations of 0.25 mM. These strains also exhibit a depressed growth rate on 10 mM urea, indicating existence of a relationship between the active transport and facilitated diffusion modes of urea uptake.

Urea transport in *Saccharomyces cerevisiae* may occur by two routes (5). The first mode is a low K_m (14 μM), energy-dependent active transport system. It is induced by allophanate or oxalurate, the native (4, 18) and gratuitous (13) inducers, respectively, of the allantoin degradative enzymes. Production of the active transport system is also subject to nitrogen repression when cultures are grown on very good nitrogen sources such as asparagine and glutamine (2). The second mode, which occurs at urea concentrations in excess of 0.5 mM, does not require an energy source inhibitable by 2,4-dinitrophenol (DNP), KCN, or arsenate, is insensitive to nitrogen repression, and is apparently present in cells grown in the absence of allantoin degradative system inducers (5).

Here we report the isolation of mutant strains that have lost their ability to transport urea by the low K_m active transport system. Additional characteristics of the wild-type active transport system are also presented. A preliminary report of these data has already appeared (R. Sumrada and T. Cooper, Genetics 80:s79).

MATERIALS AND METHODS

Strains and medium. Strains used in the physiological portions of this work were all prototrophic diploids constructed by standard genetic methods (8, 17). Rho$^-$ strains were generated by treating appropriate diploid strains with ethidium bromide (10, 12). Loss of respiratory ability in treated strains was verified by their inability to use glycerol or lactate as sole carbon sources. Wickerham medium (19) with glucose at a final concentration of 0.6% was used throughout this work. In those instances where induction was necessary, oxalurate was either included in, or added to, the medium at a final concentration of 0.5 mM. Occasions of oxalurate addition to the medium at a cell density of 25 Klett units have been indicated in the pertinent figures to distinguish this experimental format from one in which the inducer was present throughout the entire growth period. Wiame medium was prepared as described earlier (6).

Cycloheximide treatment. Kinetic experiments measuring the effect of other allantoin-related metabolites upon urea uptake require cell samples containing a constant amount of urea permease over the time period covered by the experiment (10 to 15 min). This requirement, unfortunately, may not be met with an induced and growing culture. Therefore, a search was mounted for some condition that would prevent further growth and permease production without adversely affecting transport or associated energy-generating systems. As shown in Fig. 1, addition of cycloheximide (20 μg/ml) to the culture satisfied these requirements. There is a very slow and linear loss of uptake ability (half-life of 260 min) when the cells are maintained at 30 C. At 4 C there is an abrupt decrease in uptake ability during the first 10 min. After this time, however, no further decay is observed. Since losses in accumulation activity were negligible over the first 15 min at 30 C, these conditions were used in the kinetic experiments described in Fig. 6 and Table 3.

Enzyme assays. Uptake assays were performed by the methods of Cooper and Sumrada (5). All other enzyme assay procedures have been described in detail elsewhere (2, 4, 17, 18). Radioactive ureidoglycolate was chemically synthesized from [^{14}C]urea by the methods of Valentine and Wolfe (16).

RESULTS

Isolation and characterization of urea transport mutant strains. Ureidoglycolate has been reported to serve as a nitrogen source for several strains of bacteria and yeast (3, 7, 13, 15). We also have observed growth of our wild-type strains using this compound as sole nitrogen source. In theory, this observation provides

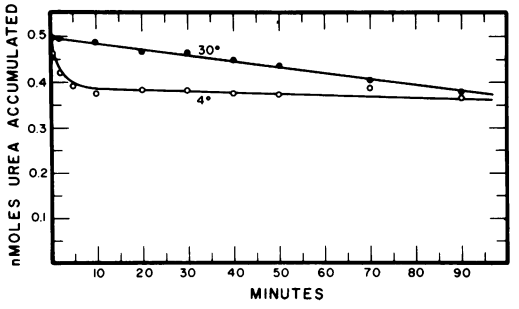

FIG. 1. *Effect of cycloheximide upon urea transport in S. cerevisiae. A urea carboxylase-defective strain of Saccharomyces (M62, dur-1) was grown to a cell density of 45 Klett units (100 Klett units is equivalent to approximately 3 × 10⁷ cells per ml of culture) on minimal ammonia medium containing 0.5 mM oxalurate. At zero time, 1.0 ml of the culture was removed for assay and 15 ml were transferred to each of two flasks containing sufficient cycloheximide to yield a final concentration of 10 μg per ml. One flask had been prewarmed to 30 C (●) and the other had been precooled to 4 C (○). At the times indicated, 1.0-ml samples were removed from each flask and permitted to accumulate [¹⁴C]urea for 4 min. Assay procedures are described in Materials and Methods.*

a means of obtaining mutant strains defective in ureidoglycolate hydrolase, the only enzyme of allantoin degradation for which we have not yet isolated defective strains. Therefore, a wild-type haploid culture (M25-12b) was mutagenized with ethylmethane sulfonate (1.5% final concentration for a period of 40 min at 30 C), and mutant strains unable to utilize ureidoglycolate as sole nitrogen source were isolated by the techniques described earlier (17). The growth characteristics of one of these mutant strains are shown in Table 1. Mutant strains provided with ammonia, arginine, or ornithine as nitrogen source grow at the same rate as wild-type cultures. However, when provided with allantoin degradative intermediates they behave quite differently. In the presence of ureidoglycolate no growth occurs. Utilization of 10 mM urea is greatly slowed, whereas growth on allantoin and allantoate is moderately decreased. The extraordinarily slow growth rate on urea was not expected because heretofore, ureidoglycolate hydrolase activity was not thought to be involved in the metabolism of urea. To verify the loss of ureidoglycolate hydrolase biochemically, all five enzymes of allantoin degradation were assayed in mutant and wild-type strains grown on either ammonia or 10 mM urea (Table 2). To our surprise, mutant strains contained all five allantoin degradative enzymes in approximately wild-type amounts. These data argued that we had not isolated a strain defective in ureidoglycolate hydrolase, but rather in some other unknown function, most probably involving permeation of a metabolite.

A report in the literature that ureidoglycolate decomposes (14) prompted us to determine the extent of ureidoglycolate decomposition that may have occurred in the medium we used to isolate the mutant strains. As shown in Fig. 2, breakdown occurs at a measurable rate. Such decomposition would provide cells with a continuous supply of urea at low concentration. This suggested that the mutant strains we isolated might be defective in the low K_m urea transport system. This was verified biochemically. As shown in Fig. 3, the mutant lesion (*dur-3*) renders strains incapable of accumulating 0.25 mM urea from the medium. This is true whether inducer is provided exogenously as oxalurate or internally by using a strain containing a defective allophanate hydrolase gene product (*dur-2*). Further verification of this phenotype was made by testing the ability of various compounds to serve as inducers of allophanate hydrolase in *dur-3* mutant strains. As shown in Fig. 4A, oxalurate performs this function well. However, the amount of hydrolase induction observed with 10 mM urea is decreased by 50% and 0.25 mM urea is totally ineffective, compared to the wild-type strains (Fig. 4B).

Isolation of urea permease-defective mutants by this method led us to determine specifically whether or not ureidoglycolate could enter cells as such, or only after its decomposition to urea. This was done by adding freshly prepared [¹⁴C]ureidoglycolate to an oxalurate-induced culture of a strain devoid of urea carboxylase activity (M62) and assaying the amount of radioactivity found in the cells. As can be seen in Fig. 5, only a small quantity of radioactivity

TABLE 1. *Growth of a strain unable to use ureidoglycolate as sole nitrogen source*

Nitrogen source[a]	Doubling time (min)	
	M25, wild type	M277, ureidoglycolate minus (*dur-3*)
Ammonia	120	122
Allantoin	150	170
Allantoate	160	189
Ureidoglycolate	157	NG[b]
Urea	128	450
Arginine	150	156
Ornithine	155	150

[a] Nitrogen sources were present at a final concentration of 0.1% except for urea, which was 10^{-2} M.

[b] NG, No growth after 72 h.

TABLE 2. *Production of allantoin degradative enzymes in wild-type and dur-3 mutant strains of S. cerevisiae*[a]

Enzyme activity	Nitrogen source	Soluble protein (nmol/min per mg)		Increase in enzyme activity[b] per 25 Klett units of growth	
		Wild type	*dur-3* mutant	Wild type	*dur-3* mutant
Allantoinase	Ammonia	4.7	3.1		
	Urea	15.3	16.9		
Allantoicase	Ammonia	5.3	8.3		
	Urea	23.8	18.9		
Ureidoglycolate hydrolase	Ammonia	23.6	25.5		
	Urea	40.3	60.6		
Urea amidolyase[c]	Ammonia			0.004	0.004
	Urea			0.59	0.34
Allophanate hydrolase	Ammonia			0.16	0.17
	Urea			3.22	1.46

[a] Diploid strains, M25 (wild type) and M277 (ureidoglycolate minus) were grown in either minimal ammonia or urea medium. Urea-amidolyase and allophanate hydrolase were assayed using permeabilized cell suspensions, whereas allantoinase, allantoicase and ureidoglycolate hydrolase were assayed using cell-free extracts.

[b] Activities are expressed as nanomoles per minute per milliliter of culture.

[c] Assayed as production of ^{14}C-labeled CO_2 from [^{14}C]urea, which requires both urea carboxylase and allophanate hydrolase activities.

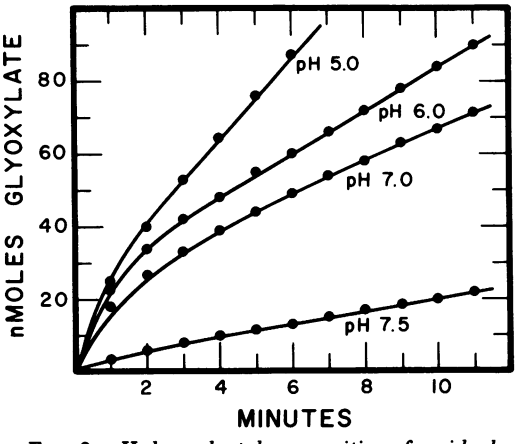

FIG. 2. *pH-dependent decomposition of ureidoglycolate. Recrystallized ureidoglycolate was dissolved in a solution containing: 0.2 M tris(hydroxymethyl)aminomethane acetate buffer at the indicated pH values and 1.5 mg of phenylhydrazine per ml. The final concentration of ureidoglycolate in solution was 1.0 mM. This solution was immediately transferred to a cuvette, and its absorbance (325 nm) was monitored with a Beckman model 25 double-beam spectrophotometer. Amounts of glyoxylate-phenylhydrazone formation were calculated with an extinction coefficient of 1.74 × 10⁴.*

is transported into cells when [^{14}C]ureidoglycolate is provided, compared to addition of an equivalent amount of [^{14}C]urea. It is also significant that urease added to the culture just before [^{14}C]ureidoglycolate decreased the amount of uptake observed to near background levels. In toto, these data argue that ureidoglycolate is not capable of entering the cells at all.

In view of the anomalous way in which the urea transport mutants were obtained, it was advisable to isolate additional mutant strains by more conventional methods. This was done by isolating strains on the basis of their inability to use 0.25 mM urea as sole nitrogen source. Mutant strains isolated in this manner displayed the same biochemical and physiological phenotypic characteristics as the ureidoglycolate-minus strains. In addition, strains isolated by the two procedures were not capable of genetic complementation, further supporting their functional identity.

Inhibition of urea transport by allantoin-related metabolites. The close structural relationships of allantoin-degradative intermediates raise the possibility of their sharing one or more permeases in common. To test this possibility in a preliminary manner, various allantoin-related compounds were assayed for their ability to compete with urea uptake. As shown in Fig. 6, only ureidoglycolate, formamide, and acetamide inhibit urea uptake competitively. The competition observed with ureidoglycolate is likely more apparent than real, due to its decomposition to urea and glyoxylate. As shown in Table 3, the presence of formamide or

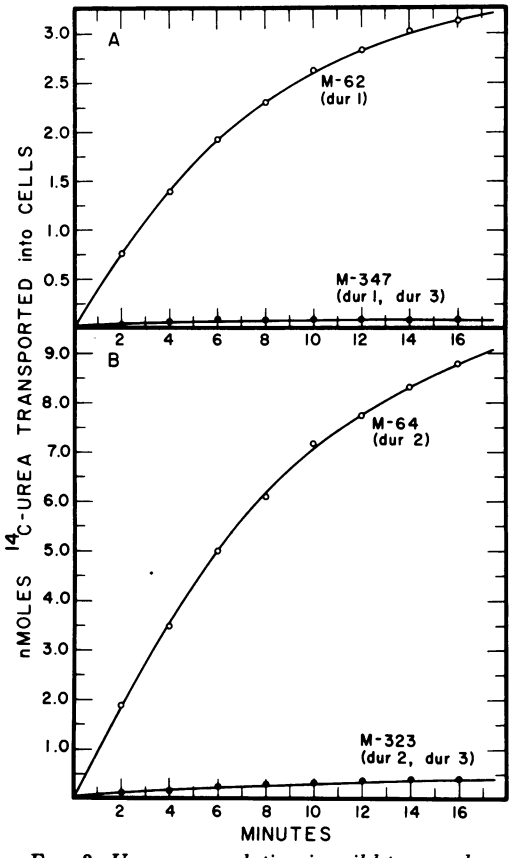

FIG. 3. *Urea accumulation in wild-type and mutant strains of Saccharomyces. All strains were homozygous diploids and contained either the dur-1 (urea carboxylase) or dur-2 (allophanate hydrolase) allele in addition to the dur-3 allele whose phenotype was being tested. The dur-1 or dur-2 alleles were included to prevent metabolism of [^{14}C]urea to ^{14}C-labeled CO_2. Strains containing the dur-1 allele were grown on minimal ammonia medium containing 0.5 mM oxalurate. In the case of dur-2-containing strains, oxalurate addition to the medium was unnecessary. At a cell density of 45 Klett units, samples were removed from each culture for assay of urea accumulation.*

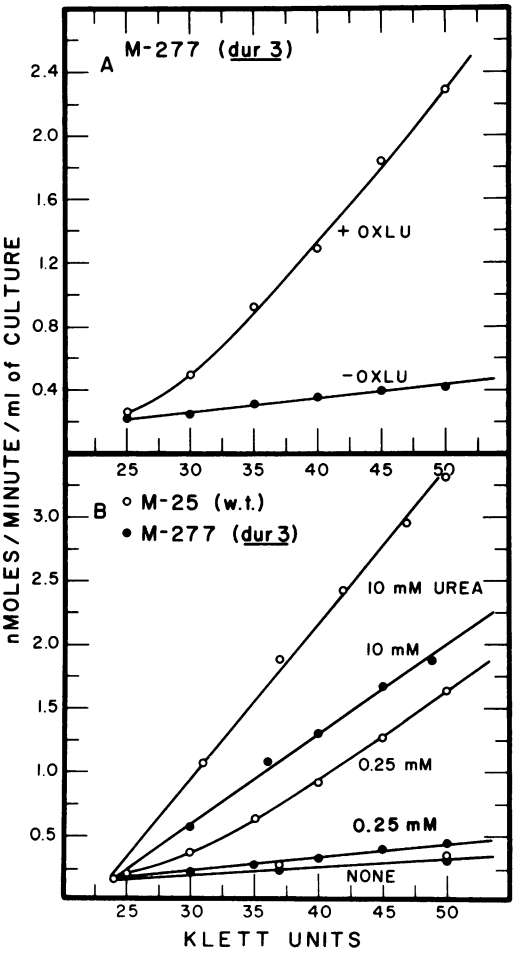

FIG. 4. *Induction of allophanate hydrolase in wild-type and dur-3 mutant strains of Saccharomyces. Wild-type (M25) and dur-3 (M277) strains were grown to a cell density of 25 Klett units on minimal ammonia medium. At that time the indicated inducer was added to each culture. Urea was provided at a final concentration of either 0.25 or 10 mM and oxalurate at a concentration of 1 mM. Subsequently samples of each culture were removed for allophanate hydrolase assay.*

acetamide in the urea uptake assay mixture increases the apparent Michaelis constant of the transport system for urea by 160- and 20-fold, respectively. Also noteworthy is a 14-fold increase in the apparent K_m value for urea brought about by the presence of allophanate. In this case, however, the observed inhibition is not competitive.

pH optimum of urea transport. It has been previously reported (2) that cultures of *Saccharomyces* growing in Wickerham and Wiame media behave differently from one another with respect to induced production of the allantoin-degradative enzymes. As shown in Fig. 7, these observations extend to urea transport also. Little, if any, transport was observed in cultures grown on Wiame medium containing the non-metabolizable inducer oxalurate. The same result was observed when a strain containing defective allophanate hydrolase (*dur-2*) was grown under these conditions. This precludes oxalurate exclusion and lack of induction as the reason behind failure of these cells to accumulate urea, because strains lacking allophanate hydrolase activity are internally induced and are, hence, phenotypically constitutive for the components of the allantoin deg-

radative system. The facts that (i) buffering Wickerham medium to pH 6 elicited the same response (Fig. 6A), and (ii) buffering either medium to pH 3.3 resulted in normal or accelerated rates of uptake suggest urea uptake is a

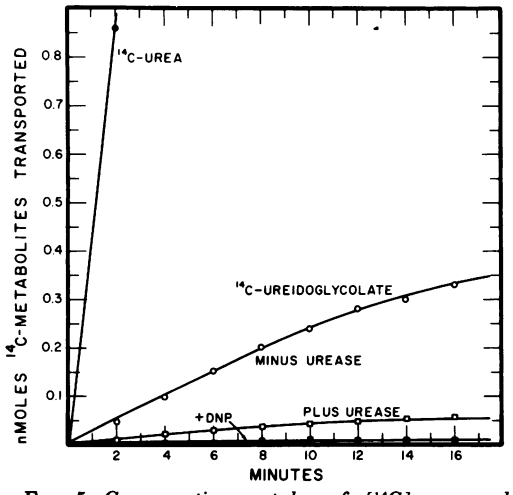

FIG. 5. *Comparative uptake of [^{14}C]urea and [^{14}C]ureidoglycolate by Saccharomyces. Four cultures of strain M62 were grown on minimal ammonia medium to a cell density of 45 Klett units. At zero time, the following additions were made to each culture: (●) urea at a final concentration of 0.36 mM and a specific activity of 4.6 mCi per mmol; (○) ureidoglycolate at a final concentration of 0.36 mM and a specific activity of 0.15 mCi per mmol; (□) 0.36 mM radioactive ureidoglycolate and 1.7 mg per ml of jack bean urease; and (■) 0.36 mM radioactive ureidoglycolate and 1 mM DNP. The pH of the medium at zero time was 3.3 to 4.0.*

TABLE 3. *Apparent K_m values of urea permease for urea in presence of various metabolites and urea analogues*

Inhibitor added	Apparent K_m, (μM)[a]
None	15.6
Allantoin	18.5
Allantoate	17.9
Ureidoglycolate	54.9
Allophanate	217.0
Oxalurate	29.4
Formamide	2,500.0
Acetamide	310.0

[a] These data were derived from the data shown in Fig. 6.

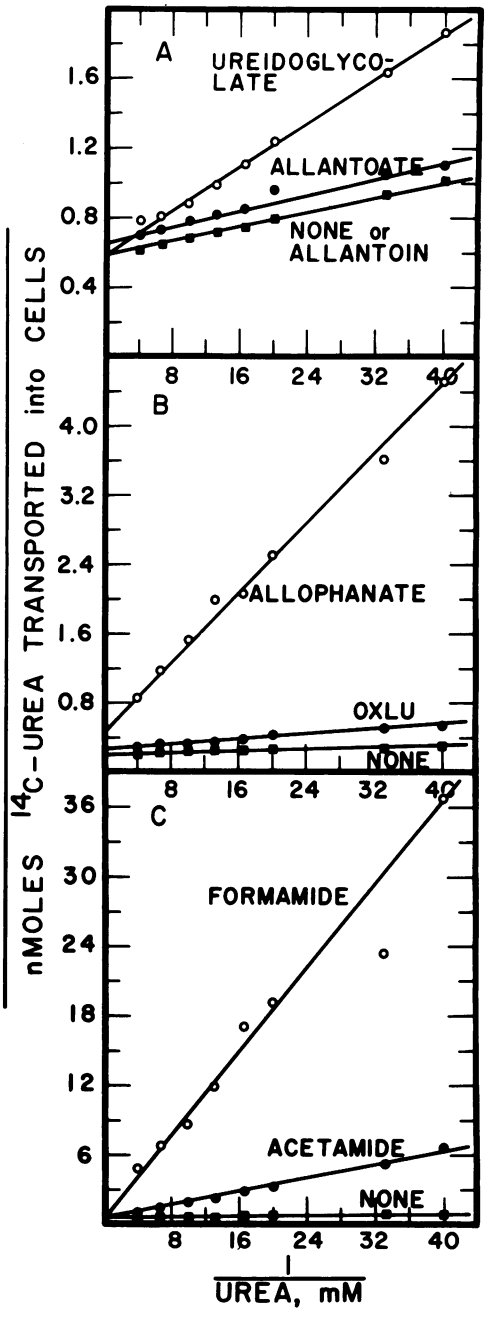

FIG 6. *Inhibition of urea uptake by allantoin-related metabolites. Either induced cultures of a urea carboxylase minus (dur-1) strain (panels A and C) or uninduced cultures of an allophanate hydrolase minus strain (panel B) were used at a cell density of 45 Klett units for these experiments. Prior to initiation of the experiment, cycloheximide was added to the cultures at a final concentration of 20 μg per ml. This was followed by a 3- to 5-min equilibration period at 30 C. The assay mixture included urea (0.025 to 0.25 mM) at a specific activity of 5 mCi per mmol and the compound being tested for inhibitory activity at a final concentration of 1 mM. Accumulation of urea was initiated by adding 1.0 ml of the cycloheximide-treated cultures to the remaining assay components. Urea was permitted to accumulate for 4 min, after which a 0.5-ml sample of reaction mixture was processed as described in Materials and Methods.*

highly pH-dependent process. This is confirmed by the pH profiles shown in Fig. 8. Urea uptake exhibits a rather broad maximum at pH 3.25. Control experiments (R. Sumrada and T. Cooper, unpublished observations) indicate that the increased ionic strength resulting from addition of a buffering agent (citrate or phosphate) to the medium does not adversely affect the cells.

Nature of the energy source driving urea transport. Inhibition of urea uptake by a variety of inhibitors that interrupt energy metabolism argues strongly for existence of an energy-dependent step in urea transport. However, the precise nature of the primary energy source could not be identified from those data. To test the need for direct participation of mitochondrially derived energy, Rho$^-$ strains were prepared. As can be seen in Fig. 9A, transport in these strains occurs at about one-third to one-

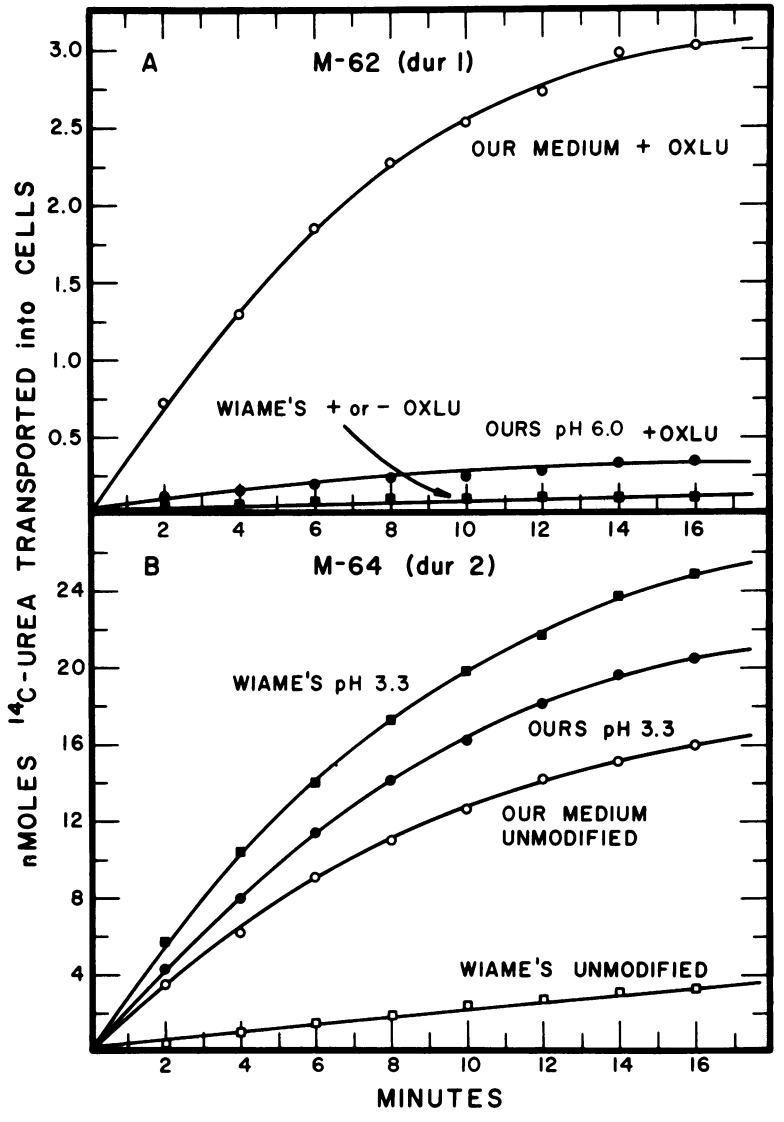

FIG. 7. *Urea accumulation in Saccharomyces strains grown on various types of media. Urea carboxylase minus (dur-1) or allophanate hydrolase minus (dur-2) strains of Saccharomyces were grown to a cell density of 45 Klett units on one of several types of media. These included: Wickerham medium in an unmodified condition (this is our standard medium), the medium normally used by Wiame and his colleagues, Wickerham medium buffered to either pH 6.0 or 3.3 using 1% citrate as the buffering agent, and Wiame medium adjusted to pH 3.3 instead of 6.1. When induction was necessary (panel A) oxalurate (OXLU) was added to the growth medium as indicated in the figure. Urea uptake was monitored with our standard assay procedures.*

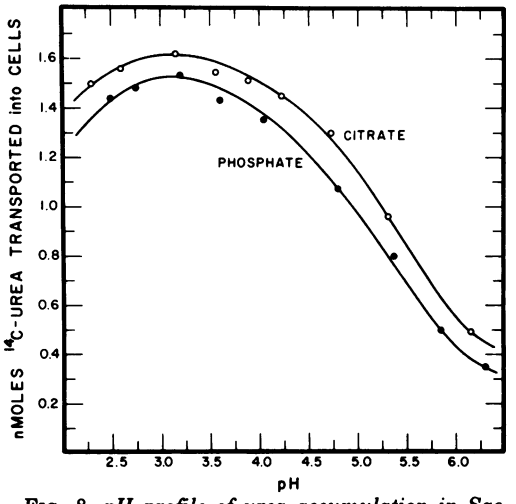

FIG. 8. *pH profile of urea accumulation in Saccharomyces. Strain M64 (dur-2) was grown to a cell density of 45 Klett units on minimal ammonia medium. A 50-ml sample of the culture was then transferred to a prewarmed flask containing cycloheximide (20 μg per ml, final concentration) and equilibrated 3 to 5 min at 30 C with shaking. A 3.6-ml sample of the cycloheximide-treated culture was transferred to a flask containing citrate or phosphate buffer at the desired pH (final concentration of buffer was 0.1 M in 4.0 ml). After an equilibration period of 1 min, a 1.0-ml sample of the buffered cell suspension was incubated at 30 C with [^{14}C]urea (0.36 mM, final concentration) for 4 min. Subsequent to this point, assay samples were processed normally. The pH of the remaining 3.0 ml of cell suspension was carefully measured at the conclusion of assay.*

half the rates observed in their Rho$^+$ counterparts. This, however, is likely the result of a general decrease in available energy as evidenced by the fact that production of allophanate hydrolase is decreased roughly the same amount whether cultures were induced by the presence of oxalurate (Fig. 9B) or 10 mM urea (Fig. 9C). The last observation is particularly significant because at this concentration, urea entry is not energy dependent. The presence of urea transport in Rho$^-$ strains suggests that KCN inhibition of urea transport (5) may arise from an inhibition of mitochondrial electron transport per se. That this is the case is shown by the data in Fig. 10A, which demonstrates that urea transport in Rho$^-$ organisms is insensitive to KCN inhibition. However, as shown in Fig. 10B and C, both Rho$^+$ and Rho$^-$ cultures remain sensitive to proton ionophores such as DNP and carbonylcyanide *m*-chlorophenylhydrazone (CCCP).

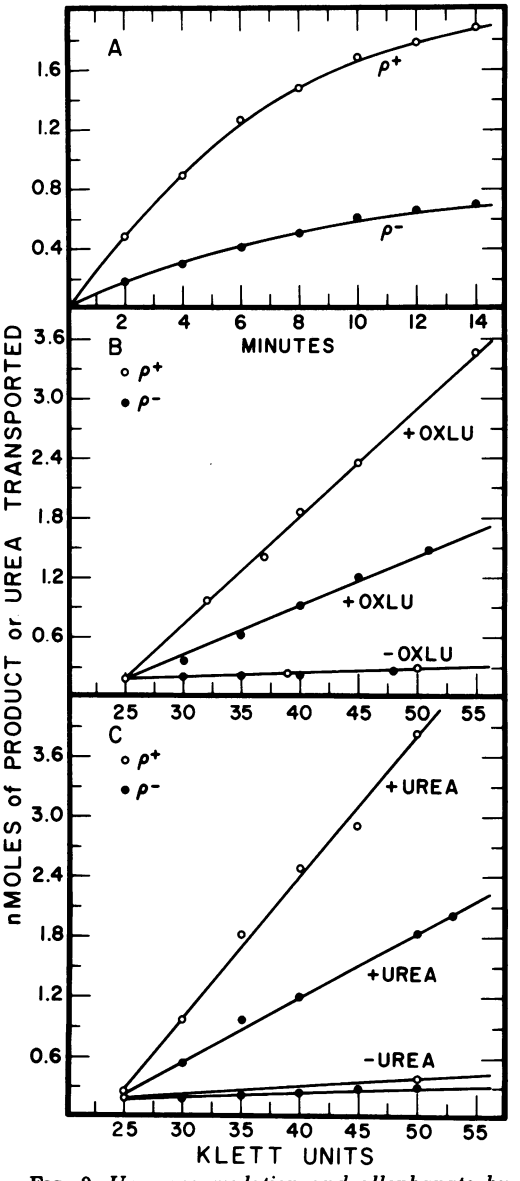

FIG. 9. *Urea accumulation and allophanate hydrolase induction in Rho$^+$ and Rho$^-$ strains of Saccharomyces. [^{14}C]urea uptake (A) was measured in Rho$^+$ and Rho$^-$ strains (derived from strain M-62) containing oxalurate (OXLU) as inducer. Uptake assays are described in Materials and Methods. Rho$^+$ and Rho$^-$ strains generated by ethidium bromide treatment of strain M25 (our standard wild type) were grown to a cell density of 25 Klett units on minimal ammonia medium (B and C). At that time, oxalurate (1 mM) or urea (10 mM) was added as indicated. The cultures were sampled thereafter as indicated for allophanate hydrolase assay.*

DISCUSSION

This work describes the isolation of mutant *Saccharomyces* strains unable to accumulate urea at low external concentrations. We propose that the genetic locus within which these mutations are found be called *dur-3*. Genetic

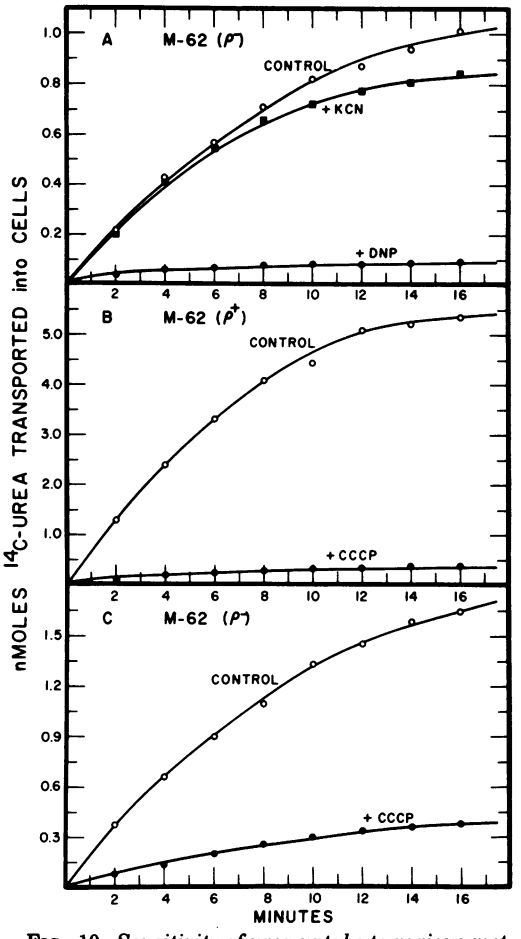

FIG. 10. *Sensitivity of urea uptake to various metabolic inhibitors in Rho⁺ and Rho⁻ strains. Rho⁺ and Rho⁻ strains prepared by ethidium bromide treatment of strain M62 (dur-1) were grown to a cell density of 45 Klett units on minimal ammonia medium containing 1 mM oxalurate. At that time, urea uptake was measured in the presence and absence of the various inhibitors. Inhibitors at concentrations of 1 mM for DNP and KCN and 0.1 mM for CCCP were added 1 min before initiation of the uptake assay.*

characterization of the *dur-3* locus has begun, with preliminary experiments indicating its centromere linkage. However, no linkage between this locus and any of the other allantoin-degradative genes can be detected.

The data presented here argue that the low K_m, energy-dependent, active transport system and the high-concentration (above 0.5 mM), energy-independent urea uptake system may share certain elements in common. This is predicated upon the observations that growth on 10 mM urea is severely decreased in *dur-3* strains and that induction of allophanate hydrolase, using 10 mM urea as inducer, yields only about one-half the expected amounts of enzyme in the mutant strains. If the two systems were totally separate, loss of the low K_m system by mutation would not have been expected to profoundly influence the remaining high-concentration uptake system. Pleotrophic effects upon the high-concentration uptake system also suggest that at high external concentrations of urea, entry occurs via carrier-mediated facilitated diffusion rather than by simple chemical diffusion. Questions concerning the relationship of allantoin and allantoate uptake systems to urea uptake are also appropriate, because growth on these compounds was also adversely affected in *dur-3* mutant strains. The significance of these questions arises from the possibility that all of the allantoin-related metabolites are transported by a single multicomponent transport system.

The driving force behind urea transport appears to be cytoplasmically generated adenosine 5'-triphosphate (ATP). This conclusion was reached on the basis of urea uptake sensitivity to inhibitors of glycolysis such as arsenate and fluoride. Further support is offered by the fact that urea transport is not seriously affected in Rho⁻ strains. Whether or not mitochondria of Rho⁺ strains make a positive energy contribution toward urea uptake or merely "spare" cytoplasmic ATP reserves cannot at present be decided. The inhibitory action of uncoupling agents and proton conductors such as DNP and CCCP in Rho⁻ strains raises the possibility that ATP generated by glycolysis is used to establish some sort of ion gradient, which in turn drives urea transport. However, the question of whether or not an adenosine triphosphatase participates in energy coupling as has been shown for several bacterial systems (1, 9, 11) remains open because thus far it has not been possible to demonstrate any decrease of urea-accumulating ability in the presence of 1 mM *N,N'*-dicyclohexylcarbodiimide, a specific inhibitor of the bacterial Mg^{2+}-Ca^{2+}-adenosine triphosphatase (1, 11).

ACKNOWLEDGMENTS

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