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Oxaluric Acid: a Non-Metabolizable Inducer of the Allantoin Degradative Enzymes in *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae degrades allantoin in five steps to ammonia, CO_2, and glyoxylate. Previously we demonstrated that allophanic acid, the last intermediate of the pathway, was required for induction of all five degradative enzymes. The data presented here indicate that oxaluric acid, an allophanate analogue, is capable of serving as a non-metabolizable inducer. Oxaluric acid brings about a high level of induction even in strains lacking urea carboxylase. Induction observed with parabanic acid was found to result from its spontaneous breakdown to oxaluric acid.

Saccharomyces cerevisiae degrades allantoin and arginine as shown in Fig. 1. This was demonstrated by isolation of a series of mutants, each lacking one of the various activities, and the growth response exhibited by each strain toward the pathway intermediates (12; R. P. Lawther, E. Reimer, and T. G. Cooper, manuscript in preparation). We have reported that synthesis of the five enzymes involved in allantoin degradation is dependent upon the presence of the last intermediate, allophanic acid (5, 6, 13). This was concluded from the facts that: (i) a large increase in all five activities was observed in wild-type strains regardless of which pathway intermediate was added to the medium, (ii) no increase in any of the activities was observed unless the added intermediate could be metabolized to allophanic acid, (iii) strains which lack allophanate hydrolase and would thus be expected to accumulate large amounts of allophanate, resulting from the metabolism of intracellular arginine pools, possessed very high amounts of the four remaining enzymatic activities even in the absence of an added intermediate, and (iv) the physiological urea analogue, formamide, was capable of bringing about synthesis of the pathway enzymes in wild-type strains, but would not perform this function in a strain lacking urea carboxylase. In many bacterial systems, the availability of a non-metabolizable inducer has greatly facilitated studies concerning the regulation of enzyme synthesis under various physiological conditions. Indeed, our present understanding of the mechanisms underlying and regulating the expression of the lactose operon in *Escherichia coli* was largely dependent upon the use of IPTG (isopropyl-thio-galactoside) as a non-metabolizable inducer (3, 7, 8). In view of the great advantages afforded by effective inducer analogues, a search was mounted for a compound that would perform this function in the allantoin degradative pathway. The results of that search were not only identification of the allophanate analogue, oxaluric acid (OXLU), as a non-metabolizable inducer of the allantoin degradative enzymes, but also the gaining of some insight into the particular atoms of allophanate which probably participtate in the induction process.

MATERIALS AND METHODS

Induction experiments. All of the strains used in this work were prototrophic diploids and have been biochemically characterized (12). Strain M-25 is wild type with respect to the allantoin degradative enzymes, whereas strain M-62 possesses a defective urea carboxylase. The culture and enzyme assay procedures employed here were identical to those reported by Cooper and Lawther (6). Cell density measurements were made turbidimetrically with a Klett-Summerson colorimeter by employing a green (500 to 570 nm spectral range) filter. One hundred Klett units is approximately equivalent to 3×10^7 cells per ml of culture.

Chromatography of oxaluric acid. A number of the commercially available intermediates such as oxaluric and oxamic acids were found to support good growth as a nitrogen source for both wild-type and various mutant strains of *Saccharomyces*. This observation led to a suspicion that these compounds might be contaminated with usable sources of nitrogen such as urea or ammonia, or both. To test this possibility, a 25-mg sample of oxaluric acid or other intermediate

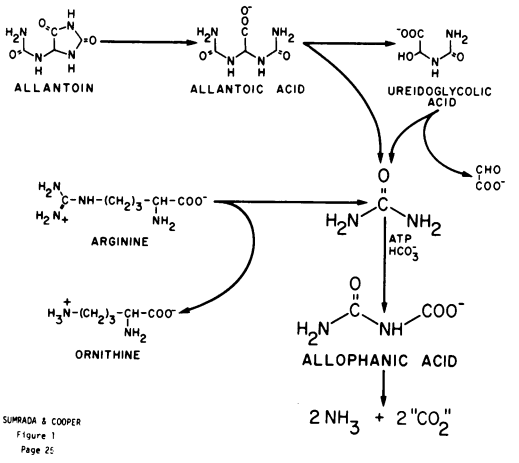

FIG. 1. *Reactions by which allantoin and arginine are degraded in Saccharomyces cerevisiae.*

was dissolved in 20 ml of glass-distilled water and carefully neutralized (final pH of 6.5) with dilute KOH. This solution was then applied to a Dowex-1-acetate (1-X10) column (1 × 16 cm) which was washed first with water and finally with a 0 to 0.33 M NaCl linear gradient (400 ml was the total gradient volume). Fractions of 4.0 ml were collected and assayed for ammonia by using the Nessler procedure (2) and for ureido groups by using the procedures of Bojanowski et al. (4). The results of such a chromatographic analysis performed on allophanic acid and commercially obtained oxaluric acid are shown in Fig. 2. A large amount of ammonia contamination is clearly evident in this chromatogram. It should also be noted that the purity of the oxaluric acid influenced the level of induction observed. Because preparations of greatest purity yielded the highest levels of induction, identification of the deleterious component present in the commercial preparations was not pursued. Analysis of our purified material by means of gas-liquid chromatography directly coupled to a mass spectrometer yielded fragmentation patterns characteristic of oxaluric acid. Notably absent were fragmentation patterns characteristic of either urea or allophanic acid. No degradation of oxaluric acid to oxamic acid and urea (1) could be detected under the conditions and periods of incubation employed in our experiments.

RESULTS

Survey of potential inducers. The search for a non-metabolizable inducer of the allantoin degradative system was initiated by screening a large number of ureido compounds with respect to their ability to serve as sole nitrogen sources and to bring about induction of allantoinase and allophanate hydrolase. The results of that survey (Table 1) demonstrate that, unlike other fungi and genera of yeast, *S. cerevisiae* will use only a very few ureido compounds as nitrogen sources. The only compounds found to support growth were intermediates related to the arginine and allantoin pathways. Formyl urea and *t*-butyl urea supported growth of the wild-type organism, but were incapable of supporting growth of a urea carboxylase minus strain. This may reflect a requirement for carboxylation of these compounds prior to their degradation, or it may suggest that these commercial preparations contain contaminating amounts of urea. This consideration must be emphasized, because oxaluric and oxamic acids were found to be up to 20% contaminated by ammonium ion. Four non-metabolizable compounds were able to bring about enzyme induction in the wild-type organism: formamide, allophanamide, parabanic acid, and oxaluric acid. Only the latter two of these compounds, however, functioned as inducers in a strain lacking urea carboxylase. The levels of induction observed with oxaluric and parabanic acids were greater than those found with urea (Table 1).

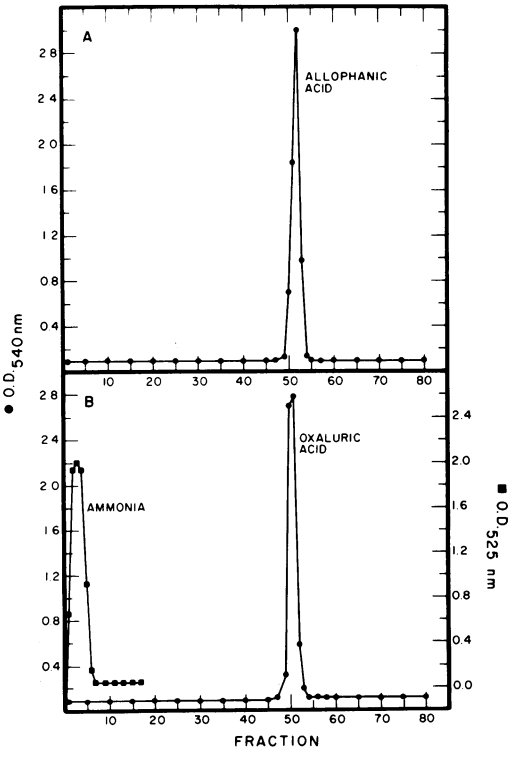

FIG. 2. *Chromatography of allophanic acid and commercially obtained oxaluric acid. The conditions of chromatography and assay are described in Materials and Methods. The linear gradient was initiated at fraction number 7.*

Characterization of oxaluric acid as a non-metabolizable inducer. In order to demonstrate more carefully the ability of oxaluric acid to serve as an inducer, the differential rate of oxaluric acid-dependent allantoinase and allophanate hydrolase synthesis was monitored in both wild-type and urea carboxylase-defective strains. The level of induction observed in

TABLE 1. *Growth-supporting and induction capabilities of various ureido group-containing compounds*[a]

Compound added	M-25, wild-type strain			M-62, urea carboxylase-defective strain		
	Doubling time (min)	Allantoinase[b]	Allophanate hydrolase[b]	Doubling time (min)	Allantoinase[b]	Allophanate hydrolase[b]
Acetamide	–	0.36	0.57		0.31	0.10
Acetyl thiourea	–	0.30	0.43		0.24	0.15
Acetyl urea	–	0.36	0.39		0.36	0.10
Allantoate	160	1.01	4.21	–		
Allantoin	150	1.01	3.02			0.10
Allophanamide	–	0.78	1.23		0.43	0.46
Ammonia	120	0.28	0.23		0.18	0.07
Barbituric acid	–	0.38	0.38			
N-butyl urea	–	0.23	0.24			
t-Butyl urea	+	0.65	0.18	–		
Carbamyl aspartic	–	0.03	0.05			
Carbamyl-β-alanine	–	0.42	1.16		0.20	0.13
2-Cl-acetamide	–	0.13	0		0	0
Citrulline	300	0	0.55		0.42	0.15
Creatinine	–	0.33	0.36			
Diacetamide	–	0.29	0.29		0.19	0.11
Ethyl carbamate	–	0.26	0.25			
Formamide	–	0.94	4.32		0.03	0.14
Formyl urea	170	1.12	2.98	–	0.30	0.12
Glyoxyl urea	143	1.05	4.54			0.13
Homoarginine	–	0.16	0.17			
Hydantoate	–	0.23	0.68		0	0.34
N-OH-urea	–	0.18	0.14			
Hypoxanthine	–	0.19	0.31			
Inosine	–	0.12	0.25			
Methyl allophanate	–	0.32	0.21			
N-methyl formamide	–	0.56	0.25			
N-methyl urea	–	0.50	0.36		0.34	0.15
Ornithine	155	0	0.47		0	0.02
Oxaluric acid	–	1.07	4.60	–	0.53	2.14
Oxamic acid	–	0.18	0.16			
Oxonic acid	–	0.42	0.18			
Parabanic acid	–	0.68	2.73		0.42	2.20
Phenyl urea	–	0.19	0.22			
Selenourea	–	0	0			
Succinimide	–	0	0.22			
2-Thio-hydantoin	–	0	0.21			
Thiourea	–	0.23	0.24			
Urea	139	0.89	3.68		0.06	0.21
Uric acid					0.02	0.12

[a] Growth experiments were conducted by inoculating either wild-type (M-25) or urea carboxylase-less (M-62) strains into minimal medium containing the indicated compound at 0.1% or its maximal solubility as sole nitrogen source. A compound was scored negative (–) only if there was insignificant growth after 48 h. Induction experiments were performed by adding the indicated compound to a concentration of 1×10^{-2} M. After one generation of growth in the presence of the compound, samples were taken and assayed for allantoinase and allophanate hydrolase activity by using the procedures described in Methods and Materials. Because a large number of independent experiments were required to complete this table, the data were normalized on the basis of fold induction over the uninduced level. Blank spaces in this table indicate determinations that were not done.

[b] Values are expressed as nmoles per minute per milliliter of culture.

both strains is essentially the same (Fig. 3 and 4). This indicates that no carboxylation of oxaluric acid is required for induction, thus distinguishing it from the urea analogue, formamide, which apparently requires carboxylation to serve as an inducer (13). The effect of oxaluric acid concentration upon the degree of induction was ascertained by adding inducer (to the final concentration indicated) to cells growing exponentially on minimal medium and by determining the level of each enzyme one generation later. Maximal induction was observed at approximately 0.1 mM for allantoinase, whereas 0.5 mM oxaluric acid was required for maximal allophanate hydrolase activity (Fig. 5). Note, however, that the level of both enzymes decreased if the concentration of oxaluric acid was increased above 1.0 mM. The reason for this decrease is not presently known.

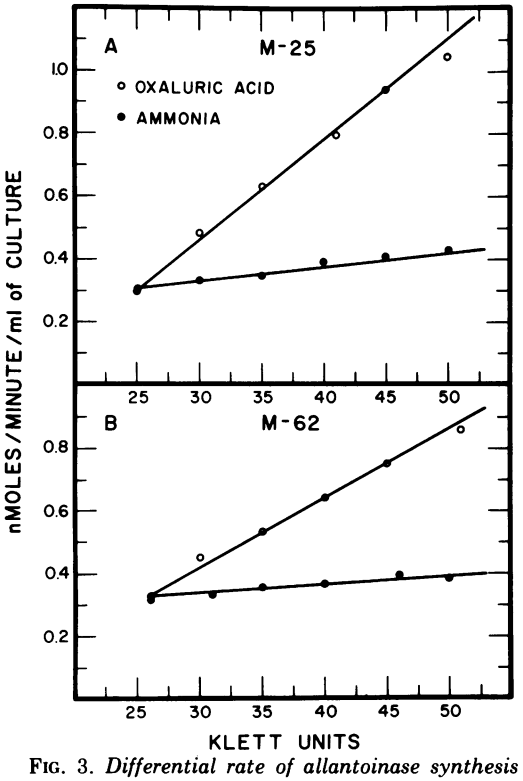

FIG. 3. *Differential rate of allantoinase synthesis in wild-type (M-25) and urea carboxylase-less (M-62) strains of Saccharomyces cerevisiae. The cells were grown to a density of 25 Klett units in minimal medium containing 0.1% ammonia as sole nitrogen source. At this cell density the culture was split; one-half received no additions (●) and the other half received oxaluric acid to a final concentration of 1 × 10⁻² M (○). The cultures were sampled at the indicated times and assayed for allantoinase activity by using the procedures described in Materials and Methods.*

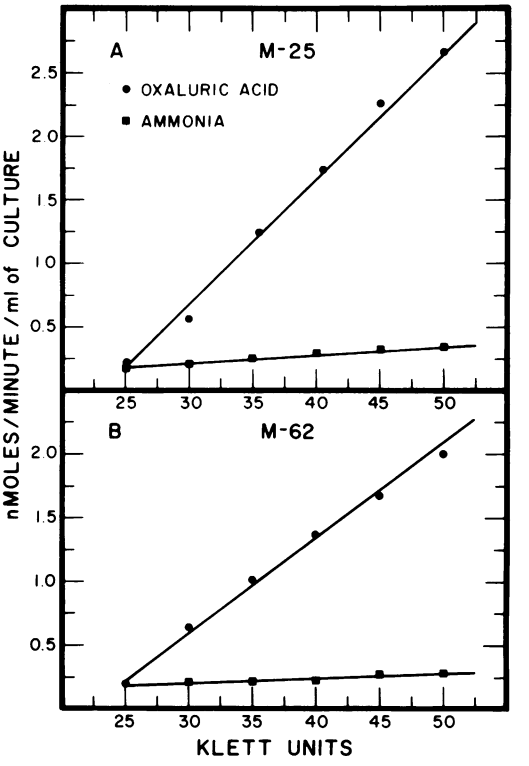

FIG. 4. *Differential rate of allophanate hydrolase synthesis in wild-type (M-25) and urea carboxylase-less (M-62) strains of Saccharomyces cerevisiae. The induction experiments were performed as described in Fig. 3, and samples were assayed for allophanate hydrolase as described in Materials and Methods.*

The great advantages of non-metabolizable inducers reside in their ability to bring about synthesis of a given enzyme while failing to participate in and hence alter the metabolism of the cells to which they are added. To verify this characteristic in the case of oxaluric acid, two experiments were performed. Two wild-type cultures of *Saccharomyces* were grown on minimal-glucose medium containing either ammonia or purified oxaluric acid as sole nitrogen source. Oxaluric acid was unable to support any growth whatsoever when provided as the only nitrogen source. If, at the conclusion of the 96-h incubation period used in this experiment, ammonia was added to the oxaluric acid containing medium, the cells multiplied at a rate identical to that of the control culture. This experiment indicates that oxaluric acid could not be metabolized to products that were metabolically useful as a source of nitrogen to the cell. However, to eliminate the possibility that

oxaluric acid was being metabolized to another unusable metabolite, this inducer was added to an exponentially growing culture for two generations. Samples of both cells and medium were taken immediately after addition of oxaluric acid and at the conclusion of the incubation period. The two samples of medium and extracts of the two cell samples were chromatographed on Dowex-1-acetate, and the appropriate fractions were assayed for ureido-containing compounds according to the procedures described in Materials and Methods. Table 2 indicates the amount of material that gave a positive ureido-group reaction and that possessed a retardation factor (R_f) value identical to that of oxaluric acid. As shown in this table the total amount of oxaluric acid in the medium is decreasing while that in the cells is increasing. Although these data suggest that oxaluric acid is not being metabolized within the cell, they cannot be considered as quantitative because: (i) there is ureido group-containing material appearing in fractions 54 to 57 (Fig. 6), and (ii) the cells contain ureido-positive compounds in fractions 51 to 54 (the expected position of oxaluric acid) at the time of oxaluric acid addition (Table 2). The only method of

TABLE 2. *Uptake of oxaluric acid by a wild-type strain of Saccharomyces*

Fraction	Amt of material cochromatographing with oxaluric acid on Dowex-1-acetate (nmol)[a]
Medium at zero time	629
Cells at zero time	256
Medium 2.5 generations later	555
Cells 2.5 generations later	386

[a] These values were arrived at by determining the area under fractions 50 to 54 inclusively and converting it to nmoles (assuming all of the material has the same extinction coefficient as oxaluric acid).

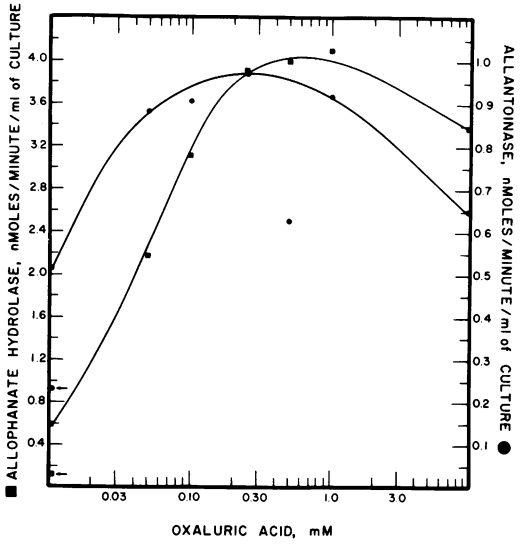

FIG. 5. *Differential rate of allantoinase and allophanate hydrolase synthesis as a function of inducer concentration. A wild-type strain (M-25) was grown to a density of 26 Klett units in minimal medium containing 0.1% ammonia as the sole nitrogen source. At this time the culture was divided into eight portions. Oxaluric acid was added to each fraction to the final concentration indicated. A sample was taken from each fraction immediately after addition of oxaluric acid and after a one-generation incubation period. These samples were assayed for allantoinase and allophanate hydrolase by using the procedures described in Materials and Methods. The enzyme activities are expressed as differential rates of synthesis. The arrows indicate the amounts of activity observed for each enzyme in the absence of added inducer.*

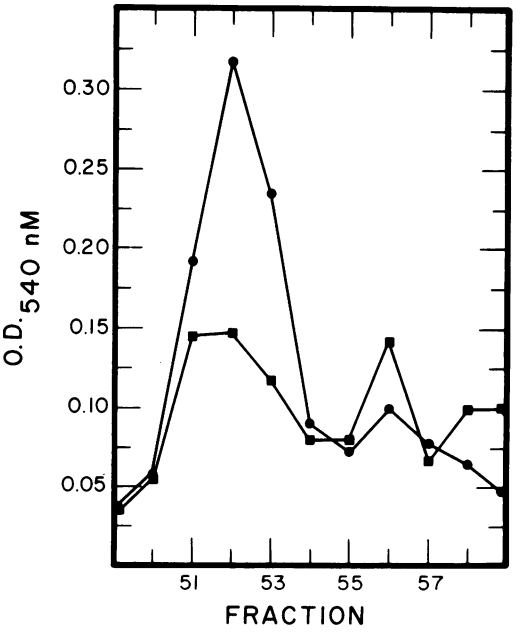

FIG. 6. *Isolation of ureido compounds cochromatographing with oxaluric acid from medium and extracts of oxaluric acid-induced cells. Samples of the medium after addition of oxaluric acid (●) and the soluble components of cells incubated for 1.5 generations in the presence of oxaluric acid (■) were chromatographed on Dowex-1-acetate, and the fractions in which oxaluric acid would be expected were assayed for ureido-positive compounds. The chromatographic and assay procedures are described in Materials and Methods.*

unequivocally demonstrating that oxaluric is not altered is through the use of uniformly labeled oxaluric acid which is not presently available.

Parabanic acid induction of the allantoin degradative system The observation (Table 1) that parabanic acid could bring about induction of the allantoin degradative enzymes in a strain lacking urea carboxylase was verified by the experimental data shown in Fig. 7. Although the level of induction seen here is somewhat less than that found in Fig. 3 and 4, it is significant. The concentration dependence of parabanic acid induction was quite similar to that observed with oxaluric acid and hence will not be presented. To ascertain whether or not this presumptive non-metabolizable inducer could be recovered intact from growing cells, an experiment similar to that described in Table 2 and Fig. 6 was performed by using parabanic acid in place of oxaluric acid. In this case, however, the small molecules were not chromatographed. Rather, the content of parabanic acid was determined by its ultraviolet absorbance spectrum. A large percentage of the parabanic acid originally present in the medium disappeared during the incubation period. Surprisingly, however, there was no concomitant increase of parabanic acid content in the cells. This result strongly suggested that parabanic acid was either being metabolized or degraded to a nonabsorbing compound. To test the latter possibility, parabanic acid was dissolved in sterile medium devoid of cells. Spectra of the solution taken at various times after its preparation indicated that parabanic acid was being spontaneously degraded. This is in agreement with an early observation of Andrews and Sell (1), who reported that parabanic acid is degraded to oxaluric acid at high pH. However, we find that the rate of degradation (half-life of 20 min at room temperature) is considerably higher than that reported earlier. This is likely the result of the present experiments being carried out in culture medium which contains high concentrations of both salts and metals. These data preclude parabanic acid being classified as an inducer of the allantoin degradative enzymes.

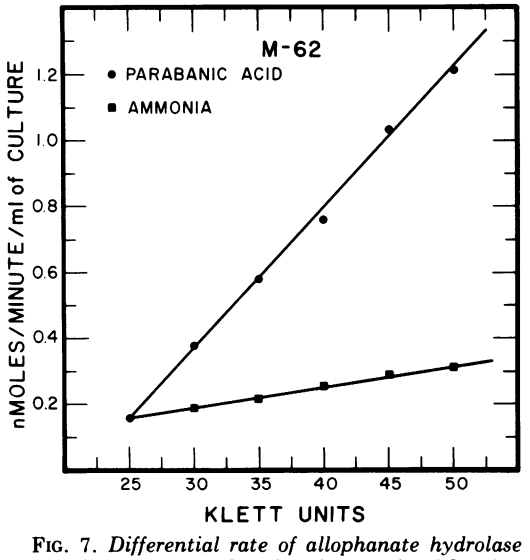

FIG. 7. *Differential rate of allophanate hydrolase synthesis in a urea carboxylase-less strain of Saccharomyces cerevisiae. The induction experiment and enzyme assays were performed as described in Fig. 3, except that parabanic acid was used in place of oxaluric acid.*

Identification of the atoms participating in the induction process. There exist a variety of compounds that are capable of inducing the allantoin degradative enzymes, and by comparing their ability to serve as inducers, some insight concerning the specific atoms of allophanate that are participating in the induction process may be gained. The experimental results shown in Table 3 and Fig. 8 compare the

TABLE 3. *Induction of allantoinase and allophanate hydrolase by analogues of urea and allophanic acid in wild-type and urea carboxylase-defective strains of Saccharomyces*[a]

Compound	Cell density	Wild-type strain[b]		Urea carboxylase-defective strain[b]	
		Allantoinase	Allophanate hydrolase	Allantoinase	Allophanate hydrolase
Ammonia	12	0.18	0.13	0.09	0.06
	20	0.32	0.15	0.14	0.12
	40	0.46	0.36	0.13	0.16
	80	0.57	0.66	0.13	0.20
Oxaluric acid	12	0.16	0.17	0.08	0.04
	20	0.44	1.02	0.23	0.45
	40	1.09	4.07	0.40	1.67
	80	1.25	6.42	0.47	2.86
Formamide	12	0.21	0.15	0.08	0.06
	20	0.50	1.55	0.12	0.11
	40	1.16	4.63	0.11	0.20
	80	1.50	8.81	0.12	0.30
Hydantoic acid	12	0.19	0.14	0.00	0.06
	20	0.20	0.25	0.00	0.15
	40	0.43	0.83	0.08	0.40
	80	0.68	1.84	0.10	0.57

[a] These induction experiments and enzyme assays were performed in a manner similar to those described in Figure 3.

[b] Values are expressed as nmoles per minute per milliliter of culture.

abilities of four compounds to serve as inducers of allantoinase and allophanate hydrolase. All of the compounds, except for hydantoic acid, yield high levels of induction in wild-type strains. In a strain lacking urea carboxylase, however, ureidoglycolate and formamide are ineffective. Hydantoic acid, on the other hand, elicits a low, but measurable, response in this mutant strain, and oxaluric acid brings about high levels of induction for both enzymes.

DISCUSSION

We have demonstrated that oxaluric acid fulfills all of the criteria of a non-metabolizable inducer for the allantoin degradative enzymes in *S. cerevisiae*. This compound will not serve as a sole nitrogen source and may be recovered intact from cells which have taken it up from the growth medium. Although being refractile to metabolism, oxaluric acid will bring about high-level synthesis of the allantoin degradative system. Similar behavior in the case of parabanic acid was, surprisingly, the result of its spontaneous decomposition to oxaluric acid. Although oxaluric acid has never been reported as participating in or influencing metabolism in yeast, it has been reported by a number of workers as an intermediate in the metabolism of allantoin by *Streptococcus allantoicus* (4, 9–11).

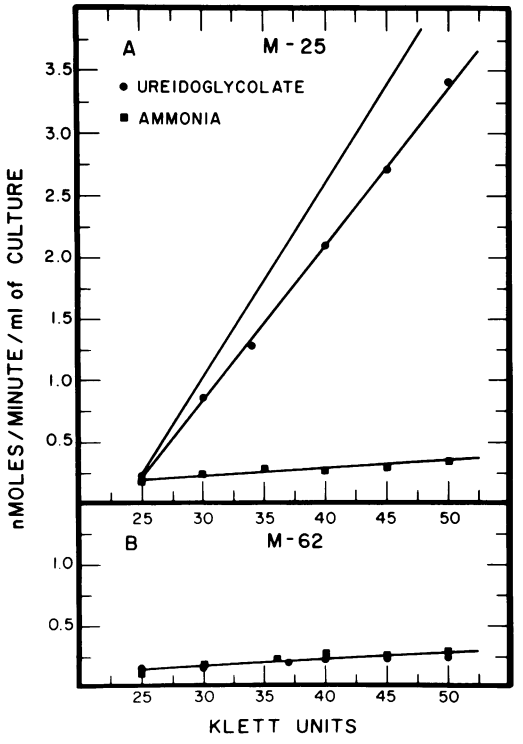

FIG. 8. *Differential rate of allophanate hydrolase synthesis in wild-type (M-25) and urea carboxylaseless (M-62) strains of Saccharomyces cerevisiae. The induction experiments were performed as described in Fig. 3, except that ureidoglycolate was used in place of oxaluric acid. Samples were assayed by using standard procedures. The theoretical line (without data points) represents the differential rate of synthesis observed when the culture was grown in the presence of urea instead of ureidoglycolate.*

By monitoring the abilities of a number of compounds to function as inducers of the allantoin enzymes it has been possible to gain insight into the specific atoms of the allophanate molecule that are necessary for the induction process. In Fig. 9, the structure of each of these compounds is depicted along with that of allophanic acid, the naturally occurring inducer. The high-level induction obtained with formyl carbamic acid (formamide after carboxylation) indicates that the free amino group of allophanic acid is not necessary for induction. Likewise, the efficiency observed with oxaluric acid is consistent with the suggestion that the hydroxyl group of allophanate is also unnecessary. On the other hand, the poor response observed with hydantoic acid would suggest that the proximity of the two carbonyl carbons is essential to induction. It may also be argued that higher levels of induction would be observed if this compound were not retarded from entering the cell. Although this argument can never be effectively eliminated, the shape of the dose response curve obtained for hydantoic acid (figure 3 of ref. 13) is not in keeping with such a suggestion. The inability of ureidoglycolate to function as an inducer suggests that: (i) a planar structure which would be lost upon reduction of the carbonyl group is required for induction, or (ii) the carbonyl oxygen must be available for hydrogen bonding of allophanate to its control element. In this case, failure of

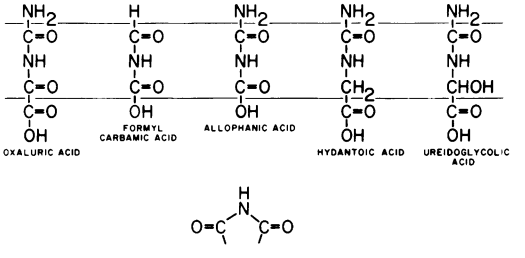

FIG. 9. *Structures of the compounds assayed for their ability to serve as inducers of the allantoin degradative enzymes.*

ureidoglycolate to penetrate the cell is not a valid explanation for its inability to bring about induction, because the wild-type cell will use this compound as a sole nitrogen source. Therefore, the six atoms shown in the lower part of Fig. 9 are suggested to be necessary for induction.

Because all five of the allantoin degradative enzymes are induced by the same compound, it is reasonable to ask whether or not the induction of these activities is coordinate. The data presented in Fig. 10 afford an opportunity to test this possibility, because all of the assays were performed on the same cultures. The allophanate hydrolase activity observed at a variety of inducer concentrations was plotted as a function of the allantoinase activity observed at that concentration. It is clear that although both activities increase, they are by no means coordinate. This fact is also emphasized by the observation (Figure 5) that saturation concentrations of oxaluric acid are attained much earlier for allantoinase activity than for allophanate hydrolase. This lack of coordinancy may be readily explained by our genetic studies (R. P. Lawther and T. G. Cooper, manuscript in preparation) which indicate that the structural genes for the five allantoin degradative enzymes

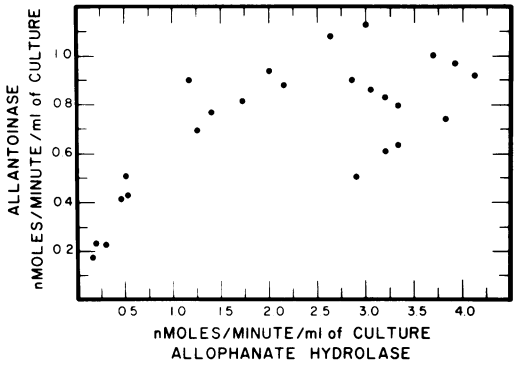

FIG. 10. *The relation between allantoinase levels and allophanate hydrolase levels at various concentrations of inducers. Each of these points was derived from the levels of the two enzymes observed at various concentrations of oxaluric acid (data from Fig. 5 were used), parabanic acid, and allophanamide.*

are situated as at least two unlinked clusters in the *Saccharomyces* genome.

ACKNOWLEDGMENTS

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